The cell cycle inhibitor p57^{Kip2} promotes cell death via the mitochondrial apoptotic pathway

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The p57^{Kip2} gene belongs to the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors and has been suggested to be a tumor suppressor gene, being inactivated in various types of human cancers. However, little is known concerning p57^{Kip2} possible interplay with the apoptotic cell death machinery and its possible implication for cancer. Here, we report that selective p57^{Kip2} expression sensitizes cancer cells to apoptotic agents such as cisplatin, etoposide and staurosporine (STS) via a mechanism, which does not require p57^{Kip2}-mediated inhibition of CDK. Translocation of p57^{Kip2} to mitochondria occurs within 20 min after STS application. In fact, p57^{Kip2} primarily promotes the intrinsic apoptotic pathways, favoring Bax activation and loss of mitochondrial transmembrane potential, consequent release of cytochrome-*c* into cytosol, caspase-9 and caspase-3 activation. In accordance, Bcl2 overexpression or voltage-dependent anion channel (VDAC) inhibition is able to inhibit p57^{Kip2} cell death promoting effect. Thus, in addition to its established function in control of proliferation, these results reveal a mechanism whereby p57^{Kip2} influences the mitochondrial apoptotic cell death pathway in cancer cells.

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Apoptosis plays a central role in the regulation of normal tissue homeostasis and participates in the elimination of potentially dangerous cells including the precursors of tumor cells. Most currently used antitumor agents kill cancer cells by stimulating the apoptotic machinery, and therapy resistance can be attributed, at least in part, to a disabled apoptotic program. Apoptotic cell death and withdrawals from the cell cycle are tightly coordinated processes. In fact, mechanisms essential for regulation of the cell cycle also influence processes of cell death.¹ An important mechanism for cell cycle control involves inhibition of cyclin-dependent kinases (CDKs) by CDK inhibitors (CKIs). p57^{Kip2} together with p21^{Cip1} and p27^{Kip1} belongs to the Cip/Kip family of CKIs, which plays a critical role in regulating cell proliferation.^{2,3} Importantly, only p57^{Kip2} has been shown to play essential roles during embryogenesis that other cell cycle inhibitors cannot compensate for. p57^{Kip2}-null mutant mice display severe developmental defects.^{4–7} Interestingly, many of the defects described are not directly linked to increased cell proliferation.^{4,5,7} In addition, p57^{Kip2} has been shown to influence cell differentiation of several cell types, implying that p57^{Kip2} may have a broader scope of cellular action than only being a cell cycle inhibitor.^{5–7} We and others have shown that p57^{Kip2}-deficient mice exhibit an increase in the rate of cell death in several cell populations during embryogenesis.^{4,6,7} Whether this increased apoptosis is a direct or indirect effect due to aberrations in cell proliferation and differentiation is not known. Importantly, the p57^{Kip2} gene, located on chromo-

some 11p15.5, has been suggested to be a tumor suppressor gene, being inactivated in various types of human cancers.^{8–12} However, little is known concerning p57^{Kip2} function during apoptotic cell death during adulthood and its possible implication for cancer.

In the present study, we show that specific p57^{Kip2} expression in HeLa cells sensitizes these cells to apoptosis induced by staurosporine (STS). Our studies reveal that p57^{Kip2} primarily promotes the mitochondrial apoptotic pathway, favoring loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), and consequent release of cytochrome-*c* (cyt-*c*) into cytosol, caspase-9 and caspase-3 activation. Inhibition of p57^{Kip2} proapoptotic function by Bcl2 overexpression or by the voltage-dependent anion channel (VDAC) inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), further confirmed the requirement for the cyt-*c*/caspase-dependent mitochondrial cell death pathway downstream of p57^{Kip2}. Thus, these results indicate that p57^{Kip2} plays a critical role in regulation of the mitochondrial apoptotic cell death pathway in cancer cells.

Results

Staurosporine-induced apoptosis is strengthened by p57^{Kip2}. To explore a putative role for p57^{Kip2} during apoptosis, we took advantage of HeLa Tet-On-derived cell lines stably transfected with the tetracycline-inducible

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Abbreviations: α Fas, agonistic anti-Fas Abs; AIF, apoptosis inducing factor; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; CP, cisplatin; cyt-*c*, cytochrome-*c*; $\Delta\Psi_m$, mitochondrial transmembrane potential; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; Dox, doxycycline; FACS, fluorescence-activated cell sorting; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; MEF, mouse embryo fibroblasts; NLS, nuclear localization signal; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; STS, staurosporine; TMRE, tetramethylrhodamine ethyl ester; VP16, etoposide; VDAC, voltage-dependent anion channel

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Figure 1 p57^{Kip2} strengthens STS-induced apoptosis. (a) Total cell extracts were prepared from HeLa-p57 and HeLa-ctrl cells treated with Dox for 24 h and subjected to immunoblotting with anti-p57^{Kip2} antibody. (b, d, e) HeLa-p57 and HeLa-ctrl cells were pretreated with Dox for 24 h before treatment with STS for 5 h. (c) HeLa Tet-On cells were treated with Dox for 48 h or 72 h. (b–c) Cells were fixed and nuclei were stained with Hoechst 33342. Apoptosis was assayed by scoring cells presenting condensed or fragmented nuclei. Three hundred nuclei were analyzed in duplicate and each experiment was repeated three times. (d) Appearance of subdiploid fragmented nuclei derived from apoptotic cells was analyzed by FACS after DNA staining with PI. Data are represented as mean $\pm$ S.E.M. (e) Total protein cell extracts were analyzed by Western blot for the presence of 17 kDa active caspase-3 fragment, 116 kDa native PARP and its 89 kDa caspase-3-cleavage product. G3PDH was used as protein loading standard

p57^{Kip2} 13 HeLa-p57 cells can be induced to selectively express p57^{Kip2} upon administration of doxycycline (Dox) (Figure 1a). HeLa-ctrl cells are control cells in which no increase in p57^{Kip2} expression level occurs following Dox treatment. To investigate the effect of selective p57^{Kip2} expression on STS-induced apoptosis, HeLa Tet-On cell lines were pretreated for 24 h with Dox (2 μ g/ml), after which STS (0.1 μ M) was added. Cells were harvested 5 h after STS addition and analyzed for apoptosis (Figure 1b). Apoptotic nuclei were observed in both cell lines after STS treatment. Interestingly in HeLa-p57 cells, whereas the induction of p57^{Kip2} expression did not provoke cell death, its induction before the addition of STS further increased the fraction of apoptotic cells. In contrast, Dox treatment of HeLa-ctrl cells had no apoptosis promoting effect.

A main issue is whether the observed death promoting effect of p57^{Kip2} could be an overexpression artifact. In order to rule out that possibility, HeLa Tet-On cell lines were treated with Dox for 48 and 72 h, and apoptosis was assessed. Importantly, none of these cell lines upon Dox treatment exhibited more than 2% apoptotic cells up to 72 h (Figure 1c). The sensitization to STS-killing effect by p57^{Kip2} in HeLa-p57 cells was confirmed by fluorescence-activated cell sorting (FACS) analysis (Figure 1d). One of the biochemical hallmarks of apoptosis is cleavage of poly(ADP-ribose)polymerase (PARP) induced by caspase-3. Appearance of the PARP cleavage product was observed in STS-treated HeLa-p57 cells. Processing of PARP correlated with caspase-3 activa-

tion (Figures 1e and 5a). Nevertheless, total disappearance of native PARP was only observed in cells treated by Dox and then STS. These cells also exhibited an increased active caspase-3 level as compared to cells treated only with STS. Altogether, these results show that selective p57^{Kip2} expression supports STS-induced apoptosis in HeLa cells. To notice, p57^{Kip2} expression also sensitized HeLa cells to apoptosis induced by DNA damaging agents such as etoposide (VP16) and cisplatin (CP), but was ineffective in promoting death receptor-mediated apoptosis stimulated with agonistic anti-Fas antibodies (α Fas) (Figure 2a). In addition, p57^{Kip2} proapoptotic effects were also observed in neuroblastoma SH-SY5Y cells (Figure 2b) and ovarian carcinoma SKOV3 cells (Figure 2c). However, the death promoting effect of p57^{Kip2} was not seen in human embryonic kidney HEK-293 cells (Figure 2d), suggesting some degree of cell type specificity.

Depletion of p57^{Kip2} inhibits staurosporine-induced apoptosis. To further characterize and reveal the function of p57^{Kip2} in apoptosis without the use of induced expression, we performed additional experiments using mouse embryo fibroblasts (MEF) derived from wild-type and p57^{Kip2}^{-/-} mice. Importantly, STS- and VP16-induced apoptosis were considerably reduced in MEF cells derived from p57^{Kip2}^{-/-} mice (Figure 3a and b). These data provide evidence that p57^{Kip2} is of importance for efficiency of the death program elicited by apoptotic stimuli such as STS and VP16.

Figure 2 p57^{Kip2} promotes VP16- and CP-induced apoptosis. (a) HeLa-p57 cells were pretreated with Dox for 24 h before treatment with VP16, CP or α Fas for additional 24 h. Nuclei were stained with Hoechst 33342 and apoptosis was assayed as described in Figure 1b. SH-SY5Y cells (b), SKOV3 cells (c) and HEK-293 cells (d) were transfected with expression vectors encoding p57^{Kip2} for 24 h and then treated with STS for 5 h (b, d), CP for 24 h (c, d) or exposed to 60 J/m² UV light and incubated further for 6 h (d). Cells were then processed for immunofluorescence using anti-p57^{Kip2} antibody. Nuclei were counterstained with Hoechst 33342. Apoptosis was assayed by scoring nuclei presenting condensed or fragmented apoptotic features. Three hundred transfected cells were analyzed in duplicate and each experiment was repeated three times. Data are represented as mean $\pm$ S.E.M.

p57^{Kip2} promotes staurosporine-induced apoptosis by means of a CDK-independent mechanism. No major cell cycle perturbations were observed in p57^{Kip2} expressing cells before the manifestation of apoptosis (Figure 4a).¹³ To further investigate if p57^{Kip2} death promoting effects also depend upon the same domain as the antiproliferative ones, we used a mutated derivative of p57^{Kip2} (p57CDK^{mut}) that is unable to inhibit CDK activity.^{7,14} p57CDK^{mut}, which contains two substitutions in the cyclin binding domain and two in the CDK binding region, has lost its capability to physically

Figure 3 Depletion of p57^{Kip2} inhibits STS- and VP16-induced apoptosis. MEF cells derived from wild-type and p57^{Kip2}^{-/-} mice were treated with 0.1 μ M STS for 5 h (a) or 2 μ g/ml VP16 for 24 h (b). Cells were fixed and nuclei were stained with Hoechst 33342. Apoptosis was assayed by scoring cells presenting condensed or fragmented nuclei. Three hundred nuclei were analyzed in duplicate and each experiment was repeated three times. Data are represented as mean $\pm$ S.E.M.

interact with CDK2 and therefore its ability to inhibit its activity (Figure 4b and c).¹⁴ Although p57CDK^{mut} is unable to induce cell cycle arrest, it retains the ability to promote STS-induced apoptosis (Figure 4d). Taken together, these data provide compelling evidence indicating that p57^{Kip2} promotes cell death by a mechanism that is independent of its ability to inhibit CDK activity.

p57^{Kip2} supports staurosporine-induced caspase-3 and caspase-9 activities. STS-induced cell death was inhibited by the caspases' inhibitor zVAD-fmk, indicating that STS-induced cell death is mediated by caspases activation (Figure 5e). To confirm caspases' activation in STS-treated HeLa cells and to evaluate the consequence of p57^{Kip2} expression, cleavage of specific fluorogenic substrates, which correspond to preferred cleavage site for caspase-3 like (Figure 5a), caspase-2 (Figure 5b), caspase-8 (Figure 5c) and caspase-9 enzyme (Figure 5d) was monitored. Caspase-2 and caspase-3 activities were detected already after 2 h STS treatment. We were not able to notice significant increase in caspase-8 and caspase-9 activities upon STS treatment alone. Interestingly, HeLa-p57 cells pretreated with Dox exhibited an increase in caspase-3 and caspase-9 activities already at 2 h after STS treatment, whereas significant effects on caspase-2 and caspase-8 were only detectable at 5 h post STS treatment. In order to further characterize which caspases are mediating the p57^{Kip2} cell death promoting effect, p57^{Kip2} expressing cells were pretreated with DEVD-fmk (caspase-3 inhibitor), LEHD-fmk (caspase-9 inhibitor) or VDVAD-fmk (caspase-2 inhibitor) before addition of STS. Each of these inhibitors leads to a reduction of STS-killing effect, implying that caspase-2, caspase-3 and caspase-9 activities account for STS-induced cell death (Figure 5e). However, whereas p57^{Kip2} was still able to promote STS-induced apoptosis in the presence of caspase-2 inhibitor, caspase-3 and

Figure 4 p57^{Kip2} promotes STS-induced apoptosis by means of a CDK-independent mechanism. (a) HeLa-p57 cells were treated with Dox for 30 h. Distribution of the cells in the G₁, S and G₂/M phases of the cell cycle was analyzed by FACS after DNA staining with PI. Data shown are representative of multiple independent experiments. (b) HeLa cells were transfected with expression vectors encoding HA-p57^{Kip2}, HA-p57CDK^{mut}, CDK2 or their combinations for 24 h. Total cell extracts from transfected cells were subjected to immunoblotting with anti-p57^{Kip2} or anti-Cdk2 antibodies (inputs, top panels). Total cell extracts were immunoprecipitated by using anti-HA antibody and immune complexes were subjected to immunoblotting by using anti-p57^{Kip2} or anti-Cdk2 antibodies (IP, lower panels). (c) HeLa cells were transfected with expression vectors encoding HA-p57^{Kip2} or HA-p57CDK^{mut} for 24 h. Total cell extracts were immunoprecipitated by using anti-CDK2 antibody and kinase activity in the CDK2 complexes was measured by the incorporation of [γ -³²P]ATP into Histone H1. Data are presented as the fold increase over basal levels. Each experiment was repeated three times. (d) HeLa cells were transfected with expression vectors encoding p57^{Kip2} or p57CDK^{mut} for 24 h and then treated with STS for 5 h. Cells were then processed for immunofluorescence using anti-p57^{Kip2} antibody. Nuclei were counterstained with Hoechst 33342 and apoptosis was assayed as described in Figure 2b

caspase-9 inhibitors abrogated p57^{Kip2} proapoptotic effects. Thus, whereas STS treatment leads to the activation of several caspases, p57^{Kip2} only supported caspase-9 and caspase-3 activities, suggesting that p57^{Kip2} acts upstream of their activation.

p57^{Kip2} promotes and accelerates staurosporine-induced mitochondrial dysfunctions. Mitochondria play an important role in the regulation of apoptosis. Specifically, the release of several proteins, including cyt-*c* and apoptosis inducing factor (AIF), normally located in the intermembrane space of mitochondria, has been observed during the early stages of apoptotic cell death.¹⁵ We therefore decide to examine if the mitochondrial cyt-*c*/caspase-9-activated pathway could be a target for the p57^{Kip2} proapoptotic

effects. As shown in Figure 6a, STS induced a drop in $\Delta\Psi_m$ measured by the reduction of tetramethylrhodamine ethyl ester (TMRE) staining in HeLa cells. HeLa-p57 cells after Dox addition displayed identical features to control cells for this parameter. In contrast, pretreatment with Dox further increases STS-induced $\Delta\Psi_m$ disruption. In addition, p57^{Kip2} expression also amplified the release of cyt-*c* into the cytosol observed upon STS treatment (Figure 6b). Whereas AIF was not detected in the cytosolic fraction 5 h after STS treatment, the protein was observed when cells were pretreated with Dox. Then, in order to characterize the pharmacological effects on a single-cell basis, time-lapse confocal microscopy was performed with TMRE-loaded GFP-p57 expressing HeLa cells (Figure 6c and d). Time-lapse recordings of mitochondrial TMRE staining in GFP expressing in HeLa

Figure 5 p57^{Kip2} supports STS-induced caspase-3 and caspase-9 activities. HeLa-p57 cells were pretreated with Dox for 24 h before treatment with STS for 2 or 5 h. Caspase-3-like activity (a), caspase-2 (b), caspase-8 (c) and caspase-9 activity (d) were determined by the cleavage of the specific peptide substrate DEVD-AMC, VDVAD-AMC, IETD-AMC and LEHD-AMC, respectively. The maximum linear rate of AMC release was estimated by linear regression. Data are represented as mean $\pm$ S.E.M. of three independent experiments performed in duplicate. (e) HeLa-p57 cells were subsequently treated with Dox for 24 h, zVAD-fmk, DEVD-fmk, LEHD-fmk or VDVAD-fmk caspases inhibitors for 1 h and finally with STS for 5 h. Nuclei were stained with Hoechst 33342 and apoptosis was scored as explained in Figure 1b

Figure 6 p57^{Kip2} promotes and accelerates STS-induced mitochondrial dysfunctions. **(a, b)** HeLa-p57 cells were pretreated with Dox for 24 h before treatment with STS for 5 h. **(a)** Cells were harvested and mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by FACS using TMRE staining. Data are represented as mean $\pm$ S.E.M. **(b)** Cytosol cell extracts were prepared and subjected to immunoblotting with anti-cyt-c and anti-AIF antibodies. G3PDH was used as standard for equal loading of protein. **(c, d)** HeLa cells expressing GFP-p57 and stained with TMRE were treated with STS and $\Delta\Psi_m$ loss was followed by time-lapse confocal microscopy as described in Materials and Methods. **(c)** Quantitative results of time-lapse confocal microscopy using TMRE. Each plot represents the fraction of TMRE-positive cells at the indicate time. **(d)** Representative sequential confocal images of GFP-p57 (green), mitochondrial TMRE staining (red) and nuclear Hoechst 33342 staining (blue) of HeLa cells after STS treatment. Similar results were obtained from three independent experiments

cells consistently failed to show difference in the kinetic of $\Delta\Psi_m$ loss upon STS treatment as compared to control cells (data not shown). In contrary, GFP-p57 expressing HeLa cells lost their $\Delta\Psi_m$ faster than the non-expressing ones (Figure 6d and Supplementary Movie S1). In fact, 147 min after STS treatment, 50% of the p57^{Kip2} expressing cells were TMRE negative, whereas it took 209 min to obtain the same effect in control cells (Figure 6c). Altogether, these data demonstrated that p57^{Kip2} not only enhances but also accelerates STS-induced mitochondrial apoptotic events and suggest that mitochondrial disruptions are corner stone for p57^{Kip2} proapoptotic effects.

p57^{Kip2} translocates to mitochondria upon staurosporine exposure and promotes Bax activation. How might p57^{Kip2} modulate the apoptotic machinery at the mitochondrial level? Using conventional immuno-

fluorescence detection and confocal microscopy on paraformaldehyde-fixed HeLa cells, a decrease of p57^{Kip2} nuclear staining was observed already after 2 h STS treatment. However, using this method, we were unable to detect cytoplasmic localization for p57^{Kip2} (data not shown). We took advantage of GFP tag, which allows tracing of protein in live cells. We used time-lapse confocal microscopy imaging of HeLa cells expressing GFP-p57 to visualize changes in the localization of p57^{Kip2} during STS-induced apoptosis. In order to follow p57^{Kip2} subcellular localization, cells were cotransfected with DsRed-mito to label the mitochondria and the nuclei were labeled with Hoechst 33342. Within about 20 min after STS exposure, a GFP-p57 punctuate cytoplasmic staining pattern with some preference for the perinuclear area was yielded (Figure 7a). The mitochondrial subcellular preference was confirmed by the fluorescence overlapping between GFP-p57 and DsRed-mito

Figure 7 p57^{Kip2} translocates to the mitochondria and promotes STS-induced Bax activation. (a–c) HeLa cells expressing GFP-p57 or GFP-NLS and the mitochondrial marker DsRed-mito were treated with STS and the distribution of GFP fluorescence was followed by time-lapse confocal microscopy, as described in Materials and Methods. (a) Representative sequential confocal images of GFP-p57 (green), mitochondrial DsRed-mito staining (red) and nuclear Hoechst 33342 staining (blue) of HeLa cells after STS treatment. Asterisks indicate the position of cells presenting a punctuate cytoplasmic staining pattern for GFP-p57. Single confocal plane images of GFP-p57 (b) and GFP-NLS (c) expressing HeLa cells respectively 1 and 2 h after STS treatment are depicted. In (b), right panel, the colocalization between GFP-p57 and DsRed-mito staining pattern was analyzed using the Zeiss Software (colocalized pixels are pseudocolored in white). Similar results were obtained from three independent experiments. (d) HeLa-p57 cells were pretreated with Dox for 24 h before treatment with STS for 2 h. Activated membrane-bound form of Bax was detected by the polyclonal anti-Bax antibody clone N20 and Alexa488-conjugated secondary antibody. Nuclei were counterstained with DAPI. Confocal images of immunostaining with active Bax (green) nuclear DAPI staining (blue) of HeLa cells after treatments is depicted

(Figure 7b). The p57^{Kip2} protein contains a nuclear localization signal (NLS).² Therefore, as control, NLS-GFP expressing HeLa cells were challenged with STS and changes in GFP fluorescence were visualized by live confocal fluorescence imaging. Up to 2 h after treatment, GFP fluorescence was restricted to the nuclei compartment, demonstrating that the translocation of GFP-p57 to the mitochondria upon STS apoptotic stimulus was specific (Figure 7c).

Mitochondrial outer-membrane permeabilization, which regulates the release of apoptosis promoting proteins, from the mitochondrial intermembrane space, is controlled by pro- and antiapoptotic members of the Bcl2 family. The antiapoptotic family members Bcl2 and BclxL have been shown to block release of apoptosis promoting proteins, whereas proapoptotic members such as Bax promote the same.¹⁶ Therefore, based on the observed relocalization of p57^{Kip2} to the mitochondria upon STS exposure, we decided to explore

the effect of p57^{Kip2} on Bax activation. Bax activation was investigated in HeLa-p57 cells by confocal immunofluorescence microscopy using antibodies against the active form of this protein.^{16,17} HeLa-p57 cells after Dox addition displayed identical features to control cells for this parameter. In contrast, pretreatment with Dox markedly increased STS-induced Bax activation and relocalization to the mitochondria (Figure 7d).

Requirement of the mitochondrial apoptotic pathways for p57^{Kip2} proapoptotic effects. A change of mitochondrial membrane permeability is essential for apoptosis, leading to translocation of apoptogenic cyt-*c* and AIF into the cytoplasm.¹⁸ The Bcl2 family of proteins regulate $\Delta\Psi_m$ and release of mitochondrial proteins by directly modulating the activity of VDAC through binding.¹⁹ Whereas, Bax alters the physical properties of the VDAC, making the VDAC permeable to cyt-*c*, Bcl2, through its BH4 domain, maintains VDAC in a closed configuration.¹⁹ Since Bcl2 is thought to protect cells from apoptotic death by preventing the dissipation of $\Delta\Psi_m$ and subsequent cyt-*c*/AIF release, we investigated the ability of Bcl2 overexpression to block p57^{Kip2} proapoptotic effects (Figure 8a). For this purpose, HeLa and Bcl2 overexpressing HeLa cells (HeLa-Bcl2) were transfected with p57^{Kip2} expression vector and then treated or not with STS for 5 h. STS-induced apoptosis was severely

reduced in Bcl2 overexpressing cells, indicating that engagement of the mitochondrial-dependent apoptotic pathway account predominantly for STS-killing effect. In addition, whereas p57^{Kip2} expression potentiated STS-induced apoptosis in HeLa cells, its expression had no effect in HeLa-Bcl2 cells. These results suggested that Bcl2 overexpression in HeLa cells inhibits p57^{Kip2} positive effect on STS-induced apoptosis. To notice, Bcl2 overexpression in HeLa cells was also able to suppress p57^{Kip2} promoting effect on VP16- and CP-induced cell death (data not shown). In order to validate that Bcl2 expression effectively prevented mitochondrial dysfunction, cyt-*c* and AIF release into the cytosol were investigated in both cell types (Figure 8b). p57^{Kip2} expression strengthened the release of these mitochondrial proteins into the cytosol in HeLa cells. On the other hand, in HeLa-Bcl2 cells, no cyt-*c* and AIF release were detected upon STS treatment alone or in combination with p57^{Kip2} ectopic expression, demonstrating that Bcl2 indeed blocked the mitochondrial apoptotic pathway. We then examined the consequence of Bcl2 overexpression on caspase-3-like activity (Figure 8c). Again, whereas p57^{Kip2} promoted STS-induced DEVDase activity in HeLa cells, its expression had no effect in HeLa-Bcl2 cells. To notice, STS-induced caspase-3 activity in HeLa-Bcl2 cell line was extensively reduced as compared to HeLa cell line. This observation suggests that STS treatment leads to the

Figure 8 Requirement of the mitochondrial apoptotic pathways for p57^{Kip2} proapoptotic effects. (a–c) HeLa and HeLa-Bcl2 cells were transfected with expression vectors encoding p57^{Kip2} for 24 h and then treated with STS for 5 h. (a) Cells were then processed for immunofluorescence using anti-p57^{Kip2} antibody. Nuclei were counterstained with Hoechst 33342 and apoptosis was evaluated as described in Figure 2b. (b) Cells were harvested after 5 h and cytosol extracts were analyzed by Western blot for the presence of cyt-*c* and AIF. G3PDH was used as standard for equal loading of protein. (c) Caspase-3-like activity was determined by the cleavage of the specific peptide substrate DEVD-AMC as described in Figure 4. (d) HeLa-p57 cells were pretreated with Dox for 24 h and then treated for 5 h with STS, DIDS or their combination. Nuclei were stained with Hoechst 33342 and apoptosis was assayed as described in Figure 1b

activation of two caspases-dependent apoptotic pathways: one dependent on mitochondrial event, which can be inhibited by Bcl2 protein and promoted by p57^{Kip2} and another independent of mitochondrial event, which can therefore not be blocked by Bcl2 or enhanced by p57^{Kip2}. Finally, to further confirm that mitochondria are the target of p57^{Kip2} proapoptotic effect, and since Bcl2 has been shown to inhibit mitochondrial-dependent apoptosis by blocking VDAC,¹⁹ the effect of DIDS, a chemical inhibitor of this mitochondrial porin, was tested (Figure 8d). Results obtained with VDAC inhibition were similar to those obtained with Bcl2 overexpression, as DIDS treatment inhibited p57^{Kip2} positive effect on STS-induced apoptosis. In summary, Bcl2 overexpression or VDAC inhibition resulted in repression of p57^{Kip2} proapoptotic effect.

Discussion

Although p57^{Kip2}-null mutant mice exhibit an increase in the rate of cell death in several cell populations during embryogenesis,^{4–7} whether this increased apoptosis is a direct or indirect effect due to aberrations in cell proliferation and differentiation is still discussed. For example, it seems that in the p57^{Kip2}-deficient retina, apoptosis precisely compensates for the inappropriate proliferation to preserve the correct proportion of the major retinal cell types.⁶ In contrast, p57^{Kip2} promotes differentiation of dopaminergic neurons *in vivo* after they have already exited the cell cycle, and occurrence of cell death in the p57^{Kip2} mutant ventral midbrain can probably be attributed to the deficient late maturation of dopamine cells.⁷ Taken together, these observations suggest that p57^{Kip2} exerts tissue-specific effects during development.

Interestingly during adulthood, it has also been reported that p57^{Kip2} expression is reduced in various tumors,^{8–12,20–24} suggesting a possible function for this protein in tumorigenesis. Importantly, decreased p57^{Kip2} expression may provide an important prognostic implication for patients with ovarian, hepatocellular and colorectal cancers, and acute lymphoblastic leukemia.^{8–10,23} In spite of frequent low/absence of p57^{Kip2} expression in various tumors, rare/no somatic mutations of p57^{Kip2} gene are found in these tumors.^{8–12} These observations suggest that inactivation of p57^{Kip2} gene expression, rather than mutations in the p57^{Kip2} gene, accounts for the involvement of p57^{Kip2} in other types of cancer. Accordingly, inactivation of p57^{Kip2} by regional promoter hypermethylation and histone deacetylation has been suggested in human tumors.^{25,26} Finally, several apoptotic stimuli, including treatment with hypomethylating agents, histone deacetylase inhibitors²⁷ or p73beta overexpression²⁸ have been shown to induce p57^{Kip2} expression. In the case of p73beta-mediated apoptosis, silencing p57^{Kip2} considerably reduced cell death, suggesting that p57^{Kip2} was required.²⁸

Although p57^{Kip2} has been suggested to be a tumor suppressor gene, being inactivated in various types of human cancers, its precise involvement in apoptosis in response to cytotoxic drugs or during tumorigenesis is unclear.

To further elucidate these issues, the effects of ectopic p57^{Kip2} expression alone or in the presence of the cytotoxic drug STS on the apoptotic execution machinery were investigated. STS-induced mitochondrial disruption leads to

the activation of the cyt-c/caspase-9/caspase-3 mitochondrial-dependent apoptotic pathway in HeLa cells. Experiments using the broad-spectrum inhibitor of caspases, zVAD-fmk, and the VDAC inhibitor, DIDS, or Bcl2 overexpression, also revealed that caspases are required for STS-induced apoptosis in HeLa cells, and that mitochondrial dysfunctions such as loss of $\Delta\Psi_m$ and cyt-c release account mostly for the STS-killing effect. Our results suggest that the CKI p57^{Kip2} bears proapoptotic properties, which are independent of its ability to inhibit CDK activity. p57^{Kip2} proapoptotic effects were observed in several cell types (HeLa, SH-SY5Y, SKOV3 and MEF). However, these effects could be cell type specific. In fact, it has previously been reported that p57^{Kip2} expression inhibits green tea polyphenol-induced apoptosis in oral carcinoma cells²⁹ or suppresses UV- and MEKK1-induced apoptotic cell death in HEK-293 cells.³⁰ However, to our surprise, in our hands, p57^{Kip2} expression did not prevent UV-, STS- and CP-induced apoptotic cell death in HEK-293 cells. The cell type-specific effect may be related to differences in the stability of p57^{Kip2} within cells or the number of cell survival pathways that are active in a particular cell type.

As apoptosis is a cell type-specific process, the ability to resist p57^{Kip2} death promoting effect may, at least, be partially, attributed to cell- and tissue-specific differences in the function of apoptotic and antiapoptotic signal transduction pathways. The abundance of p57^{Kip2} is thought to be controlled by the ubiquitin–proteasome pathway. Phosphorylation at threonine residue (Thr-310) of human p57^{Kip2} is required for Skp2-mediated ubiquitylation and proteolysis.³¹ Therefore, potential differences in p57^{Kip2} post-translational modifications could in principle also influence its function in a cell type-specific manner. To notice, cell type specificity effects on apoptosis have also been reported for another member of the Cip/Kip family, p27^{Kip1}. Indeed, overexpression of this CKI has been shown not only to protect HeLa cells from STS-induced apoptosis but also to induce apoptosis in several transformed mammalian cell lines.³²

Furthermore, it comes into view that p57^{Kip2} exerts its proapoptotic effect on the mitochondrial-dependent apoptotic pathways and has no direct effect on the mitochondrial-independent ones. The conclusion that p57^{Kip2} sensitizes cells to mitochondrial-dependent stimulus of apoptosis is supported by several observations. First, p57^{Kip2} expression improved STS-induced cyt-c release into the cytosol. In addition, whereas STS caused the activation of several caspases, p57^{Kip2} expression only supported at early time point caspase-9 and caspase-3 like activities. These data suggest an improvement of the mitochondrial cyt-c/caspase-9/caspase-3-activated apoptotic pathway in p57^{Kip2} expressing cells. Second, p57^{Kip2} expression not only increased the fraction of cells presenting a low $\Delta\Psi_m$ after STS treatment but also accelerated this mitochondrial apoptotic event, as p57^{Kip2} expressing cells lose their $\Delta\Psi_m$ faster than the non-expressing ones. Third, translocation of p57^{Kip2} from the nucleus to the mitochondria was detected in live HeLa cells within 20 min after STS exposure. In fact, cytoplasmic localization for p57^{Kip2} has been reported in non-small-cell lung carcinoma²⁰ and esophageal squamous cell carcinoma.³³ In addition, during osteoblast differentiation, cytoplasmic p57^{Kip2} was found to inhibit LIM-kinase 1, a kinase involved in actin

filament dynamics, by translocating and sequestering it in the nucleus.^{34,35} Possible cytoplasmic functions for the other Cip/Kip CKIs have also been described.^{32,34} For example, p21^{Cip1} and p27^{Kip1} also appear to be implicated in actin dynamics, at least partially, through regulation of the RhoA pathway.³² Another important role for cytoplasmic p21^{Cip1} in the protection of cells against apoptosis has been proposed.³⁴ p21^{Cip1} can relocate to the mitochondria, where it interacts with procaspase-3 to inhibit caspase 3 activation and to resist Fas death receptor-induced cell death.^{32,36}

Finally, p57^{Kip2} expression appears to facilitate Bax activation and translocation to the mitochondria upon STS treatment. Whether the translocation of p57^{Kip2} to the mitochondria is directly related to the activation of Bax activation still awaits additional experiments. However, in agreement, Bcl2 overexpression, which is thought to protect cells from apoptotic death by preventing the dissipation of $\Delta\Psi_m$ and subsequent cytochrome c/AIF release, totally abrogated p57^{Kip2} proapoptotic property. Since Bcl2 has been shown to prevent mitochondrial-dependent apoptosis by blocking VDAC,¹⁹ the effect of VDAC inhibition by DIDS was looked at. DIDS treatment inhibited p57^{Kip2} positive effect on STS-induced apoptosis in HeLa cells. Altogether, these results demonstrated that the presence of p57^{Kip2} sensitizes cells to apoptotic stimuli involving mitochondrial pathways.

The cytotoxic effects of many anticancer drugs are mediated via the apoptotic pathways. Chemoresistant tumor cells have acquired the ability to evade the action of multiple classes of anticancer drugs. Mitochondrial membrane permeabilization is a critical event in the process leading to physiologic or chemotherapy-induced apoptosis.³⁷ Since mitochondria are critical 'gatekeepers' to the apoptosis process, uncovering their biological regulators and better understanding of mitochondrial cell death pathways may provide insights into sensitivity or resistance of tumor cells. Moreover, this knowledge could be further exploited to restore efficient apoptotic pathways in order to increase sensitivity of tumor cells to therapy.

Materials and Methods

Plasmids. pcDNA₃-HA-p57^{Kip2} and pcDNA₃-HA-p57CDK^{mut} were gifts from Y Xiong (University of North Carolina). The CDK/cyclin binding mutant used (p57^{CKmut}) contains two substitutions (R31A and F34D) in the cyclin binding domain and two (W61A and F65D) in the CDK binding region. GFP-p57^{Kip2} NLS-GFP, DsRed-mito and CDK2 were gifts from, respectively, JC Cross (Samuel Lunenfeld Research Institute), A Vartapetian (Moscow State University), G Kochs (University of Freiburg) and LG Larsson (Swedish University of agricultural sciences). GFP plasmid was from Clontech.

Cell culture, transfection and treatments. Cervical carcinoma HeLa, HeLa-Bcl2, ovarian carcinoma SKOV3, neuroblastoma SH-SY5Y and human embryonic kidney HEK-293 cells were cultured and transfected as described.^{7,38} MEF cells derived from wild-type and p57^{Kip2}^{-/-} mice (gifts from CL Stewart, National Cancer Institute) were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml). The cell line derived from HeLa cells in which p57^{Kip2} expression is under control of tetracycline, HeLa-p57, and HeLa-ctrl, used as negative control, have been previously described (gifts from S Okret, Karolinska Institute). A 2 μ g/ml volume of Dox was added to the culture medium to induce expression of p57^{Kip2} in Tet-On cell line. HeLa-Bcl2, a stable, Bcl2 expressing cell line derived from HeLa cells transfected with a Bcl2 expression plasmid, has been described previously (gift from DJ McConkey, MD Anderson Cancer Center). Transfections were performed with LipofectAMINE

PLUS according to the manufacturer's protocol. As control, cells were transfected with an empty vector. Twenty-four hours after transfection, cells were treated with 0.1 μ M STS (Sigma), 20 μ g/ml etoposide (VP16, Bristol-Meyers), 10–20 μ M CP (Bristol-Meyers) or 200 ng/ml agonistic anti-Fas Abs clone CH-11 (α Fas, Medical & Biological Laboratories Ltd.) for the indicated time period. For time-lapse experiments and cytosol extract preparation, cells were treated with 0.2 μ M STS. Caspase inhibitors zVAD-fmk, DEVD-fmk, LEHD-fmk and VDVAD-fmk, used at 10 μ M, were purchased from Enzyme Systems Products and added 1 h before STS treatment. The VDAC blocker, DIDS, used at 300 μ M, was obtained from Sigma. The cell density was maintained at levels allowing exponential growth.

Detection of apoptosis. After treatment, cells were fixed in 4% paraformaldehyde and subsequently DNA was stained with Hoechst 33342 (0.1 μ g/ml, Molecular Probes). The number of apoptotic cells was measured quantitatively by assessing the percentage of cells with fragmented or condensed nuclei.

Measurement of caspase activity. The measurement of DEVD-AMC, VDVAD-AMC, IETD-AMC or LEHD-AMC (Peptide Institute Inc.) cleavage was performed in a standard reaction buffer using a modified version of a fluorometric assay reported previously.³⁸ Cleavage of the fluorogenic peptide substrates was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems) using 355 nm excitation and 460 nm emission wavelengths.

Assessment of mitochondrial depolarization. For detection of mitochondrial membrane potential change, harvested cells were stained by TMRE, a lipophilic, cationic fluorochrome dye that is only taken up by mitochondria having an intact electrochemical gradient (Molecular Probes), for 30 min at 37°C and then analyzed by flow cytometry on a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson).

Subcellular fractionation, immunoprecipitation and Western blot analysis. Total, cytoplasmic and nuclear protein extracts were prepared as reported.⁷ For immunoprecipitation, protein Sepharose-purified total protein extracts were incubated with the indicated antibody in nuclear extract buffer for overnight at 4°C. Immunocomplexes bound to protein A-Sepharose or protein G-Sepharose were collected by centrifugation and washed in radio-immunoprecipitation assay (RIPA) buffer. For immunoblot analysis, cell extracts were resolved on SDS-PAGE and blotted; protein fragments were detected with anti-AIF, anti-p57^{Kip2} C20 (Santa Cruz Biotechnology), anti-cleaved caspase-3 (Cell Signaling), anti-PARP or anti-cyt-c (BD Pharmingen) antibody. Immunoblot with anti-glyceraldehyde-3-phosphate dehydrogenase (anti-G3PDH) antibody (Trevigen) was used for standardization of protein loading. Bands were visualized by enhanced chemiluminescence (ECL Plus) following the manufacturer's instructions (Amersham).

CDK activity assay. For CDK2 kinase assay, cell lysates (500 μ g) were immunoprecipitated with anti-CDK2 M2 antibody (Santa Cruz Biotechnology). The immunoprecipitate was incubated with histone H1 (CDK kinase assay kit; Upstate Biotechnology) in the presence of p32-labeled γ -ATP for 10 min at 30°C. The reaction products were separated by SDS-PAGE and phosphorylated histone H1 proteins were detected by autoradiography and quantified by ImageJ software.

Cell cycle distribution analysis by flow cytometry. The distribution of cells in the G1, S and G2/M cell cycle phases was determined by DNA flow cytometry as described earlier.³⁸ Cells were harvested and fixed with ice-cold 70% ethanol for 1 h. Samples were then washed with PBS and stained with propidium iodide (PI) in presence of RNase A. FACS analysis was carried out using on a FACSCalibur.

Immunofluorescence, confocal microscopy and time-lapse recording. Anti-p57^{Kip2} C20 and anti-active Bax N20 antibodies (Santa Cruz Biotechnology) were used for immunodetection. Alexa Fluor 488-conjugated anti-IgG was used as secondary antibody (Molecular Probes). Paraformaldehyde-fixed cells and sections were blocked in Hepes/3%BSA/0.3%triton and successively incubated with primary (4°C, 16 h) and secondary antibodies (room temperature, 1 h). Cell nuclei were counterstained with 1 μ g/ml Hoechst 33342. Mounting was performed with VECTASHIELD (Vector Laboratories).

For time-lapse recording of $\Delta\psi_m$ in live cells, HeLa cells were transfected with GFP or GFP-p57, then mixed with control cells (ratio 1:1) and placed on glass chamber slide. Mitochondria presenting an intact $\Delta\psi_m$ were labeled with the potential-dependent dye TMRE. To investigate the subcellular localization of p57^{Kip2} in live cells, HeLa cells were transfected with GFP-p57 or GFP-NLS, together with DsRed-mito. In both experiments, cell nuclei were counterstained with 0.1 μ g/ml Hoechst 33342. Chambers were then placed in the POC-chamber/CTI Controller/Heating insert P system for live cell imaging. Time-lapse images were then acquired with 5 min intervals. Samples were analyzed with a Zeiss 510 Meta confocal laser scanning microscope equipped with an inverted Zeiss Axiovert 200 m microscope. Mix dyes were acquired by sequential multiple channel fluorescence scanning to avoid bleeds through. In addition, for accurate colocalization, each pinhole was adjusted so that each channel has the same optical slice. Pixel colocalization was analyzed using the colocalization module from the Zeiss LSM Image Examiner software.

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