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

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Fas receptor–Fas ligand interaction appears to be important in carcinogenesis, tumour outgrowth and metastasis. Emerging evidence suggests that CDK11 (cyclin-dependent kinase 11) plays a role in apoptosis and melanoma development. Here, we show that CDK11^{p110} protein kinase was cleaved after induction of apoptosis by Fas. The N-terminal portion of CDK11^{p110}, CDK11^{p60}, was translocated from the nucleus to the mitochondria. The targeting of CDK11^{p60} to mitochondria occurred as early as 12 h after treatment. Overexpression of EGFP (enhanced green fluorescent protein)-tagged CDK11^{p60} could partially break down the mitochondrial membrane potential, induce cytochrome *c* release and promote apoptosis. Reduction of endogenous CDK11^{p110} protein

levels with siRNA (small interfering RNA) resulted in the suppression of both cytochrome *c* release and apoptosis. In addition, subcellular fractionation studies of Fas-mediated apoptosis demonstrated that CDK11^{p60} was associated with the mitochondrial import motor, mitochondrial heat shock protein 70. Taken together, our data suggest that CDK11^{p60} can contribute to apoptosis by direct signalling at the mitochondria, thereby amplifying Fas-induced apoptosis in melanoma cells.

Key words: apoptosis, cyclin-dependent kinase 11 (CDK11), mitochondria, mitochondrial heat-shock protein (Hsp70).

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 p34^{cdc2} family members, can influence apoptosis [2–6]. CDK11 (cyclin-dependent kinase 11; formerly known as PITSLRE) binds to cyclin L, and is a member of the extended family of p34^{cdc2}-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 ectopical 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 effector in apoptotic signalling pathways [15,19,20]. During apoptosis induced by Fas, tumour necrosis factor or staurosporine, CDK11^{p110} 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 CDK11^{p46} 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 CDK11^{p46} inhibits protein synthesis and induces apoptosis [16]. These data suggest that CDK11^{p46} might play a role in mediating apoptosis. However, the function of CDK11^{p60} has not been fully explored.

In the present study, we evaluate the pro-apoptotic effects of the CDK11^{p60} fragment. We show that, during apoptosis, CDK11^{p60} redistributes from the nucleus to the mitochondria. Ectopic expression of CDK11^{p60} can partially disrupt the mitochondrial membrane potential, induce cytochrome *c* release and apoptosis. Taken together with the effect of CDK11^{p46} 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 (1×10^6) 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 CDK11^{p110} was obtained by PCR using forward primer 5'-TCAAATGGGTG-ATGAAAAGG-3' and reverse primer 5'-ATGCCTCCTCA-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 4×10^5 cells/well and grown in antibiotic-free medium. After 24 h, cells were transfected with 0.4 μ g of siRNA and 5 μ l 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 CDK11^{p60}, RT-PCR (reverse transcriptase-PCR) was performed to obtain CDK11^{p60} fragment using an N-terminal primer containing an EcoRI site (5'-GAATTCCTCTCTCTGGCTC-CGGGACCG-3') and a C-terminal primer containing a BamHI site (5'-CGGGATCCTGAGCTCGATGG-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. For constructing Myc-tagged CDK11^{p60}, CDK11^{p60} was generated by RT-PCR with the following pair of primers: 5'-GG-AATTCAAATGGGTGATGAAAAGGACTCTT-3' and 5'-GGG-GTACCTAGGTACTTGGGCAGCTCCTG-3', in which EcoRI and KpnI restriction sites were added to the forward and reverse primers respectively. The 1.2-kb fragment was then cloned in-frame into a mammalian expression vector pCMV-Myc (ClonTech) with Myc tag at the N-terminus of fusion protein Myc-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 1×10^6 cells/well. After 24 h, cells were transfected with complexes containing 10 μ l of LF2000 and 4 μ g of EGFP-tagged CDK11^{p60} or Myc-tagged CDK11^{p60}. For preparing samples for immunofluorescent microscopy images, 12-mm coverslips were seeded with 1×10^6 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 enriched with Extraction Buffer III. Components of the cytoskeleton are finally solubilized with Extraction Buffer IV.

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 13 000 *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. mt-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 μ l 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-Pharmingen, San Diego, CA, U.S.A.), rabbit polyclonal PARP [poly(ADP-ribose) polymerase; 1:1000 dilution, Upstate Biotechnology], mouse monoclonal mt-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. After 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 1×10^4 cells/well. After 24 h, cells were treated with 0.5 μ g/ml anti-Fas antibody. Caspase-Glo 3/7 reagent (100 μ l) 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).

Cytofluorimetric determination of $\Delta\psi_m$ (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 $\Delta\psi_m$. A375 cells were transfected with EGFP or EGFP-p60 constructs for 24 h. Approx. 0.5×10^6 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 μ l and incubated in the dark for 15 min at 24°C. Samples were analysed by flow cytometry immediately after the addition of 400 μ l of $1 \times$ 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 μ g/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 $\pm$ 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 μ g/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) staining followed by FACS analysis (results not shown).

Knockdown of CDK11^{p110} by siRNA partially attenuates Fas-induced apoptosis in A375 cells

We next examined the role of endogenous CDK11^{p110} 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 CDK11^{p110} 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 CDK11^{p110} 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 CDK11^{p110} occurs early following Fas treatment, and the N-terminal CDK11^{p60} fragment is targeted to mitochondria

We previously demonstrated that the CDK11^{p110} 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 CDK11^{p110} following Fas treatment are not known. Typically, full-length CDK11^{p110} is located in the nucleus of tumour cell lines, including A375 cells [15,16]. To assess the location of CDK11^{p60} fragment upon Fas treatment, whole cell lysates and subcellular fractions of A375 control and Fas-treated cells were isolated and examined. The cleaved CDK11^{p60} 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 CDK11^{p60} fragment translocated from the nucleus to mitochondria at 12 h, and the amount of CDK11^{p60} 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, CDK11^{p110} is found in the nucleus. We have also shown that the caspase-processed CDK11^{p46} isoform can be found in the nucleus [16], whereas the N-terminal portion generated by caspase cleavage of CDK11^{p110} (i.e. CDK11^{p60}) is in the nucleus, and translocates through the cytoplasm to the mitochondria. Collectively, these results indicate that the two CDK11^{p110} fragments, generated by caspase cleavage upon treatment with Fas, have different subcellular locations.

Based on the fact that: (1) CDK11^{p110} is a substrate of caspase-3, and the cleavage occurs at 6 h after treatment (Figure 3A), and (2) CDK11^{p60} 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 $0.5 \mu\text{g/ml}$ anti-Fas antibody for 24 h. Nucleus 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 $0.5 \mu\text{g/ml}$ 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 later 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^{P110} during Fas-induced apoptosis (Figure 3A and [15]). In addition, the increase in the caspase-3 activity and the translocation of CDK11^{P60} fragment into mitochondria over time were associated with cytochrome *c* release (Figure 3D).

Ectopically expressed CDK11^{P60} targets to mitochondria and leads to increased apoptosis in A375 cells

The role of mitochondrial targeting by CDK11^{P60} in modulating apoptosis was investigated further by overexpression of CDK11^{P60}. We engineered an expression vector by fusing CDK11^{P60} with an EGFP epitope tag at the C-terminus. We first overexpressed this EGFP-p60 in A375 cells and assessed

Figure 2 Silencing of CDK11 blocks cytochrome *c* release and inhibits apoptosis

A375 cells were transfected with LacZ siRNA and CDK11 siRNA. **(A)** Reduced CDK11^{p110} expression in the presence of CDK11 siRNA is shown; Western blot analysis of total cell lysates of A375 cells transfected with LacZ siRNA or CDK11 siRNA was carried out for 48 h. **(B)** Quantification of apoptotic cells in mock-transfected, LacZ siRNA or CDK11 siRNA transfected cells treated with Fas for 24 h. Apoptosis was scored according to nuclei morphology in 300 cells stained with Acridine Orange and ethidium bromide. The results are presented as the percentage apoptosis (means $\pm$ S.D. for three independent experiments).

its mitochondrial localization. Western blot analysis of mitochondrial fractions confirmed mitochondrial targeting of the EGFP-tagged CDK11^{p60} fusion protein (Figure 4A). Equal loading of mitochondrial fractions was confirmed by re-probing the same membrane with mitochondrial Hsp60 protein (Figure 4A). Absence of the tubulin and PARP indicated that the mitochondrial fractions were not contaminated with cytosolic and nuclear proteins (Figure 4A). Similar results were observed in A375 cells transfected with Myc-tagged CDK11^{p60} (results not shown). In addition, to confirm the subcellular localization of EGFP-p60 in A375 cells, confocal microscopy was used to examine the mitochondria (stained with MitoTracker Red) and EGFP-p60 (green). A375 cells transfected with EGFP alone displayed no particular localization in the cells (Figure 4B). However, EGFP-p60 fusion protein showed predominant mitochondrial localization. These results indicate that ectopically expressed CDK11^{p60} targets to mitochondria in A375 cells.

Next, we evaluated whether mitochondria-localized CDK11^{p60} was sufficient to induce cytochrome *c* release and apoptosis using immunofluorescent microscopy. Transient expression of EGFP did not cause release of cytochrome *c* from mitochondria and did not induce apoptosis. However, expression of EGFP-p60 resulted in the release of cytochrome *c* from mitochondria and condensation of nuclei is not shown. To quantify the apoptosis induced by overexpression of CDK11^{p60}, EGFP or EGFP-p60 was transiently transfected into A375 cells. After 24 h, apoptosis was characterized by scoring condensed and fragmented nuclei of EGFP-positive cells. Compared with empty vector, expression of EGFP-p60 could induce higher apoptosis. The quantification of apoptosis induced by EGFP-p60 is shown in Figure 5. Increased apoptosis was also observed in Hela cells transfected with EGFP-p60 (results not shown). Higher apoptosis was also obtained using 7-AAD staining, followed by flow cytometry as well as Acridine Orange/ethidium bromide staining (results not shown). These results demonstrate that CDK11^{p60} can promote apoptosis.

Overexpression of CDK11^{p60} partially breaks down $\Delta\psi_m$

Our data suggest that overexpressed CDK11^{p60} targets to mitochondria and leads to increased apoptosis in A375 cells. We next asked whether overexpressed CDK11^{p60} could perturb mitochondrial function by disrupting $\Delta\psi_m$ (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 ($\Delta\psi_m$) across the mitochondrial membrane collapses. To investigate changes in $\Delta\psi_m$, we transiently transfected A375 cells with EGFP or EGFP-p60 construct and stained the cells with the $\Delta\psi_m$ -sensitive fluorescent probe MitoTracker Red (CMXRos). The lipophilic cation CMXRos accumulates in the intact mitochondrial matrix as a consequence of $\Delta\psi_m$. While in apoptotic cells, $\Delta\psi_m$ 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 $\Delta\psi_m$. 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 CDK11^{p60} 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 CDK11^{p60}, 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 CDK11^{p60}, immunoprecipitation assays were performed. We observed an interaction of cleaved CDK11^{p60} with mt-Hsp70 *in vivo* from cells undergoing Fas-induced apoptosis (Figure 7A). We confirmed the association by conducting CDK11^{p60} 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 CDK11^{p60} 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 CDK11^{p110} 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

Figure 3 Cleavage of CDK11^{p110} and subcellular redistribution of the CDK11^{p60} fragment to mitochondria upon Fas treatment

(A) Cleavage of CDK11^{p110} in Fas-treated A375 cells. Western blot analysis of total cell lysates prepared from A375 cells treated with Fas for the indicated time points. (B) Subcellular distribution of CDK11^{p60} in A375 cells treated with Fas for the indicated time points. Mitochondrial fractions were prepared and analysed for accumulation of CDK11^{p60} in mitochondria by Western blot analysis. The same membrane was also blotted with anti-Hsp60, anti-PARP or anti-tubulin antibodies (C) Activation of caspase-3 in A375 cells treated with Fas. Cells were treated with Fas for the indicated times and measured for caspase-3 activity. The results are presented as means $\pm$ S.D. for three independent experiments. (D) Release of cytochrome *c* to the cytosol in A375 cells treated with Fas. Cytosolic and mitochondrial fractions of Fas-treated cells at the indicated times were analysed by Western blotting using anti-cytochrome *c* antibody.

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 p46 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

Figure 4 Ectopic expression of CDK11^{p60} 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 $\pm$ S.D. for three experiments; ** $P < 0.01$).

and enhance cell death. Similar to our observations, combined activation of death receptor and mitochondrial pathways results in downstream activation of effector caspases and cleavage of

Figure 6 Overexpression of CDK11^{p60} induces disruption of $\Delta\psi_m$

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 y-axis shows the mean red fluorescence intensity for EGFP-positive cells only. The data shown are the means $\pm$ S.D. for three independent experiments (* $P < 0.05$).

Figure 7 mt-Hsp70 forms *in vivo* complexes with CDK11^{p60}

(A) Endogenous CDK11^{p60} interacts with mt-Hsp70. Mitochondrial fractions from A375 cells untreated and treated with Fas for 24 h were immunoprecipitated (IP) with IgG or anti-CDK11 antibodies and immunoblotted (IB) for co-precipitating proteins with mt-Hsp70 antibody. (B) Overexpressed CDK11^{p60} interacts with mt-Hsp70. A375 cells were transfected with Myc or Myc-p60 for 24 h. Cells were immunoprecipitated with mt-Hsp70 and immunoblotted with anti-Myc antibody.

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 CDK11^{p60} 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 $\Delta\psi_m$, cytochrome *c* release and procaspase-3 activation [31]. Similarly, cofilin 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 to translocate from the nucleus to mitochondria to induce cytochrome *c* release and apoptosis [33]. In the present study, CDK11^{p60}, which does not contain the kinase domain but has several regulatory domains, also translocates to mitochondria and is associated with disruption of $\Delta\psi_m$ (Figure 6). Furthermore, overexpression of CDK11^{p60} seems to be associated with cytochrome *c* release and promotes apoptosis (Figure 5). However, unlike p53 or cofilin, the redistribution of CDK11^{p60} to mitochondria occurs after caspase-3 activation. After targeting of CDK11^{p60} 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 CDK11^{p110} 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 CDK11^{p60} fragment is imported into mitochondria by the TOM and TIM complex as a specific *in vivo* complex is formed between endogenous CDK11^{p60} 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 CDK11^{p60} compromises mitochondria will require further study.

For CDK11^{p110}, the different subcellular localization of the two CDK11^{p110} fragments generated by caspase cleavage suggests a different function for these apoptotic signalling effectors. Whereas activation of CDK11^{p46} kinase domain may result in the phosphorylation and regulation of downstream substrates (such as eIF3f) involved in the final stages of apoptosis, the CDK11^{p60} fragment lacking the kinase domain may act as a regulator of mitochondrial function and amplify the death signal. Thus the CDK11^{p110} protein kinase 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 therapeutic value for treating cancer cells.

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