Cyclin-dependent kinase 11 (CDK11; also named \(\text{PITSLRE}\)) is part of the large family of \(\text{p34}^{\text{cdc2}}\)-related kinases whose functions appear to be linked with cell cycle progression, tumorigenesis, and apoptotic signaling. However, substrates of CDK11 during apoptosis have not been identified. We used a yeast two-hybrid screening strategy and identified eukaryotic initiation factor 3 \(\text{p47}\) protein (eIF3 \(\text{p47}\)) as an interacting partner. We demonstrate that the eIF3 \(\text{p47}\) can interact with CDK11 \textit{in vitro} and \textit{in vivo}, and the interaction can be strengthened by stimulation of apoptosis. eIF3 \(\text{p47}\) contains a Mov34/JAB domain and appears to interact with CDK11\(\text{p46}\) through this motif. We show \textit{in vitro} that the caspase-processed CDK11\(\text{p46}\) can phosphorylate eIF3 \(\text{p47}\) at a specific serine residue (Ser\(^{46}\)), and that eIF3 \(\text{p47}\) is phosphorylated \textit{in vivo} during apoptosis. Purified recombinant CDK11\(\text{p46}\) inhibited translation of a reporter gene \textit{in vitro} in a dose-dependent manner. In contrast, a kinase-defective mutant CDK11\(\text{p46m}\) did not inhibit translation of the reporter gene. Stable expression of CDK11\(\text{p46}\) \textit{in vivo} inhibited the synthesis of a transfected luciferase reporter protein and overall cellular protein synthesis. These data provide insight into the cellular function of CDK11 during apoptosis.

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 \(\text{PITSLRE}\)) protein kinases are members of the cyclin-dependent kinase superfamily. Two distinct but closely related human CDK11 genes (\(\text{Cdc2L1}\) and \(\text{Cdc2L2}\)) express several CDK11 isoforms (1, 2). CDK11 homologues exist in several species including humans, mice, chickens, \(\text{Drosophila melanogaster}\), and \(\text{Xenopus}\) (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\(\text{p110}\)) 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 (6, 7). Furthermore, CDK11\(\text{p110}\) associates with cyclin \(\text{L}\) (8). Cyclin \(\text{L}\) is an RS domain protein that may function in pre-mRNA splicing (8). These observations suggest that CDK11\(\text{p110}\) kinases play some role in the production of translatable RNA transcripts in proliferating cells.

The CDK11\(\text{p110}\) isoforms contain an internal ribosome entry site, which leads to the generation of a CDK11\(\text{p58}\) isoform during the G2/M phase of the cell cycle (9). Elevated expression of CDK11\(\text{p58}\) in eukaryotic cells alters normal cytokinesis and can delay cells in late telophase (10). In contrast, reduced expression of CDK11\(\text{p58}\) increases DNA replication and enhances cell growth (11). This p58 isoform of CDK11 can interact with cyclin D3 (12). This suggests that CDK11\(\text{p58}\)/cyclin D3 may play role in mitosis.

In regard to apoptosis, increased expression of CDK11\(\text{p58}\) reduces cell growth due to apoptosis (1). In addition, our group and others have shown that the CDK11\(\text{p110}\) isoform and the CDK11\(\text{p58}\) isoform are cleaved by caspases to generate a smaller 46-kDa protein that contains the catalytic portion of the protein (2, 13, 14). This smaller CDK11\(\text{p56}\) isoform can be triggered by Fas, tumor necrosis factor \(\alpha\), or staurosporine and phosphorylate histone H1. Caspase inhibitors can modulate the kinase activity of the caspase-processed isoform of CDK11 (2). Collectively, these observations suggest that CDK11 may play a role in apoptotic signaling. However, substrates potentially regulated by CDK11 during apoptosis have not been identified. The present study was performed to address this issue. We identify the p47 subunit of eukaryotic initiation factor 3 (eIF3\(\text{p47}\)) as a protein associated with the caspase-processed isoform of CDK11 (CDK11\(\text{p46}\)) using a yeast two-hybrid screen. The interaction between CDK11\(\text{p46}\) and eIF3 \(\text{p47}\) occurs \textit{in vitro} and \textit{in vivo}. In addition, CDK11 kinase isolated from cells undergoing apoptosis can phosphorylate eIF3 \(\text{p47}\), and serine phosphorylation of eIF3 \(\text{p47}\) occurs in cells during apoptosis. Taken together, these results strongly support our hypothesis that CDK11 may be involved in apoptotic signaling by interacting with eIF3 \(\text{p47}\).
Construction of eIF3 p47 Vectors  
Construction of eIF3 p47 Vectors—pcCMV-HA-eIF3 p47, containing the full-length coding sequence to the eIF3 p47 with a HA tag at the N terminus, was constructed. Full-length eIF3 p47 was isolated from the original fetal brain cDNA clone we obtained by yeast two-hybrid assay using EcoRI/NotI restriction enzymes. The full-length eIF3 p47 was cloned into pGEX-4T-2 to generate GST-eIF3 p47 fusion protein expressing vector for DNA fragment, and 16–360, 248–360, 1–113, and 1–113 was obtained by PCR amplification and cloned into pGEX-4T-2 vector to generate deletion GST-p47 fusion proteins.  

Construction of CDK11p47 Mutants—The CDK11p47 mutation was generated by polymerase chain reaction using a QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) with CDK11p47 wild-type construct as template. Sequencing analysis was used to confirm the mutation. This mutation has been known to cause CDK11 to lose its kinase activity (1). Therefore, we created this mutation in the ATP binding domain to inactivate the kinase. The mutagenesis primers were 5’-GAGAAACCCGGCGCTGAGGAGG-3’ and 5’-CCATCTCAGAGGCTGAGGACTATCTTTGTT-3’.  

Immunoprecipitation and Western Blotting—Cells were harvested, washed twice with cold PBS, and lysed by lysis buffer (10 mM Hepes, pH 7.2, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% Nonidet P-40) containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture (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 lysate (50 μg) was precleared with protein A- or G-agarose beads (Oncogene, La Jolla, CA) and goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 h. Myc-CDK11p110 fusion protein or eIF3 was then immunoprecipitated using c-Myc monoclonal antibody (Sigma) or goat eIF3 polyclonal antibody (15) and protein A- or G-agarose overnight at 4°C. The immune complex was 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 different antibodies. A secondary probe with horseradish peroxidase-labeled antibodies (Sigma) was detected by ECL (Amersham Biosciences) or ECL Plus (Amersham Biosciences).  

Immunofluorescence Confocal Microscopy—pCMV-HA-eIF3 p47 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. 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 in PBS for 10 min at room temperature, and then the bovine serum albumin was removed. The coverslips were then incubated with CDK11p110-specific antibody P1C (1:500 dilution) and rabbit anti-HA antibody (1:50 dilution) for 1 h at room temperature. Coverslips were washed three times with PBS for 5 min each and then incubated with secondary antibodies fluorescein isothiocyanate-conjugated anti-mouse IgG and 4′,6-diamidino-2-phenylindole (Invitrogen) according to the manufacturer’s instructions. The wild type and K451M substituted CDK11 C-terminal portion (CDK11p110) were used as baits. In the K451M altered CDK11p46, one lysine residue that is associated with phosphatase 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 × 10⁶ transformants from a human fetal brain Matchmaker cDNA library (Clontech) were screened in the yeast strain AH109 (Clontech), and 28 colonies were obtained by hybridization with wild-type CDK11p46 as bait. One of these clones (clone 4) was reisolated, named CDK11p46/WT, and the percentage of p47-subclone-containing nuclei was determined. Nuclei were collected at 2000 rpm for 10 min. The supernatant contained the cytoplasm.  

Purification of Recombinant Protein from E. coli—GST, GST-CDK11p46, GST-CDK11p110, and GST-p47 were induced by 0.2 mM isopropyl-1-thio-β-p-galactosidase 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 instructions (Amersham Biosciences). Purified proteins were concentrated using Centricron 30 (Amicon Inc.) to an appropriate concentration and stored as aliquots at −70°C.  

GST Pull-down Assay—The assay was performed as described previously (12) with a few modifications. Briefly, GST or GST fusion proteins were expressed in BL21 cells, and equal amounts of bacterial lysates were 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 [35S]methionine-labeled CDK11p46 overnight at 4°C. [35S]Methionine-labeled CDK11p46 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 CDK11p46 protein was analyzed by autoradiography after resolved by SDS-PAGE.  

Kinase Assay—A375 cells were treated with anti-Fas (Upstate Biotechnology) or with both anti-Fas and caspase-3 inhibitor DEVd-FMK (Zymed, South San Francisco, CA) at 37°C overnight at room temperature. Cells were harvested and the supernatant and the pellet were subjected to kinase activity assay. Quant software was used to quantitate the relative phosphorylation level of the substrates.

Identification of eIF3 p47 Phosphorylation Site—Caspase-processed CDK11p46 was immunoprecipitated from anti-Fas-treated A375 cells. 5′-CTTCAGCCGCCTTTACGAGTGG-3′ and 5′-CCAATCCAGGAGGCTGAGGACTATCTTTGTT-3′ were used as primers.

CDK11 Interacts with eIF3 p47 during Apoptosis  
(Mediatech, Inc., Herndon, VA), supplemented with 5% fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), 1% t-glutamine, and 1% penicillin/streptomycin (Invitrogen). For induction of apoptosis, cells were treated with either 0.5 μg/ml anti-Fas antibody (Upstate Biotechnology, Inc., Lake Placid, NY) or 10 ng/ml staurosporine (Sigma) from the second day of seeding for the indicated time. HEK293 (human embryonic kidney 293) cell line was cultured in Dulbecco’s modified Eagle’s medium (Meditech) supplemented with 10% fetal bovine serum, 1% t-glutamine, and 1% penicillin/streptomycin at 37°C with 5% CO₂. All transfections were carried out using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions.

Antibodies—GIN is an affinity-purified rabbit polyclonal antibody raised by injection of rabbit with purified recombinant glutathione S-transferase (GST)-p47 fusion proteins. CTTCAGCCGCCTTAGAGCCACAATTTC-3’ was a gift from Drs. Vincent Kidd and Jill Lahti (St. Jude Children’s Research Hospital, Memphis, TN) (7). EGF-R antibody was constructed by cloning the PCR amplified CDK11 p46 with an in frame K451M substitution into the pGEX-4T-2 vector (Amersham Biosciences). pcDNA3-CDK11 p46 was constructed by cloning the PCR amplified CDK11 p46 with an in frame K451M substitution into the pGEX-4T-2 vector (Amersham Biosciences). Purified proteins were concentrated using Amicon Ultra-3000 centrifugal filter devices (Millipore) and were a gift from Drs. Vincent Kidd and Jill Lahti (St. Jude Children’s Research Hospital, Memphis, TN) (7). EIF3 p47 antibody is a monclonal antibody and monoclonal anti-phosphoserine antibody was purchased from Sigma.

Immunoprecipitation and Western Blotting—Cells were harvested, washed twice with cold lysis buffer (10 mM Hepes, pH 7.2, 142.5 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% Nonidet P-40) containing 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture (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 lysate (50 μg) was precleared with protein A- or G-agarose beads (Oncogene, La Jolla, CA) and goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 h. Myc-CDK11p110 fusion protein or eIF3 was then immunoprecipitated using c-Myc monoclonal antibody (Sigma) or goat eIF3 polyclonal antibody (15) and protein A- or G-agarose overnight at 4°C. The immune complex was 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 different antibodies. A secondary probe with the goat eIF3 polyclonal antibody was detected by ECL (Amersham Biosciences).
CDK11 Interacts with eIF3 p47 during Apoptosis

**RESULTS**

The Kinase Domain of the CDK11 Protein Kinase (CDK11^{p110}) Interacts with the Mov34/JAB_MPN Domain of eIF3 p47—The yeast two-hybrid method was used to screen a human fetal brain cDNA library using the caspase processed C-terminal portion containing the kinase domain of CDK11^{p110} (CDK11^{p110} as bait, and 13 CDK11^{p46}-interacting cDNA clones were isolated. DNA sequencing and data base searching revealed that the nucleotide sequence of clone 4 encoded the yeast two-hybrid method was used to screen a human fetal brain cDNA library using the caspase processed C-terminal portion containing the kinase domain of CDK11^{p110} (CDK11^{p110} as bait, and 13 CDK11^{p46}-interacting cDNA clones were isolated. DNA sequencing and data base searching revealed that the nucleotide sequence of clone 4 encoded the

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In Vitro Translation Inhibition Assay—First, a time course study and a dose response study were performed to determine the linear range of luciferase production using the rabbit reticulocyte lysate system. 50 ng of luciferase mRNA were added into the system and incubated at 30 °C for 0–120 min followed by measurement of luciferase activity every 15 min to determine the luciferase production rate over the time course. The production of luciferase is in a linear range up to 75 min of incubation. 20–50 ng of luciferase mRNA were incubated with the system at 30 °C for 60 min followed by measurement of luciferase activity. The production of luciferase is in a linear range between 20 and 50 ng of luciferase mRNA. Therefore, 40 ng of luciferase mRNA was added to 50 μl of a rabbit reticulocyte lysate in vitro translation reaction (Promega, Madison, WI) in the presence of recombinant purified 18–70 nM of CDK11^{p46}, CDK11^{p46}, 70 nM GST, or elution buffer. The reaction mixtures were incubated at 30 °C for 1 h. Luciferase activity was measured with a luminometer. In addition, newly synthesized 35S-labeled luciferase proteins were analyzed by subjecting equal amounts of reaction mixtures to 10% SDS-PAGE and autoradiography.

In Vivo Reporter Synthesis Assay—25 ng of pG53 control vector (Promega) containing SV40 promoter-driven luciferase gene was transfected into A375 cells that were stably transfected with either pCDNA3 (A375-pCDNA3), pcDNA3-CDK11^{p46} (A375-CDK11^{p46}), or pcDNA3-CDK11^{p46} in 24-well plates in triplicate. After 24 h, cells were lysed in passive lysis buffer (Promega), and luciferase activity was measured using the luciferase reporter assay system (Promega).

RT-PCR and Quantitative Real Time One-step RT-PCR—Total RNAs were extracted from A375-pCDNA3 and A375-CDK11^{p46} cells transfected with pG53 control vector and A375 cells untransfected with pG53 in triplicate using RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed with 2 μg of total RNA using the Omniscript RT kit (Qiagen). 2 μl of the cDNA mix was added to 20 μl of the PCR containing 2.5 units of Taq polymerase (TaKaRa, Otsu, Shiga, Japan), 1× buffer, and 1 pmol of primers. For histone, PCR was carried out at 95 °C for 5 min followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s with a final extension at 72 °C for 5 min using the primers Histone-5′-CCACGGAACCTCTGATTCGC-3′ and Histone-R 5′-GGTGCTAGCTGGATGTCTT-3′. For luciferase, PCR was carried out at 95 °C for 5 min followed by 30 cycles at 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 10 min using the primers Lucifer 5′-ATTGTGTTCTCTTGCGGCT-3′ and Lucifer 5′-GGTGCTAGCTGGATGTCTT-3′. The amplified fragments were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide. Real time RT-PCR was performed using SYBR® Green PCR Core Reagents (PerkinElmer Life Sciences) and Omniscript RT kit (Qiagen) and amplified in a PerkinElmer Life Sciences Prism 7000 sequence detection system according to the manufacturer’s instructions. Dissociation curves were performed to determine the specificity of the amplicon. Each sample was completed in triplicate, and no template control was included. Threshold cycle (Ct) during the exponential phase of amplification was determined by real time monitoring of fluorescent emission. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control gene. Primers for GAPDH were purchased from PerkinElmer Life Sciences. Primers for luciferase are 5′-CGCTTTAAATGATAGTTAGTGAAGAAG-3′ and 5′-TGGCGAAGAGGGAGATAAG-3′. Luciferase mRNA levels were demonstrated as the absolute number of copies normalized against GAPDH mRNA. Difference in amplification was indicated as ΔΔCt (ΔCt luciferase – Ct GAPDH) (19, 20).

Overall Cellular Protein Synthesis Inhibition Assay—A375 or A375-CDK11^{p46} cells were plated at 2 × 10^4 cells/well in 96-well plates in RPMI 1640 medium without leucine (U.S. Biological, Swampscott, MA) in triplicate and incubated at 37 °C for the indicated time. Cells were either treated with ethanol or treated with 10 ng/ml of staurosporine for the indicated time. 6 h before the end of the incubation, 1 μCi of 1-[3,4,5-^3^H]Hilucine (PerkinElmer Life Sciences) per well was added. The cells were collected onto UniFilter by cell harvester, and the incorporated ^3^Hilucine was measured by a microplate scintillation counter (Packard, Meriden, CT).

Apoptosis and Cell Survival Assays—Cells were stained with 7-aminoactinomycin D (7-AAD) and analyzed for apoptosis using a FACStar flow cytometer (Becton Dickinson, San Jose, CA) as described (2). Cell morphology analysis was performed by cytoospin preparation of cells followed by methanol fixation for 6 min. Slides were air dried and stained for 15 min in Giemsa stain. Slides were then rinsed in deionized water and air-dried followed by light microscopy analysis at ×15 magnification. Cell viability was measured with a 5(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (2).
Region in eIF3 p47 interacting with CDK11p46, we deleted specific sequences within eIF3, made fusion proteins, and performed GST pull-down assays (Fig. 1A). In the GST pull-down assay, in vitro translated [35S]-labeled CDK11p46 protein could be pulled down by GST-p47 amino acids 106–360 and GST-p47 amino acids 170–360 but not by GST-p47 amino acids 248–360 or GST-p47 amino acids 1–113 (Fig. 1, A and B). This suggests that the region within eIF3 p47 that directly interacts with

Fig. 2. The co-localization of CDK11 and eIF3 p47 in human cells. A, co-localization of CDK11p110 and eIF3 p47 in the nucleus. A375 cells were transfected with pCMV-HA-p47, and 24 h post-transfection, a confocal immunofluorescence microscopy assay was performed using P1C antibody and anti-HA antibody. The subcellular location of CDK11p110 (green) and eIF3 p47 (red) and their co-localization (yellow) are shown (magnification, ×100). B, localization of CDK11p110 and eIF3-p47 by cell fractionation and Western blot. 20 μg of fractionated A375 cell lysates were used for each lane and CDK11p110-specific antibody GN1 or anti-eIF3 antibody was used for immunoblotting. C, the interaction between transfected CDK11p46 and eIF3 p47 in human cells. HEK293 cells were cotransfected with pCMV-Myc-CDK11p46 and pCMV-HA-p47. 48 h post-transfection, cells were harvested, and cell extracts were made. Immunoprecipitation was performed with either mouse IgG or anti-Myc antibody followed by Western blotting with anti-Myc or anti-HA antibody.

Fig. 3. The interaction between endogenous CDK11p46 and eIF3 p47 during apoptosis. A375 cells were treated with 0.5 μg/ml anti-Fas antibody to stimulate apoptosis for 0, 24, 36, and 48 h, and then the cells were lysed and used for immunoprecipitation. CDK11p46 overexpressed A375 cells were used in lanes 5 and 11. In lanes 1–4, immunoprecipitation was performed with goat anti-GST IgG, whereas in lanes 5–9, immunoprecipitation was performed with goat anti-eIF3 antibody followed by immunoblotting with CDK11p110-specific antibody, GN1. 20 μg of cell lysates were used in lanes 10 and 11 for Western blot.

CDK11p46 is between amino acids 113 and 248, which contains the Mov34/JAB_MPN domain. Therefore, the interaction of the caspase-processed isoform of CDK11 (CDK11p46) with eIF3 p47 may be mediated through the Mov34 domain, and the major region of interaction appears to be the C-proximal half of the Mov34 domain.

To further demonstrate the interaction in human cells, an expression vector with an HA epitope-tagged full-length eIF3 p47 was constructed and transfected into A375 cells. Immuno-fluorescence confocal microscopy using an anti-HA antibody revealed that p47 localized both in nucleoplasm and cytoplasm, especially the region around nucleus (Fig. 2A). CDK11p110 localizes predominantly to the nucleoplasm as revealed by P1C antibody. No fluorescence was seen with the HA antibody in untransfected cells (data not shown). Merging of the two images showed a yellow color in the nucleoplasm (Fig. 2A), indicating that a portion of eIF3 p47 co-localizes in the nucleus with CDK11. To confirm this observation, we performed cell fractionation and Western blot analysis. Again, we observed both a cytoplasmic and nuclear pool of eIF3 p47, whereas CDK11 was seen in the nucleus (Fig. 2B). Thus, CDK11 and a portion of eIF3 p47 can both be found in the nucleus. In addition, reciprocal immunoprecipitation and Western analysis with Myc-tagged CDK11p46 and HA-tagged eIF3 p47 from transfected HEK293 cells showed that CDK11p46 and eIF3 p47 could associate in cells (Fig. 2C). These results together with

Fig. 4. Time course study of apoptosis induced by anti-Fas antibody or staurosporine. A, A375 cells were treated with 0.5 μg/ml of anti-Fas antibody for 0, 24, 36, 48, and 72 h and analyzed for apoptosis by 7-AAD staining followed by flow cytometry. The y axis indicates the percentage of total apoptotic cells. B, A375 cells were treated with 10 ng/ml staurosporine for 0, 24, 48, and 72 h, and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *, p < 0.05.
the following endogenous interaction data suggest that CDK11ph46 interacts with eIF3 p47 in human cells.

**Endogenous eIF3 p47 Interacts with CDK11 during Apoptosis**—Next we tested whether p47 is a potential substrate of CDK11 during apoptosis. During apoptosis, the p110 isoform of CDK11 is cleaved by caspase-3 into a p46–50 C-terminal fragment containing the kinase domain and a p60 N-terminal fragment (2). We saw a weak interaction between the endogenous CDK11p110 isoform and eIF3 in untreated A375 cells (Fig. 3, lane 6). Interestingly, when apoptosis is induced by anti-Fas, the caspase-processed C-terminal fragment of CDK11 p110 interacted strongly with eIF3. The interaction was strongest at 36 h after stimulation of apoptosis (Fig. 3, lanes 7–9). The specificity of the interaction was confirmed by including non-specific goat IgG as negative controls (Fig. 3, lanes 1–4), and the correct sizes of the expected protein bands are shown in lanes 5, 10, and 11 (Fig. 3). In CDK11ph46 stable transfected cells, the interaction between eIF3 and CDK11ph46 (p46-p50) was strong (Fig. 3, lane 5), which means that the overexpressed CDK11ph46 associates with eIF3, presumably through its p47 subunit. In order to prove that the cells did undergo apoptosis after anti-Fas or staurosporine treatment during the time course, the percentage of apoptotic cells was measured by flow cytometry (Fig. 4A), and the cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 4B). In addition, apoptosis was confirmed by light microscopy analysis (data not shown). The results indicated that the cells underwent apoptosis gradually after stimulation and the percentage of apoptotic cells approached 100% by 72 h after treatment (Fig. 4A). These observations are consistent with the previous findings by our group (2).

**CDK11ph46 Phosphorylates eIF3 p47 during Apoptosis in Vitro**—Since CDK11ph46 contains the kinase domain of CDK11 and it has been shown by our group and others that the kinase activity of CDK11 is stimulated during apoptosis (1, 2), we hypothesized that CDK11ph46 may phosphorylate eIF3 p47 during apoptosis. CDK11 was immunoprecipitated from anti-Fas-treated A375 cell lysates, and GST control or GST recombinant p47 protein were used as substrates in kinase assays. Immunoprecipitated CDK11 from A375 cells undergoing apoptosis was capable of phosphorylating recombinant eIF3 p47 protein (Fig. 5A). In contrast, CDK11ph46 did not phosphorylate GST alone. Furthermore, the addition of a caspase-3 inhibitor (DEVD-FMK) diminished the degree of eIF3 p47 phosphorylation (Fig. 5B, lane 3), and A375 cells stably transfected with kinase mutant CDK11ph46m were used. Cells were then lysed for the kinase assay as described for A. 5 μg of GST-p47 were used as substrate.

**Identification of the Phosphorylation Site in eIF3 p47 and the in Vivo Phosphorylation Status of p47 during Apoptosis**—To determine the phosphorylation site in the eIF3 p47 protein phosphorylated by CDK11, recombinant eIF3 p47 was incubated with caspase-processed CDK11 that immunoprecipitated...
from anti-Fas-treated cell lysate in kinase buffer. After the kinase reaction, recombinant eIF3 p47 protein was separated by SDS-PAGE. EIF3 p47 was digested with pepsin in gel and subjected to liquid chromatography/MS/MS analysis. The spectrum for the p47 phosphopeptide (residues Ala-41 to Pro-49), [M-H3PO4]+ = 784 m/z is shown in Fig. 6A. Identification of a phosphorylated Ser46 is seen by analysis of b ions and y ions. A gain of phosphate at the y4 ion, 485 m/z, derived from fragmentation between Ser-45 and Ser-46 was observed. The reason that y ions are not completely shown is that multiple internal cleavages instead of sequential cleavages from the carboxyl to amino terminus occurred. Once we found that Ser-46 within...
eIF3 p47 was phosphorylated by CDK11 in vitro, we investigated the serine phosphorylation status of eIF3 p47 in cells undergoing apoptosis in vivo. A375 cells were treated with staurosporine to trigger apoptosis, and cell lysates were immunoprecipitated with eIF3-specific antibody followed by Western analysis with phosphoserine-specific antibody or eIF3 p47-specific antibody. In A375 cells stimulated to undergo apoptosis, serine phosphorylation of eIF3 p47 was seen, and the maximum phosphorylation level was seen at 36 h after treatment (Fig. 6B). These results are consistent with our in vitro kinase assay and show that serine phosphorylation of eIF3 p47 occurs during apoptosis in vivo.

CDK11p46, but not the Kinase Mutant CDK11p46M, Inhibits in Vitro Translation—We further investigated the functional consequences of the association of caspase-processed CDK11p110 with eIF3 p47. We determined the effect of CDK11p46 on protein synthesis in vitro. A rabbit reticulocyte in vitro translation system was used for this purpose. In this system, a given mRNA is translated into protein. For our studies, we choose 40 ng of luciferase mRNA and incubated for 60 min in vitro, and luciferase activity was measured. The incubation time or ng of luciferase mRNA was plotted against luciferase activity as a measurement of protein production. First, we could use the luciferase gene product for two reasons. First, we could monitor the luciferase activity as a measurement of protein synthesis. For our studies, we established that our assay conditions were within the linear range (Fig. 7A) ($R^2 = 0.9906$, $p < 0.01$). Next, we investigated whether the dose response of luciferase mRNA to translation in vitro is linear. We found that adding luciferase mRNA between 20 and 50 ng is within the linear range (Fig. 7A) ($R^2 = 0.9791$, $p < 0.01$). Therefore, we chose 40 ng of luciferase mRNA and incubated for 60 min in subsequent experiments. Luciferase mRNA was translated in the presence or absence of added recombinant CDK11p46 or CDK11p46M protein. Both luciferase activity and incorporated [35S]methionine were measured. CDK11p46 significantly inhibited the synthesis of luciferase (Fig. 7B). The specificity of this observation was verified by adding GST, another purified Escherichia coli expressed protein, to the reaction, and it had no effect on translation compared to the buffer (Fig. 7B, top panel). This observation was further confirmed by performing a dose-response assay with recombinant CDK11p46 protein. A concentration-dependent inhibitory effect of CDK11p46 protein on translation was observed (Fig. 7B). Moreover, the phosphorylation of eIF3 p47 by CDK11p46 appears to be required for the inhibition of translation, since the kinase-defective mutant CDK11p46M did not inhibit the synthesis of luciferase (Fig. 7B).

CDK11p46, but Not Kinase Mutant CDK11p46M, Inhibits Translation in Vivo—To examine the ability of CDK11p46 to inhibit translation in human cells, a luciferase reporter plasmid was transiently transfected into A375 cells stably transfected with either pcDNA3, CDK11p46, or kinase-defective mutant CDK11p46M. Luciferase activity was then measured 24 h after transfection. Luciferase synthesis was significantly inhibited in A375 cells that overexpress CDK11p46 compared with the vector-transfected cells (Fig. 8A, left panel). In contrast, luciferase activity was slightly elevated in the CDK11 kinase-
defective mutant CDK11<sup>p46M</sup>-transfected cells relative to vector-transfected cells (Fig. 8A, right panel). To rule out the effect of CDK11<sup>p46</sup> on transcription of the luciferase gene, RT-PCR was performed on three different transfected clones from either vector alone- or luciferase reporter gene-transfected cells. The histone gene served as a control (246 bp). The luciferase mRNA of vector- or CDK11<sup>p46</sup>-transfected cells was the same (Fig. 8B, 454 bp), indicating that CDK11<sup>p46</sup> inhibited the translation of the luciferase reporter gene and not transcription. To further quantify the luciferase mRNA, real time PCR was performed from pcDNA3 vector- or CDK11<sup>p46</sup>-transfected cells. The Ct of luciferase was normalized against GAPDH. No difference was observed between vector- and CDK11<sup>p46</sup>-transfected A375 cells (Fig. 8C). Untransfected A375 cells and no template control served as negative controls (Fig. 8C). These results suggest that the caspase-processed CDK11<sup>p110</sup> isoform inhibits protein synthesis <em>in vivo</em> at the translational level.

**CDK11<sup>p46</sup> Suppresses Overall Protein Synthesis**—To further investigate the impact of CDK11<sup>p46</sup> on overall cellular protein synthesis, we established two stably transfected cell lines. One was stably transfected with pcDNA3 vector, and the other was stably transfected with CDK11<sup>p46</sup>. Overall protein synthesis, as measured by pulse labeling with <sup>[3H]</sup>leucine <em>in vivo</em>, was significantly lower in CDK11<sup>p46</sup>-overexpressing cells compared with vector-transfected cells (Fig. 9A). Therefore, CDK11<sup>p46</sup> can also inhibit the overall rate of cellular protein synthesis. Since it was reported that the rate of protein synthesis is rapidly down-regulated in mammalian cells following the induction of apoptosis (21–24), we also measured protein synthesis in A375 cells after staurosporine treatment. Consistent with these findings, the overall cellular protein synthesis decreased during apoptosis (Fig. 9B).

**Overexpressed CDK11<sup>p46</sup> Causes Higher Apoptosis Rate**—We next addressed whether overexpression of CDK11<sup>p46</sup> increases apoptosis. Vector control or CDK11<sup>p46</sup>-overexpressing A375 cells were stained with 7-AAD and subjected to flow cytometry. Results showed increased apoptosis in cells stably transfected with CDK11<sup>p46</sup> compared with cells transfected with vector only (Fig. 10A). This observation was confirmed by cell morphological analysis using light microscopy and Giemsa staining, which showed that CDK11<sup>p46</sup>-overexpressing cells had more apoptotic cells than vector only-transfected cells (Fig. 10B). These results demonstrate that CDK11<sup>p46</sup> promotes apoptosis.

**DISCUSSION**

CDK11 appears to be downstream of caspase-3 in apoptotic signaling (2). Upon apoptotic stimulation, CDK11 is cleaved by caspases and activated (1, 13, 14). However, the potential substrates of CDK11 that may be involved in cell death are not known. In the present study, we identify that eIF3 p47 interacts with the caspase-processed CDK11 isoform using a yeast two-hybrid screening strategy. The eIF3 p47 is a subunit of the mammalian eIF3 multipro-
In this study, we show that endogenous CDK11p46 and eIF3 p47 protein can interact and that stimulation of apoptosis enhances this interaction. We also show that the kinase activity of CDK11 is activated during apoptosis, that the kinase obtained can phosphorylate eIF3 p47 apparently at serine 46, and that the phosphorylation of eIF3 p47 by CDK11 can be blocked by caspase-3 inhibitor (DEVD-FMK). In addition, mutation of the phosphate transfer site in CDK11p46 abrogates the phosphorylation of eIF3 p47 protein. Finally, we show that serine phosphorylation of eIF3 p47 occurs in cells undergoing apoptosis, with maximal phosphorylation occurring at 36 h both in vivo and in vitro. Although not definitive, these observations strongly suggest that eIF3 p47 may be a substrate of the caspase-processed CDK11p110 isoform during apoptosis. We noticed that the protein level of eIF3 p47 also increased during apoptosis with maximal expression at 36 h after stimulation. The biological mechanism of this phenomenon is under investigation.

When cells are committed to apoptosis, a remarkable inhibition of the rate of overall protein synthesis is observed in a variety of cell types (24, 34, 35). This down-regulation of protein synthesis may either protect cells against noxious agents and ensure the conservation of resources needed for survival or activate apoptosis (36). This inhibition of protein synthesis occurs at the level of polypeptide chain initiation and is accompanied by the phosphorylation of the α subunit of eIF2 and the caspase-dependent cleavage of eIF4GI, eIF4GI, eIF4G, p35 subunit of eIF3, minor proportions of the α subunit of eIF2, and the eIF4E-binding protein 4E-BP1 (21, 37). The rate of protein synthesis is regulated by the phosphorylation status of several eIFs. eIF4E phosphorylation was reported to strongly decrease following anti-CD3 and anti-CD4 stimulation in immature DP thymocytes, which resulted in rapid decreases in protein synthesis and apoptosis (38). Phosphorylation of eIF2α by double-stranded RNA-dependent serine/threonine protein kinase was also correlated with down-regulation of translation initiation and apoptosis (21, 36, 39, 40). The data from our study suggest that phosphorylation of eIF3 p47 may also contribute to the alteration of protein synthesis during apoptosis. The biological consequences of the phosphorylation of eIF3 p47 during apoptosis are under investigation.
In A375 cells stably expressing either the caspase-processed carboxyl-terminal domain of CDK11p46 or a phosphate transfer site mutant CDK11p44-46M, we show that luciferase activity and the production of luciferase protein are compromised in CDK11p46 but not in CDK11p44-46M cells. In addition, overexpressed CDK11p46 from stably transfected cells are capable of interacting with eIF3 in vivo. These data suggest that the function of the caspase-processed CDK11p110 isoform may be to inhibit translation during apoptosis. However, whether or not this inhibition of protein translation occurs in an eIF3 p47-dependent or -independent manner remains to be clarified.

In summary, the CDK11 protein kinase may be involved in apoptotic signaling. This study demonstrates that the caspase-dependent or -independent manner remains to be clarified.

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