Death-signal-induced relocalization of cyclin-dependent kinase 11 to mitochondria

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INTRODUCTION

Apoptosis is a genetically controlled event that plays an important role in the regulation of tumour progression and metastasis [1]. It is becoming increasingly clear that cell cycle regulators, for example, the p34cdc2 family members, can influence apoptosis [2–6]. CDK11 (cyclin-dependent kinase 11; formerly known as PITSRE) binds to cyclin L, and is a member of the extended family of p34cdc2-related kinases [7]. CDK11 appears to regulate different cellular functions, including RNA splicing, transcription, cell cycle and apoptosis [8–13].

Previous studies have shown that ectopic expression of certain CDK11 isoforms reduces cell growth via apoptosis among several cell lines [14–17]. CDK11 transcription and translation is substantially induced during apoptosis of human T-cell lines after treatment with anti-Fas antibody [17]. Previous studies by our group have shown an association of decreased expression of CDK11 proteins in melanoma cell lines and surgical malignant melanoma specimens [18]. In addition, studies have indicated that proteolytic processing of CDK11 might serve as an effecter in apoptotic signalling pathways [15,19,20]. During apoptosis induced by Fas, tumour necrosis factor or staurosporine, CDK11p110 isoforms are cleaved by caspase-3 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 [15,19,20]. With respect to the CDK11p100 fragment, our group and others have demonstrated an association with at least four other proteins [eIF3f (eukaryotic initiation factor 3f), PAK (p21-activated kinase 1), RanBPM and Hsp90 (heat-shock protein 90)] during apoptosis [16,21–23]. Overexpression of CDK11p100 inhibits protein synthesis and induces apoptosis [16]. These data suggest that CDK11p100 might play a role in mediating apoptosis. However, the function of CDK11p110 has not been fully explored.

In the present study, we evaluate the pro-apoptotic effects of the CDK11p110 fragment. We show that, during apoptosis, CDK11p110 redistributes from the nucleus to the mitochondria. Ectopic expression of CDK11p110 can partially disrupt the mitochondrial membrane potential, induce cytochrome c release and apoptosis. Taken together with the effect of CDK11p100 on apoptosis, these data suggest that CDK11 proteins are downstream effectors in apoptotic signalling pathways, acting at two different levels within a cell.

MATERIALS AND METHODS

Cell culture and treatment

Human melanoma cell lines were obtained from A.T.C.C. (Manassas, VA, U.S.A.). A375 cells were grown as monolayers in RPMI 1640 (Mediatech, Herndon, VA, U.S.A.) supplemented with 5% (v/v) fetal bovine serum (Omega Scientific, Tarzana, CA, U.S.A.), 1% L-glutamine and 1% penicillin/streptomycin. A375 cells (1 × 10⁶) were seeded in 10 cm plates and incubated overnight at 37°C in a humidified 5% CO₂ environment and treated with 0.5 µg/ml anti-Fas monoclonal antibody CH-11 (Upstate Biotechnology, Lake Placid, NY, U.S.A.). After 3, 6, 12, 24, 48 and 72 h at 37°C, control and treated cells were harvested by low-speed centrifugation, washed twice with PBS and lysed as described previously [15].

siRNA (small interfering RNA)

Blocking of CDK11 gene expression was performed by using a BLOCK-iTTM Dicer RNAi Transfection Kit (Invitrogen, Carlsbad, CA, U.S.A.). The siRNA sequence targeting CDK11p110 was obtained by PCR using forward primer 5’-TCAATGC-ATGAAAAGG-3′ and reverse primer 5’-ATGCGCTCCTCA-GAGTTGC-3’. Briefly, A375 cells were seeded in six-well plates

Abbreviations used: 7-AAD, 7-aminoactinomycin; CDK11, cyclin-dependent kinase 11; DAPI, 4,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; eIF3f, eukaryotic initiation factor 3f; Hsp60/90, heat-shock proteins 60 and 90 respectively; mt-Hsp70, mitochondrial Hsp70; PARP, poly(ADP-ribose) polymerase; RT-PCR, reverse transcriptase-PCR; siRNA, small interfering RNA; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

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at a density of 4 × 10⁵ cells/well and grown in antibiotic-free medium. After 24 h, cells were transfected with 0.4 µg of siRNA and 5 µl of Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h, cells were harvested and analysed by Western blot analysis and immunofluorescent microscopy.

**Plasmid construction and transient transfection**

For constructing EGFP (enhanced green fluorescent protein)-tagged CDK11p60, RT-PCR (reverse transcriptase-PCR) was performed to obtain CDK11p60 fragment using N-terminal primer containing an EcoRI site (5′-GAATTCCTCTTCTGGCTCGGGACCG-3′) and a C-terminal primer containing a BamHI site (5′-CCGGATCCTGAGCTCGATGG-3′). The PCR product was then cloned in-frame with pEGFP-N1 vector (BD Biosciences/Clontech, Palo Alto, CA, U.S.A.) to generate EGFP–p60. Transient transfections of A375 cells were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, A375 cells were seeded in six-well plates at a density of 1 × 10⁶ cells/well. After 24 h, cells were transfected with complexes containing 10 µl of LF2000 and 4 µg of EGFP-tagged CDK11p60 or Myc-tagged CDK11p60. For preparing samples for immunofluorescent microscopy images, 12-mm coverslips were seeded with 1 × 10⁶ cells/well in six-well plates.

**Subcellular fractionation**

Subcellular fractionation was performed with Proteo-Extract™ Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer’s instructions. This kit yields the total proteome fractionated into four sub-proteomes. With Extraction Buffer I, cytosolic proteins are released. Subsequently, membranes and membrane organelles are solubilized with Extraction Buffer II, without impairing the integrity of nucleus and cytoskeleton. Next, nuclear proteins are released. Subsequently, membranes and membrane organelles are solubilized with Extraction Buffer II, without impairing the integrity of nucleus and cytoskeleton. Finally, nuclear proteins are released. Subsequently, membranes and membrane organelles are solubilized with Extraction Buffer II, without impairing the integrity of nucleus and cytoskeleton. Extracts were taken in in-frame with pEGFP-N1 vector (BD Biosciences/Clontech, Palo Alto, CA, U.S.A.) to generate EGFP–p60. Transient transfections of A375 cells were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, A375 cells were seeded in six-well plates at a density of 1 × 10⁶ cells/well. After 24 h, cells were transfected with complexes containing 10 µl of LF2000 and 4 µg of EGFP-tagged CDK11p60 or Myc-tagged CDK11p60. For preparing samples for immunofluorescent microscopy images, 12-mm coverslips were seeded with 1 × 10⁶ cells/well in six-well plates.

**Immunoprecipitation and Western blot analysis**

A375 cells treated with anti-Fas antibody or transfected with desired constructs were harvested, washed twice with cold PBS, and lysed in lysis buffer (50 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/10 mM NaF/0.1% Nonidet P40) containing 1 mM sodium orthovanadate, 1 mM PMSF and 1% protease inhibitor cocktail (Sigma) for 30 min on ice. Following lysis, cells were centrifuged at 13000 g for 15 min at 4°C. The protein content was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) with BSA as the standard. m-Hsp70 (mitochondrial heat-shock protein 70) or CDK11 proteins were immunoprecipitated using mouse monoclonal anti-mt-Hsp70 and CDK11 GN1 affinity-purified polyclonal antisera respectively, and 20 µl of Protein G–agarose beads (Oncogene, La Jolla, CA, U.S.A.). The immunocomplexes were then washed three times with lysis buffer and separated by SDS/PAGE (10% gels). For Western blot analysis, cytosolic, mitochondrial, nucleic protein or total cell lysates were separated by SDS/PAGE gels and transferred on to PVDF membranes (Bio-Rad). The following antibodies were used for Western blot analyses: rabbit polyclonal anti-cytochrome c antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), mouse monoclonal mitochondrial Hsp60 (heatshock protein 60; 1:1000 dilution; BD Biosciences–Pharmlingen, San Diego, CA, U.S.A.), rabbit polyclonal PARP [poly(ADP-ribose) polymerase; 1:1000 dilution, Upstate Biotechnology], mouse monoclonal m-Hsp70 (Affinity BioReagents, Golden, CO, U.S.A.) and monoclonal mouse anti-tubulin (1:1000; Oncogene). Anti-CDK11 antibody CDK11 GN1 (1:2000) and P2N100 (1:1000) affinity-purified polyclonal antisera were also used as described previously [15,18]. The membranes were incubated with primary and secondary antibodies (Sigma) and finally developed with enhanced chemiluminescence (Amersham Biosciences). Membranes probed with more than one antibody were stripped before re-probing.

**Immunofluorescent microscopy**

A375 melanoma cells were grown on 12-mm coverslips and transfected with desired plasmids or treated with anti-Fas antibody. For treatment or transfection for the desired time, coverslips were washed three times with PBS and incubated with MitoTracker Red CMXRos (250 nM, Molecular Probes) for 30 min at 37°C. Coverslips were then washed twice with PBS, fixed in 4% (w/v) paraformaldehyde for 20 min at room temperature and permeabilized with 100% methanol at −20°C for 6 min. Immunostaining was performed using the following antibodies: polyclonal rabbit GN1 (1:10) followed by anti-rabbit IgG conjugated with FITC (1:100; Jackson ImmunoResearch, West Grove, PA, U.S.A.), monoclonal mouse anti-cytochrome c (1:100; BD-Pharmingen) followed by anti-mouse IgG conjugated with Cy5 (1:100; Jackson ImmunoResearch). Coverslips were mounted with one drop of Prolong Gold anti-fade reagent with DAPI (4,6-diamidino-2-phenylindole; Molecular Probes). Confocal and fluorescent images were collected and analysed with Nikon PCM 2000 and Nikon VFM microscopes.

**Caspase-3 activation assay**

Caspase-3 activation assays were performed using a Caspase-Glo™3/7 assay kit (Promega, Madison, WI, U.S.A.) according to the manufacturer’s instructions. Briefly, A375 cells were seeded in 96-well plates at a density of 1 × 10⁴ cells/well. After 24 h, cells were treated with 0.5 µg/ml anti-Fas antibody. Caspase-Glo 3/7 reagent (100 µl) was then added to each well including medium alone, untreated control cells or cells treated with anti-Fas antibody for 3, 6, 12, 24, 48, 60 and 72 h. The plate was then incubated at room temperature for 1 h and the luminescence of each sample was measured with a Sirius Luminometer (Berthold Detection System).

**Cytosensorometric determination of Δψ<sub>m</sub> (mitochondrial transmembrane potential)**

The lipophilic cationic dye, MitoTracker Red (CMXRos), which accumulates in intact mitochondria, was used along with flow cytometry analysis to detect changes in Δψ<sub>m</sub>. A375 cells were transfected with EGFP or EGFP–p60 constructs for 24 h. Approx. 0.5 × 10⁶ cells were resuspended in 1 ml pre-warmed growth medium containing 100 nM CMXRos and incubated for 30 min at 37°C. Following centrifugation at 500 g for 5 min, the pellet was resuspended in 0.5 ml of PBS and kept on ice. The cells were then analysed by flow cytometry (FACScan, Becton Dickinson).
Apoptosis detection

Apoptosis assays of Fas-treated A375 cells were determined over time by the TACS™ Annexin V–FITC apoptosis detection kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer’s instructions. Adherent and floating cells were collected by centrifugation at 500 g for 5 min at room temperature and washed with cold PBS. Cells were then gently resuspended in the Annexin V Incubation Reagent at a concentration of 1×10^6 cells/100 µl and incubated in the dark for 15 min at 24°C. Samples were analysed by flow cytometry immediately after the addition of 400 µl of 1× binding buffer. The x-axis (FL1) of the dot plot reflects the logarithm of Annexin V–FITC fluorescence and the y-axis (FL2) reflects the propidium iodide fluorescence.

For analysis of nuclear morphological changes in EGFP–p60-transfected cells, A375 cells grown on coverslips and transfected with EGFP or EGFP–p60 were washed with PBS and fixed with 4% (w/v) paraformaldehyde. Coverslips were then mounted with Prolong Gold anti-fade reagent with DAPI (Molecular Probes) to visualize the nuclei. Apoptosis was characterized by scoring condensed and fragmented nuclei. Each set of experiments was repeated at least three times, with at least 200 EGFP-positive cells counted in each instance.

For analysis of nuclear morphological change in CDK11 siRNA-transfected cells, A375 cells were trypsinized and stained with the dye mix containing 100 µg/ml each of Acridine Orange and ethidium bromide. Apoptosis was characterized by scoring condensed and fragmented nuclei. Each set of experiments was repeated at least three times, with at least 300 cells counted in each instance.

Statistical analysis

The data were presented as means ± S.D. Statistical significance was determined using the Student’s t test.

RESULTS

Fas induces apoptosis in A375 melanoma cells

We investigated the Fas-induced apoptosis in melanoma A375 cells by incubating A375 cells with 0.5 µg/ml anti-Fas antibody. Treatment of A375 cells with Fas could induce apoptosis after 24 h as observed by DAPI staining (Figure 1A). To delineate the time course and occurrence of early and late apoptosis, we used Annexin V/propidium iodide staining followed by flow cytometry analysis. We measured both early and late stage apoptosis from four different experiments. In A375 cells, activation of the Fas signalling pathway led to a rapid increase in early apoptotic cells at 12 h and throughout the entire time course (Figures 1B and 1C). There was a marked increase in the number of cells entering into late apoptosis after 24 h of treatment (Figures 1B and 1C). The maximum apoptosis was observed at 60 h after Fas treatment. Similar results were seen using 7-AAD (7-aminoactinomycin D) staining followed by FACS analysis (results not shown).

Knockdown of CDK11p110 by siRNA partially attenuates Fas-induced apoptosis in A375 cells

We next examined the role of endogenous CDK11p110 on Fas-induced apoptosis in A375 cells. We used BLOCK-it Dicer RNAi Kit to perform our siRNA experiments. The kit can enzymatically generate a pool of 21–23 nt d-siRNA (diced siRNA duplexes) that covers a larger portion of the target CDK11 gene. The pool of diced siRNA duplexes was transfected into A375 cells. In addition, we generated LacZ siRNA to use as the negative control for non-specific, off-target effects in A375 cells. Endogenous CDK11p110 protein levels were efficiently and specifically reduced 48 h after transfection, as determined by Western blot analysis (Figure 2A) and immunofluorescence microscopy (results not shown). To examine the effect of CDK11p110 on Fas-induced apoptosis, we incubated control and CDK11 knockdown A375 cells with anti-Fas antibody. After 24 h, apoptosis was measured and quantified. As shown in Figure 2(B), cells with reduced CDK11 expression were partially protected from the effects of Fas treatment, suggesting that CDK11 plays a role in apoptosis induced by Fas. Similar results were observed using immunofluorescent microscopy. Control cells with normal levels of CDK11 underwent apoptosis, displaying typical morphological changes such as diffuse cytochrome c staining in the cytoplasm, and nuclear condensation after Fas treatment. However, CDK11 knockdown cells did not show any apoptotic nuclear morphology.

The cleavage of CDK11p110 occurs early following Fas treatment, and the N-terminal CDK11p60 fragment is targeted to mitochondria

We previously demonstrated that the CDK11p110 isoform was cleaved by caspases into p60 and p46 fragments at 24 h, and its kinase activity stimulated in response to Fas or staurosporine prior to maximum levels of apoptosis [15]. However, the exact time course and the subcellular localization of p60 fragment generated by caspase cleavage of CDK11p110 following Fas treatment are not known. Typically, full-length CDK11p110 is located in the nucleus of tumour cell lines, including A375 cells [15,16]. To assess the location of CDK11p60 fragment upon Fas treatment, whole cell lysates and subcellular fractions of A375 control and Fas-treated cells were isolated and examined. The cleaved CDK11p60 fragment was detected as early as 6 h after Fas treatment, and increased with time (Figure 3A). The cleavage was partially attenuated by a specific caspase-3 inhibitor (results not shown). Analysis of subcellular fractions from control and Fas-treated cells by Western blotting revealed that the CDK11p60 fragment translocated from the nucleus to mitochondria at 12 h, and the amount of CDK11p60 fragment in the mitochondrial fraction increased at 24 h (Figure 3B). Equal loading of mitochondrial fractions was confirmed by assessment of the mitochondrial Hsp60 protein levels (Figure 3B). Absence of tubulin indicated that the mitochondrial fractions were not contaminated with cytosolic proteins (Figure 3B). Analysis of the mitochondrial fractions with antibody against the nuclear PARP protein indicated further that the mitochondria were not contaminated with nuclear proteins (Figure 3B). Immunofluorescent microscopy analysis of Fas-treated A375 cells corroborated the fractionation studies. Under normal physiological conditions, CDK11p110 is found in the nucleus. We have also shown that the caspase-processed CDK11p60 isoform can be found in the nucleus [16], whereas the N-terminal portion generated by caspase cleavage of CDK11p110 (i.e. CDK11p60) is in the nucleus, and translocates through the cytoplasm to the mitochondria. Collectively, these results indicate that the two CDK11p60 fragments, generated by caspase cleavage upon treatment with Fas, have different subcellular locations.

Based on the fact that: (1) CDK11p110 is a substrate of caspase-3, and the cleavage occurs at 6 h after treatment (Figure 3A), and (2) CDK11p60 translocates to mitochondria at 12 h (Figure 3B), we then wanted to investigate the time courses of caspase-3 activity and cytochrome c release during Fas-induced apoptosis. As shown in Figure 3(C), Fas treatment induced a time-dependent activation of caspase-3 in A375 cells. The caspase-3 activity remained the
Figure 1  Time course of apoptosis induced by anti-Fas antibody in A375 cells

(A) Induction of apoptosis by anti-Fas antibody in A375 cells. Cells were treated with 0.5 µg/ml anti-Fas antibody for 24 h. Nuclei were stained with DAPI and the morphological changes associated with apoptosis were examined using fluorescence microscopy. (B) Time course of apoptosis induced by anti-Fas antibody in A375 cells. A375 cells were treated with 0.5 µg/ml anti-Fas antibody for the indicated times and analysed for apoptosis by Annexin V–FITC and propidium iodide. The percentages of the viable, early apoptotic (Annexin V–FITC-positive), and late apoptotic/necrotic cells are listed to the right of the histograms. (C) Quantification of early and late apoptotic cells in control and treated cells, as shown in (B).

same 1 h after treatment, but started to increase steadily up to 6 h. A second significant increase was observed after 12 h with a maximal activation at 60 h. These data are consistent with apoptosis time-course experiments (Figure 1C) and the cleavage of CDK11<sup>p110</sup> during Fas-induced apoptosis (Figure 3A and [15]). In addition, the increase in the caspase-3 activity and the translocation of CDK11<sup>p60</sup> fragment into mitochondria over time were associated with cytochrome c release (Figure 3D).

Ectopically expressed CDK11<sup>p60</sup> targets to mitochondria and leads to increased apoptosis in A375 cells

The role of mitochondrial targeting by CDK11<sup>p60</sup> in modulating apoptosis was investigated further by overexpression of CDK11<sup>p60</sup>. We engineered an expression vector by fusing CDK11<sup>p60</sup> with an EGFP epitope tag at the C-terminus. We first overexpressed this EGFP–p60 in A375 cells and assessed
Overexpression of CDK11p60 partially breaks down Δψm

Our data suggest that overexpressed CDK11p60 targets to mitochondria and leads to increased apoptosis in A375 cells. We next asked whether overexpressed CDK11p60 could perturb mitochondrial function by disrupting Δψm (i.e. the mitochondrial permeability transition). The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical gradient (Δψm) across the mitochondrial membrane collapses. To investigate changes in Δψm, we transiently transfected A375 cells with EGFP or EGFP–p60 construct and stained the cells with the Δψm-sensitive fluorescent probe MitoTracker Red (CMXRos). The lipophilic cation CMXRos accumulates in the intact mitochondrial matrix as a consequence of Δψm. While in apoptotic cells, Δψm collapses, and CMXRos cannot accumulate within the mitochondria. A375 cells were transfected with EGFP or EGFP–p60 for 24 h. The cells were stained with 100 nM MitoTracker Red (CMXRos) and analysed by flow cytometry. The mean red fluorescence intensity was calculated from EGFP-positive cells only. Indeed, compared with cells transfected with EGFP empty vector, the mean red fluorescence intensity of EGFP–p60-transfected cells was reduced (Figure 6), indicating that EGFP–p60 induces a disruption of the Δψm. The decrease of fluorescence intensity was approx. 45% arbitrary units for positive control cells (i.e. A375 cells treated with Fas for 24 h with approx. 15% of the cells undergoing apoptosis), indicating that the fluorescence intensity changes for EGFP–p60-transfected cells are significant.

Mitochondrial-import protein mt-Hsp70 is a potential candidate for translocation of CDK11p60 to mitochondria

Translocation of nuclear-encoded preproteins into mitochondria requires the co-ordinated action of two translocases: one [TOM (translocase of the outer membrane)] is located in the outer mitochondrial membrane, and the other [TIM (translocase of the inner membrane)] is located in the inner mitochondrial membrane [25,26]. To investigate a potential mechanism that might explain the mitochondrial location of the CDK11p60, we examined the possibility of a physical interaction between the TIM component protein mt-Hsp70 and the CDK11 p60 fragment. mt-Hsp70 is the major translocation motor that chaperones proteins into mitochondria and refolds them before they are sorted into their final compartments within the membranes or the matrix. In order to determine the existence of a potential interaction between mt-Hsp70 and CDK11p60, immunoprecipitation assays were performed. We observed an interaction of cleaved CDK11p60 with mt-Hsp70 in vivo from cells undergoing Fas-induced apoptosis (Figure 7A). We confirmed the association by conducting CDK11p60 transfection studies. Again, we saw an interaction between the two proteins (Figure 7B). These data provide direct evidence of a specific in vivo complex between CDK11p60 and mt-Hsp70.

DISCUSSION

The tumour-necrosis-factor member Fas has a central role in the physiological regulation of apoptosis, and has been implicated in the pathogenesis of various malignancies [28]. Previous studies by our group have demonstrated that CDK11p110 protein kinases can be cleaved by caspase-3 during Fas-induced apoptosis, and inhibitors of caspase-3 and caspase-8 can block Fas-induced apoptosis in melanoma A375 cells [15]. In the present study, we show further that CDK11 is implicated in apoptosis. Silencing of CDK11 by siRNA resulted in an approx. 80% reduction in CDK11 protein (Figure 2A). However, only a partial protection...
from Fas-induced apoptosis in A375 cells transiently transfected with CDK11 siRNA oligomers was observed (Figure 2B). We interpret these data to suggest that abrogation of CDK11 may be necessary but not sufficient to protect cells from apoptosis. It is also possible that a sufficient amount of CDK11 is still present in the cells and participates in apoptosis, since siRNA leads to short-term silencing of a specific RNA population and transient transfection occurs in only a portion of the cells. Further studies with shRNA (short hairpin RNA) constructs will help to clarify this issue. In addition, studies by our group have showed that geldanamycin-triggered degradation of CDK11 slows down the progression of apoptosis, which also supports the pro-apoptotic function of CDK11 [23].

Previous studies have shown that following Fas-induced apoptosis, CDK11\(^{p110}\) is cleaved by caspase-3 to generate an N-terminal portion of p60 fragment (which contains several regulator motifs), and a 46 C-terminal peptide (which has kinase activity). Recently, we have demonstrated that the C-terminal portion of CDK11\(^{p46}\) can phosphorylate eIF3f during apoptosis and participate in the inhibition of protein translation [16]. In addition, we found that CDK11\(^{p46}\) interacts with Hsp90 and its co-chaperone cdc37, which can stabilize CDK11 kinase and therefore is crucial for its pro-apoptotic function [23]. However, the functions of the p60 fragment of CDK11 during Fas-mediated cell death are not known.

The death-receptor-initiated pathway can diverge in two pathways. In type I cells, stimulation of Fas activates caspases-8/3, and this is considered the mitochondria-independent, "extrinsic pathway". In type II cells, caspase-8 is not sufficient to activate caspase-3. Instead, Bid is cleaved by caspase-8 and is then translocated to the mitochondria to activate the "intrinsic pathway", thus amplifying the death-receptor apoptotic signal. In A375 cells, CDK11\(^{p110}\) is cleaved by caspase-3 after Fas treatment. In the present study, we have shown that this cleavage occurs as early as 6 h after Fas treatment (Figure 3A), suggesting that a certain amount of caspase-3 has been activated at this time point via the "extrinsic pathway". This result is consistent with the caspase-3 activation we observed after Fas treatment (Figure 3C). In addition, we observed the translocation of CDK11\(^{p60}\) to the mitochondria at 12 h by subcellular fractionation studies and confocal microscopy (Figure 3B). Consistent with this event, significant increases in caspase-3 activity and cytochrome \(c\) release were observed after 24 h (Figures 3C and 3D). Taken together, these observations suggest that A375 cells may be type II apoptotic cells upon stimulation of the Fas-signalling pathway. As a result of CDK11\(^{p60}\) targeting to mitochondria, and in concert with other events occurring in mitochondria, more cytochrome \(c\) is released to induce the activation of caspase-3, which in turn produces more CDK11\(^{p60}\). Translocation of more CDK11\(^{p60}\) into mitochondria could help to further amplify the apoptotic cascade.
Translocation of CDK11p60 to mitochondria during apoptosis

**Figure 4** Ectopic expression of CDK11p60 targets it to mitochondria

(A) A375 human melanoma cells were transfected with EGFP or EGFP–p60. After 24 h, mitochondrial fractions were prepared and assayed by Western blotting using anti-CDK11 antibody. The same membrane was also blotted with anti-mt-Hsp60, anti-PARP or anti-tubulin antibodies. (B) A375 cells were transfected with EGFP or EGFP–p60. After 24 h, cells were loaded with MitoTracker Red and fixed. EGFP or EGFP–p60 expression and mitochondria were visualized using confocal microscopy, and the two images were overlaid.

**Figure 5** Induction of apoptosis by overexpression of EGFP–p60

A375 cells were transfected with EGFP or EGFP–p60 for 24 h and nuclei were stained by DAPI. EGFP–p60 expression and nuclear morphology were visualized using fluorescence microscopy. Apoptosis was scored according to nuclear morphology in 200 GFP-positive cells. The data are shown as the percentage apoptosis (means ± S.D. for three experiments; **P < 0.01). cellular substrates in many type II cells. For example, reovirus-induced apoptosis in HEK293 cells requires both death receptor- and mitochondrial-mediated caspase-dependent pathways [30]. In summary, Fas-induced apoptosis in A375 cells is initiated by death receptor pathways, but requires mitochondrial amplification, and targeting of CDK11p60 to mitochondria may contribute to the amplification of the apoptotic signal.

Increasing evidence suggests that movement of key proteins into or out of mitochondria during apoptosis is essential for the regulation of apoptosis. For example, a fraction of p53 protein localizes to mitochondria at the onset of p53-dependent apoptosis in tumour cells, and the accumulation of p53 in mitochondria...
precedes changes in ΔΨm, cytochrome c release and pro-caspase-3 activation [31]. Similarly, coflin was found to translocate to mitochondria before the release of cytochrome c, and has an important function during the initiation phase of apoptosis [32]. In addition, TR3, a nuclear transcription factor, was also found important function during the initiation phase of apoptosis [32]. Furthermore, overexpression of CDK11p46 seems to be associated with cytochrome c release and promotes apoptosis (Figure 5). However, unlike p53 or coflin, the redistribution of CDK11p46 to mitochondria occurs after caspase-3 activation. After targeting of CDK11p46 to mitochondria, more cytochrome c appears to be released and results in increased cell death. We propose that the redistribution of the N-terminal fragment of CDK11p110 to mitochondria may be an additional mechanism to amplify the initial death signal. However, several important questions need to be explored further. For example, which domain is necessary for mitochondrial targeting?; and is translocation of the p60 fragment dependent on any modification?

We have shown in the present study that the CDK11p110 fragment is imported into mitochondria by the TOM and TIM complex as a specific in vivo complex is formed between endogenous CDK11p110 and mt-Hsp70 following Fas-induced cell death (Figure 7). Mt-Hsp70 is essential for the translocation of cytosolic proteins across the two mitochondrial membranes. Together with two essential partner proteins, Tim44 and Mge1, mt-Hsp70 forms a membrane-associated import motor complex responsible for vectorial polypeptide movement and unfolding of proteins [34]. A similar interaction between p53 and mt-Hsp70 has been observed previously [31]. However, knowledge of the nature of the interaction is still preliminary in terms of its functional significance. The exact molecular mechanism by which CDK11p110 compromises mitochondria will require further study.

For CDK11p110, the different subcellular localization of the two CDK11p110 fragments generated by caspase cleavage suggests a different function for these apoptotic signalling effectors. Whereas activation of CDK11p46 kinase domain may result in the phosphorylation and regulation of downstream substrates (such as elf3F) involved in the final stages of apoptosis, the CDK11p46 fragment lacking the kinase domain may act as a regulator of mitochondrial function and amplify the death signal. Thus the CDK11p110 kinase protein appears to have two distinct roles in cell death signalling: (i) phosphorylation of potential death substrates and (ii) modulation of mitochondrial function. A further understanding of the pathways regulated by CDK11 may have potential therapeutical value for treating cancer cells.

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