PP2A: B56ε, a Substrate of Caspase-3, Regulates p53-dependent and p53-independent Apoptosis during Development*

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Protein phosphatase 2A (PP2A) is one of the most abundantly expressed serine/threonine protein phosphatases. A large body of evidence suggests that PP2A is a tumor suppressor and plays critical roles in regulating apoptosis. PP2A is a heterotrimeric protein complex. Its substrate specificity, localization, and activity are regulated by regulatory subunits of PP2A. A recent study has demonstrated that single nucleotide polymorphism in B56ε (PPP2R5E), a B56 family regulatory subunit of PP2A, is associated with human soft tissue sarcoma. This raises the possibility that B56ε is involved in tumorigenesis and plays important roles in regulating apoptosis. However, this hypothesis has not been tested experimentally. Our previous studies revealed that B56ε regulates a number of developmental signaling pathways during early embryonic patterning. Here we report novel functions of B56ε in regulating apoptosis. We provide evidence that B56ε has both anti- and pro-apoptotic functions. B56ε suppresses p53-independent apoptosis during neural development, but triggers p53-dependent apoptosis. Mechanistically, B56ε regulates the p53-dependent apoptotic pathway solely through controlling the stability of p53 protein. In addition to its function in regulating apoptosis, we show that B56ε undergoes proteolytic cleavage. The cleavage of B56ε is mediated by caspase-3 and occurs on the carboxyl side of an evolutionarily conserved N-terminal “DKXD” motif. These results demonstrate that B56ε, a substrate of caspase-3, is an essential regulator of apoptosis. So far, we have identified an alternative translation isoform and a caspase cleavage product of B56ε. The significance of post-transcriptional regulation of B56ε is discussed.

After decades of research since the discovery of protein phosphorylation, it is now appreciated that reversible protein phosphorylation is one of the major mechanisms for regulating cellular behavior. Reversible protein phosphorylation is executed by protein kinases and protein phosphatases. The human genome contains more than 500 protein kinase genes (1), but only a small number of protein phosphatases. Interestingly, many protein phosphatases are multimeric enzymes, with a few catalytic subunits being associated with many different regulatory subunits. This gives rise to sufficient numbers of protein phosphatases with different catalytic activity, substrate specificity, or intracellular localization to counteract the activities of protein kinases. Protein phosphatases 2A (PP2A) is one of the most abundantly expressed Ser/Thr protein phosphatases. PP2A holoenzymes consist of a catalytic subunit (PP2Ac), a structural subunit (PP2A α), and a regulatory subunit (B). Although PP2Ac exhibits fairly poor specificity in vitro, its activity in vivo is tightly regulated by multiple families of PP2A regulatory subunits, including B55/B, B56/B′, and PR72/B′. Investigation into individual regulatory subunits is central to understanding the mechanisms by which PP2A functions in a variety of cellular events (2–4).

PP2A is a tumor suppressor and is targeted by many tumor inducers (5–8), ranging from endogenous or viral proteins, such as SET (9) or SV40 small t antigen (10–13), to chemical compounds, including okadaic acid (14). These tumor inducers either inhibit the activity of PP2A or disrupt holoenzyme assembly of PP2A. Consistently, mutations in the structural A subunit have been identified in a range of tumors (15–21). The role of PP2A in tumor suppression appears to be complicated. Accumulating evidence demonstrates that interfering with PP2A may result in errors in chromosome segregation (22–25), genomic instability (26), and insensitivity to apoptotic signals (5–8). At the molecular level, one of the most important pathways regulated by PP2A is the p53-dependent apoptotic pathway. In this pathway, p53 regulates the balance between the pro- and anti-apoptotic BCL family members through transcription-independent (27, 28) or transcription-dependent (29, 30) mechanisms. In the presence of a variety of stress signals, p53 protein is post-translationally modified and becomes stabilized, leading to the activation of pro-apoptotic BCL family members. This results in mitochondrial membrane permeabilization, cytochrome c leakage, and ultimately activation of executioner caspases. PP2A regulates this pathway at multiple levels. It was noted that PP2A could dephosphorylate BCL2 and prevent its proteasome-dependent degradation (31). PP2A could also function upstream of mitochondria and regulate p53 directly (32, 33) or indirectly through controlling the activity of MDM2 (34–36), the E3 ubiquitin ligase that triggers the proteasomal degradation of p53.

B56ε belongs to the B56/B′ regulatory subunit family (37), which plays critical roles in regulating apoptosis (38–43). Our

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2 The abbreviations used are: PP2A, protein phosphatases 2A; SNP, single nucleotide polymorphism; EGT, early gastrulation transition; p53 mor, morpholino against p53.
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previous studies demonstrate that B56e regulates a number of patterning events, including axis specification, eye induction, eye field separation, midbrain-hindbrain boundary formation, and floor plate development. We were able to assign these functions of B56e into the Wnt, Hedgehog, and insulin-like growth factor pathways (44–46). Strikingly, a recent study, which investigated human single nucleotide polymorphisms (SNP) that affect p53 signaling, identified a link between B56e and human soft tissue sarcoma (47). Although it remains unclear how e-SNP2 alters the expression of B56e, it raises the possibility that B56e plays an important role in regulating the p53-dependent apoptotic pathway. Interestingly, it has been noted that depletion of both B56 family members in Drosophila induces apoptosis in a p53-dependent manner (38, 39). Triggered by these observations, we analyzed the function of B56e in regulating apoptosis during Xenopus development. Our data provides the first direct experimental evidence that vertebrate B56e is involved in apoptotic pathways. We show that B56e has anti- and pro-apoptotic functions during development. B56e suppresses p53-independent apoptosis, but plays a positive role in stabilizing p53 and triggering p53-dependent apoptosis. Interestingly, B56e undergoes caspase-3-dependent cleavage. The cleavage occurs on the carboxyl side of an evolutionarily conserved N-terminal “DKXD” motif and results in a less stable form of B56e. These results demonstrate that B56e, a substrate of caspase-3, is an essential regulator of apoptosis.

EXPERIMENTAL PROCEDURES

Plasmids and Morpholinos—Myc-EGFP, ε-FLAG, and myc-ε-FLAG were described previously (44, 46). To construct ε-cle, PCR was performed using ε-FLAG as the template. Primers were T3 primer and a specific B56e primer (5’-acagaattcag-gatcttcggaattcagcata-3’). The PCR product was digested with EcoRI/NotI and cloned into pcS2-MT. D15A and D53A were generated by the standard site-directed mutagenesis. pcS2-myc-p53 and pcS2-myc-MDM2 were generated by the standard PCR-based cloning method. The open reading frames of Xenopus p53 were PCR amplified (5’-ctcgaattcaggaaccttt-cttctc-3’ and 5’-tctgctgaatcattcgag-3’) and cloned between EcoRI and XbaI sites of pcS2-MT. The ORF of human MDM2 was amplified (5’-gacatcggacctgaatcattcag-3’ and 5’-ggggaatcggacctgaatcattcag-3’) and cloned between EcoRI and XbaI sites of pcS2-MT. Morpholinos against B56e and p53 (Gene Tools, Philomath, OR) were previously reported (46, 48).

Embryo and Oocyte Manipulations and Cell Culture—Xenopus embryos and oocytes were obtained and microinjected as described (49). The dosage of RNAs and morpholinos for microinjection are listed in the figure legends or text. For hydroxyurea treatment, embryos were cultured in 0.2× MMR (49), containing 30 mM hydroxyurea. To inhibit transcription in embryo, fertilized eggs were injected with 100 ng of α-amanatin. To block caspase activation in embryo, various doses of Ac-DEVD-CHO (Sigma) was injected at the one-cell stage. HeLa, Neuor2A, SW 480, and NIH3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Cells were cultured at 37 °C in a humified, 5% CO2 atmosphere. For cisplatin treatment, cells were exposed to 75 μg cisplatin for various times and harvested for Western blot analysis.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay and RT-PCR—Whole mount TUNEL assay was performed as described (50, 51). RT-PCR was performed according to the standard protocol. ODC primers were reported in Ref. 45; primers for p53 are: 5’-CCCCAGACCTCAACAAACTCTAC-3’ (up) and 5’-GGGCATCTCTTACCACCTAC-3’ (down).

Microcystin Pulldown, Co-immunoprecipitation, and Western Blots—Microcystin pulldown was performed as described (52). Co-immunoprecipitation and Western blots were performed according to standard protocols (44, 45). Antibodies used were: anti-FLAG (M2, Sigma, 1:1,000), anti-Myc (9E10, Sigma, 1:1,000), anti-B56e (1:500) (44), anti-PP2Aα (Upstate 05-421, 1:2,000), anti-PP2Aα (Upstate number 05-657, 1:500), anti-Tubulin (Sigma T5293, 1:1,000), anti-P53 (X77) (Santa Cruz sc-56186, 1:200), anti-P53 (DO1) (Santa Cruz sc-126, 1:500); anti-GAPDH (Chemicon mAB374, 1:1,000), and anti-Caspase-3 (Cell Signaling number 9662, 1:1,000). The quantification of Western blots was done by densitometric analysis using TotalLab Nonlinear Dynamic Image analysis software (Nonlinear Dynamics Inc., Durham, NC).

RESULTS

B56e Suppresses Apoptosis during Xenopus Head Development—B56e regulates multiple signaling pathways during neural development (45, 46). We previously noticed that depletion of B56e in the neural ectoderm led to lethality at the tadpole stage (46). Triggered by this observation, we investigated the functions of B56e in regulating cell survival and death during development.

To knockdown B56e, we took advantage of e-mor, a morpholino antisense oligo that blocks the translation of Xenopus B56e (46). At the 16-cell stage, 5 ng of e-mor was bilaterally injected into both dorsal animal blastomeres, which later give rise to the anterior neural ectoderm (53). At the tadpole stage, brain development was severely disrupted in the majority of e-mor-injected embryos. Morphologically, 61.5% of embryos exhibited obvious cell death phenotype as judged by the appearance of a large number of cells shedding from the head region. Many of these embryos had large holes in their heads as the consequence of cell death (Fig. 1B, arrow). Some embryos (17.9%) were affected less severely. Although they did not show the cell death phenotype, these embryos formed very small heads (Fig. 1C). These phenotypes were rescued by co-injection of 20 pg of ε-c, which lacks the e-mor binding sequence (46). Among 34 injected embryos, 61.7% formed relatively normal heads (Fig. 1C) and 14.7% exhibited small heads (Fig. 1C’). Only 23.5% of embryos showed the death phenotype. This demonstrates that these phenotypes are specific to the loss of B56e. A TUNEL assay was performed to confirm that these embryos indeed underwent apoptosis. At the tailbud stage, many TUNEL-positive cells were detected in the developing head region in e-mor-injected embryos (Fig. 1E), whereas only a
few cells were positive for TUNEL staining in control embryos (Fig. 1D). These results indicate that B56ε is required for cell survival during head development.

B56ε Stabilizes p53 and Induces p53-dependent Apoptosis during Xenopus Embryonic Development—In Drosophila, depletion of B56ε family members induces p53-dependent apoptosis (38, 39). We thus examined the effect of B56ε depletion on p53. Fertilized eggs were injected with 20 ng of e-mor. Control and injected embryos were harvested at various stages and subjected to Western blot analysis of endogenous p53. Surprisingly, knockdown of B56ε resulted in a clear decrease in the level of endogenous p53 (Fig. 2A). This effect was observed in all stages of embryos analyzed. This suggests that B56ε stabilizes p53 during embryonic development. Our previous studies demonstrate that depletion of B56ε impairs embryonic patterning (45, 46). It is possible that down-regulation of p53 in B56ε-depleted embryos is an indirect consequence of defective pattern formation. We thus examined the effect of B56ε depletion on p53 in Xenopus oocytes. Because the expression level of endogenous p53 in oocytes is below the limit of detection, we overexpressed a myc-tagged p53. Full-grown oocytes were injected with e-mor (20 ng) and cultured for 2 days. Subsequently, a mixture of myc-p53 and myc-EGFP was injected into control or e-mor-injected oocytes. Oocytes were harvested 24 h later and analyzed by Myc Western. As shown in Fig. 2B, depletion of B56ε decreased the level of myc-p53 in oocytes. These results demonstrate that B56ε protein levels affect the stability of p53.

In addition to the above loss of function studies, we performed gain of function experiments. Indeed, overexpression of B56ε increased the level of endogenous p53 protein dose dependently (Fig. 2C). This effect was observed as early as the late blastula stage (stage 9). To determine whether B56ε regulates the transcription of p53, we measured the level of p53 mRNA by RT-PCR. As shown in Fig. 2D, overexpression of B56ε had no effect on the transcription of p53. In fact, we were able to show that injection of α-amanitin, an inhibitor of RNA polymerase II, did not block the effect of overexpressed B56ε on p53 (Fig. 2E). Taken together, we conclude that B56ε plays a positive role in stabilizing p53.

Given that depletion of B56ε causes cell death in the developing head at the tadpole stage, the observation that B56ε stabilizes p53 was unexpected. Accumulation of p53 often induces apoptosis. We thus analyzed the effect of B56ε overexpression on cell survival. RNA encoding myc-e-FLAG (250 pg) was injected into fertilized eggs. At the late gastrula stage (stage 13), all injected embryos exhibited typical apoptosis phenotypes and lost their structural integrity. Morphologically, cells in these embryos lost intercellular contacts. Cells from the interior of the embryo were deteriorated.

FIGURE 1. B56ε suppresses apoptosis during neural development. A, control un-injected tadpole. B and B’, e-mor-injected tadpoles. e-mor was injected into both dorsal animal blastomeres at the 16-cell stage. In a representative experiment, 24 injected embryos exhibited cell death phenotypes (B), as judged by shedding of cells from the head of the embryo (indicated by the arrow). 7 injected embryos developed very small heads (B’). The size of the remaining 8 embryos was relatively normal (not shown). C and C’, tadpoles injected with e-mor and e-C, which lacks the e-mor binding sequence. Among 34 injected embryos, 8 embryos showed cell death phenotypes (not shown). 21 embryos developed normal heads (C) and 5 embryos had small heads (C’). D and E, TUNEL staining results show apoptosis in a control embryo (D) and an embryo injected with e-mor (E). The arrowhead in E points to TUNEL-positive cells in the anterior neural ectoderm in an e-mor-injected embryo.

FIGURE 2. B56ε stabilizes p53. A, Western blot result shows that depletion of B56ε decreased the protein level of endogenous p53. Fertilized eggs were injected with 20 ng of e-mor. Control and injected embryos were harvested at various stages and subjected to Western blot analysis for the expression of p53, B56ε, and tubulin. B, Western blot results show that depletion of B56ε decreased the protein level of overexpressed myc-p53 in oocytes. Full-grown Xenopus oocytes were injected with 20 ng of e-mor. Two days later, a mixture of myc-p53 and myc-EGFP was injected into control or e-mor-injected oocytes. Oocytes were harvested 24 h later and analyzed by Myc Western blots. Myc EGFP was a control for injection and loading. C, Western blot results show that overexpression of a C-terminal FLAG-tagged B56ε enhances the expression of p53 in Xenopus embryos. D, RT-PCR shows that overexpression of B56ε had no effect on the mRNA levels of p53. E, Western blot results show that inhibition of RNA polymerase II by α-amanitin does not block B56ε-induced p53 accumulation.
extruded. These embryos contained a large number of TUNEL-positive cells (Fig. 3A), indicating that overexpression of B56ε indeed triggers apoptosis. Because overexpression of B56ε stabilizes p53 (Fig. 2), we further determined whether depletion of p53 could rescue apoptosis induced by B56ε. To knock down p53, 20 ng of morpholino against p53 (p53 mor) (48) was injected into fertilized eggs. Subsequently, myc-e-FLAG (250 pg) was injected into control and p53 mor-injected embryos. As expected, all myc-e-FLAG-injected embryos (n = 41) underwent apoptosis at the late gastrula stage. In contrast, knockdown of p53 suppressed apoptosis in 56% (n = 48) of injected embryos (Fig. 3B). This demonstrates that apoptosis induced by B56ε is p53-dependent. It is important to mention that injection of p53 mor failed to rescue the cell death phenotype in e-mor-injected embryos (data not shown). Thus, despite B56ε being required for suppressing p53-independent apoptosis during head development, it plays a positive role in triggering p53-dependent apoptosis in the embryo. Because depletion of B56ε destabilizes p53, we evaluated the effect of B56ε knockdown on p53-induced apoptosis. At the one-cell stage, p53 RNA was injected into control embryos and embryos previously injected with 20 ng of e-mor. p53-injected embryos began to show an apoptotic phenotype ~45 min after the beginning of gastrulation (stage 10). At the midgastrula stage (2 h after stage 10), all p53-injected embryos exhibited an apoptotic phenotype. To better quantify p53-induced apoptosis, we assayed A50, the time at which 50% of embryos exhibit an apoptotic phenotype. p53-injected embryos reached A50 at 55 min after stage 10. Depletion of B56ε clearly delayed the onset of apoptosis induced by p53, with the A50 of 130 min after stage 10 (Fig. 3D). These results, together with the observation that B56ε stabilizes p53, demonstrate that B56ε is an important regulator of the p53-dependent apoptotic pathway.

Caspase-dependent Cleavage of B56ε—Strikingly, during the above studies, we noticed that the full-length B56ε protein could be cleaved and that the cleavage is differentially regulated during development. Consistent with our previous report (44), when analyzed at the blastula stage, overexpression of Myc-e-FLAG in Xenopus embryo produced one polypeptide. The molecular mass of the polypeptide is ~75 kDa, which corresponds to the calculated molecular mass of Myc-e-FLAG. However, when analyzed at the early neurula stage, the amount of the 75-kDa polypeptide decreased and an additional polypeptide was detected. This polypeptide migrated slightly faster than the 56-kDa full-length B56ε protein. Based on its migration rate, the molecular mass of this polypeptide is 54 kDa. We named this 54-kDa polypeptide as e-cle. Myc-e-FLAG carries 6 copies of myc tags at the N-terminal of B56ε. To rule out the possibility that addition of myc tags causes the cleavage of B56ε artificially, we analyzed e-FLAG, which has the native N terminus of B56ε (Fig. 4A). Prior to gastrulation, we detected only the full-length e-FLAG and e-s, the alternative translation isoform of B56ε (44). At the stage 14, however, e-cle was detected, demonstrating that this cleavage event is not an artifact (Fig. 4B). Because the cleavage of B56ε is differentially regulated, we analyzed the time course of B56ε cleavage. Myc-e-FLAG RNA (100 pg) injected embryos were harvested at various time points from the beginning of gastrulation and assayed by FLAG Western blotting. As shown in Fig. 4C, e-cle became detectable 45 min after the beginning of gastrulation. This demonstrates that proteolytic processing of B56ε begins at the beginning of gastrulation.

Elegant studies by Newport and colleagues (54, 55) have established that the early gastrulation transition (EGT) occurs at the beginning of gastrulation. The EGT is a critical developmental stage at which regulation of embryonic cellular processes is transferred from maternal to zygotic control (54, 55). Prior to the EGT, the apoptotic pathway is maintained in an
in inactive state by maternally deposited apoptosis inhibitors (54, 56–58). The EGT represents the earliest time during Xenopus development at which caspases can be activated by stresses (54, 56–58). Because the cleavage of B56ε occurs at the EGT, we hypothesized that the cleavage of B56ε is caspase-dependent.

Caspases can be activated by the intrinsic or extrinsic pathways. Stress and other pro-apoptotic stimuli cause mitochondrial leakage and ultimately result in activation of caspase-9 and caspase-3. Activation of caspase-9 and caspase-3 can be prevented by overexpression of BCL2 (59). In the case of the extrinsic pathway, caspase-8 becomes activated when death receptors bind to their ligands. Activation of caspase-8 can be inhibited by cFLIP (60). To determine whether the cleavage of B56ε is caspase-dependent, we first examined the effects of BCL2 or cFLIP overexpression on the production of ε-cle. As shown in Fig. 5A, overexpression of 250 pg of BCL2 blocked the production of ε-cle completely, whereas overexpression of cFLIP had little if any effect. This raises the possibility that ε-cle is a product of caspase-9/3-dependent cleavage. To test this hypothesis, we asked whether cleavage of B56ε could occur in the presence of Ac-DEVD-CHO, a peptide inhibitor of caspase-3/7 that also inhibits caspase-9 with a lower potency. Thus, Myc-ε-FLAG RNA (100 pg) was injected into embryos that were previously injected with various amounts of Ac-DEVD-CHO (0.01, 0.1, 1, and 10 ng). Indeed, production of ε-cle after the EGT was blocked by Ac-DEVD-CHO in a dose-dependent manner (Fig. 5B). In addition to these in vivo studies, we also carried out an in vitro caspase assay. Myc-ε-FLAG-injected embryos were harvested at stage 9. Cleared lysate, which contained overexpressed Myc-ε-FLAG protein, was incubated with purified human caspase-3 protein. After incubation at room temperature for 30 min, a FLAG Western blot was performed. As shown in Fig. 5C, incubating Myc-ε-FLAG protein with caspase-3 results in the cleavage of B56ε. Based on the above in vivo and in vitro studies, we conclude that B56ε is a substrate of caspase-3.

Knowing that B56ε undergoes caspase-dependent cleavage, we wanted to identify the cleavage site of B56ε. The N terminus of B56ε is highly conserved from Drosophila to mammals. Two putative caspase cleavage sites were found at the N terminus of B56ε, including “DKL/VD” and “LLKD” (Fig. 5D). Because the aspartic acid residue is essential for caspase-dependent cleavage, we replaced Asp-15 and Asp-53 in each motif with alanine residues. RNAs encoding D15A and D53A were injected into the embryo. Interestingly, B56ε was no longer cleaved when D15A was overexpressed, whereas overexpression of D53A still resulted in the production of ε-cle (Fig. 5E). This demonstrates that the DKL/VD motif at the N-terminal of B56ε is the cleavage site.

The above studies were performed in Xenopus embryos. We further extended our investigation into mammalian cells. Indeed, when overexpressed in HeLa cells, Myc-ε-FLAG, but not D15A, was proteolytically processed. As expected, overexpression of Myc-ε-FLAG or D15A increased the level of p53 (Fig. 5F). This indicates that caspase-dependent cleavage of B56ε is evolutionarily conserved.

Cleavage of Endogenous B56ε—To determine whether endogenous B56ε is cleaved, we examined expression of the endogenous B56ε protein in Xenopus embryo and mammalian cell lines. Using an affinity purified polyclonal anti-B56ε antibody, we analyzed the expression of B56ε during Xenopus development. Consistent with our previous report (44), we detected the expression of the full-length form (ε-fl, 56 kDa) and the alternative translation isoform (ε-s, 48 kDa) of B56ε. Surprisingly, ε-cle was not detected from the one-cell stage to tadpole stages (Fig. 6A). Because the cleavage of B56ε is caspase-dependent, we asked whether cleavage of endogenous B56ε could be induced by activation of the intrinsic apoptotic pathway. Three approaches were used to activate the intrinsic apoptotic pathway in Xenopus embryos. These include injection of α-amanitin (100 ng) (57), hydroxyurea (30 mM) treatment (54), and overexpression of p53 (500 pg). As expected, embryos were healthy morphologically prior to the EGT. When analyzed at stage 9, endogenous B56ε remained intact in control and experimental groups. After the EGT (stage 12), however, a large amount of ε-cle was detected in embryos injected with α-amanitin or p53, or treated with hydroxyurea (Fig. 6B). This demonstrates that activation of the intrinsic apoptotic pathway induces caspase-dependent cleavage of endogenous B56ε.
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Cleavage of endogenous B56ε cannot be detected in embryos under physiological conditions, whereas much of the overexpressed B56ε undergoes caspase-dependent cleavage. Interestingly, B56ε plays a positive role in stabilizing p53, which is capable of activating caspase-3. This raises the possibility that cleavage of overexpressed B56ε is caused by accumulation of p53 in B56ε-overexpressed cells. Thus, we asked whether cleavage of overexpressed B56ε could be blocked by p53 interference. To interfere with p53, we overexpressed MDM2 (200 pg) or knocked down endogenous p53 with a p53 mor (20 ng). Overexpression of p53, which induces cleavage of endogenous B56ε, served as a positive control in this experiment. Indeed, caspase-dependent cleavage of B56ε was blocked by overexpression of MDM2 and injection of p53 mor (Fig. 6C). This result indicates that overexpressed B56ε, through stabilizing p53, activates caspase-3, which cleaves B56ε itself.

Parallel to the above studies in Xenopus embryo, we investigated the cleavage of endogenous B56ε in mammalian cell lines, including HeLa, Neuro-2a, NIH3T3, and SW480. To activate the apoptotic pathway, cells were treated with cisplatin, a platinum-based chemotherapy drug widely used to treat various types of cancers. SW480 was included in this experiment because it carries R273H and P309S mutations in p53 (61). This mutant form of the p53 protein has only a residual transcriptional activation activity (62). Similar to what was observed in Xenopus embryo, whereas cleavage of endogenous B56ε cannot be detected in untreated cells, ε-cle was induced in cisplatin-treated cells. As expected, only a very low level of ε-cle was detected in SW480 cells (Fig. 6D). In addition, we analyzed the cleavage kinetics of B56ε in HeLa cells. We observed that ε-cle became detectable only when caspase-3 was activated (Fig. 6E). This, again, supports the idea that cleavage of B56ε is caspase-3-dependent.

Proteolytic cleavage often alters the stability of protein. To determine whether cleavage of B56ε has an impact on its stability, we compared the turnover rate of wild type B56ε and the cleavage-resistant D15A mutation. Thus, D15A-transfected HeLa cells were treated with cisplatin and harvested at various time points. Myc and B56ε Western blots were performed to evaluate the turnover rates of D15A and endogenous B56ε, respectively. Indeed, D15A was degraded at a relatively slower rate than the wild type B56ε (Fig. 6, F and G), suggesting that caspase-dependent cleavage decreases the stability of B56ε.

B56ε-fl and ε-cle, but Not B56ε-s, Form Complexes with PP2Ac—B56ε is a regulatory subunit of PP2A. So far, we have identified B56ε-s, the alternative translation isoform (44), and the caspase cleavage product ε-cle. To gain insight into the biological significance of post-transcriptional regulation of B56ε, we determined whether B56ε-s and ε-cle form complexes with the catalytic C subunit of PP2A (PP2Ac). Microcystin is a chemical inhibitor of PP2A that directly binds to PP2Ac (63). Following a protocol described by Saraf et al. (52), we performed a microcystin pulldown assay to determine whether B56ε-fl, ε-cle, and B56ε-s could be co-purified with PP2Ac. As shown in Fig. 7A, both B56ε-fl and B56ε-s were produced in ε-FLAG-injected embryos at stage 9. As expected, microcystin beads pulled down both PP2Ac and PP2Aa. However, B56ε-fl, but not B56ε-s, was precipitated by microcystin beads. This indicates that B56ε-s either does not interact with PP2Ac, or forms a complex with PP2Ac with a greatly reduced affinity. To determine whether ε-cle forms a complex with PP2Ac, embryos injected with Myc-ε-FLAG (100 pg) were harvested after the

FIGURE 5. B56ε is a substrate of caspase-3. A, Western blot results show that the cleavage of myc-ε-FLAG is blocked by overexpression of BCL2, but not by overexpression of cFLIP. B, Western blot results show that the cleavage of myc-ε-FLAG is blocked by injection of DEVD-CHO, a peptide inhibitor of caspase-3. C, Western blot results show the cleavage of myc-ε-FLAG by human caspase-3 in an in vitro caspase cleavage assay. D, alignment of N-terminal sequences of vertebrate B56ε. Two potential cleavage sites, DK and LLKD, are underlined. E, Western blot results show that D15A, which carries a Asp > Ala mutation in the DKI motiff, could not be cleaved. F, Western blot results show the cleavage of myc-ε-FLAG in HeLa cells. D15A serves as a negative control.
EGT (stage 14). The lysate, which contains both the full-length Myc-H9280-FLAG and H9280-cle, was used in the microcystin pulldown assay. Microcystin beads were able to precipitate both Myc-H9280-FLAG and H9280-cle (Fig. 7A), demonstrating that H9280-cle is capable of forming a complex with PP2Ac. Because we failed to detect complex formation between B56-s and PP2Ac in the microcystin pulldown assay, we carried out co-immunoprecipitation experiments to determine whether B56-s interacts with PP2Ac. Lysates from ε-FLAG-injected embryos (stage 9) were incubated with an anti-PP2Ac antibody and protein G beads. After wash, proteins co-precipitated with PP2Ac were subjected to FLAG Western blot analysis. Consistent with the result from the microcystin pulldown assay, only B56ε-fl was co-immunoprecipitated with PP2Ac (Fig. 7B). These results demonstrate that B56ε-fl and ε-cle are capable of forming complexes with PP2Ac. Currently, we do not know whether B56-s forms a very low affinity complex with PP2Ac, or does not interact with PP2Ac at all.

DISCUSSION

Results presented here demonstrate that B56ε has both anti- and pro-apoptotic functions. The anti-apoptotic effect of B56ε was observed in the developing neural ectoderm. Apoptosis induced by B56ε knockdown was not rescued by depletion of p53, suggesting that B56ε is responsible for suppressing p53-independent apoptosis during neural development. Currently, the mechanism by which B56ε suppresses apoptosis in the developing neural ectoderm remains unclear. Given that B56ε

FIGURE 6. Activation of the p53-dependent apoptotic pathway induces cleavage of B56ε. A, Western blot results showing the expression of B56ε during Xenopus early embryonic development. Of note, ε-cle is not detected throughout these stages. B, Western blot results showing that activation of apoptosis induces cleavage of endogenous B56ε. To induce apoptosis, we treated embryos with hydroxyurea (Hu), or injected embryos with α-amanitin or p53. C, Western blot results show that depletion of p53 prevented the cleavage of overexpressed myc-ε-FLAG. D, Western blot results show that activation of the apoptotic pathway by treating cells with cisplatin induces cleavage of endogenous B56ε in mammalian cells. E, Western blot results showing the correlation between the cleavage of B56ε and activation of caspase-3 during cisplatin-induced apoptosis of HeLa cells. Note that ε-cle is detected only in the presence of activated caspase-3. F, Western blot results showing that D15A, the uncleavable mutant of B56ε, is more stable during apoptosis. D15A was transfected into HeLa cells. Transfected cells were treated with cisplatin. Cells were harvested at various time points after the treatment. After Myc Western blot, the same blot was probed with an anti-B56ε antibody. It is important to note that the analysis was in the linear range of the antigenic response (data not shown). G, the relative intensity of D15A and B56ε bands. The intensity of the first time point (6 h) was set as 100%. In the case of B56ε, the number represents the intensity of both the full-length and cleaved forms of B56ε.
family members play a negative role in regulation of p53. The Drosophila genome contains two B56 regulatory subunits. Although knockdown of individual B56 genes had minimal effect, knockdown of both B56-1 and B56-2 induced apoptosis in S2 cells and embryos, and impaired embryonic development prior to the extended germ-band stage (38, 39). The effect of B56 knockdown on cell survival can be rescued by depletion of Drosophila p53 (38), suggesting that B56 regulates p53 negatively. Similar to these observations, knockdown of all mammalian B56 family members elevates the level of p53 in PC12 cells.3

To explain this, we hypothesize that p53 or a key regulator of p53 is a substrate shared by all B56-containing PP2As. Dephosphorylation of this protein by B56-containing PP2As is essential for maintaining p53 protein at a low level under normal conditions. Due to the redundancy among B56 family members, this p53 inhibitory mechanism remains functional when individual B56 is depleted. However, the effect of loss of B56s or B56γ is obscured when all B56 family members are knocked out. It will be of interest to test this hypothesis in the future.

What is the molecular mechanism through which B56s regulate the stability of p53? Knockdown of B56s has no effect on the steady-state level of p53 in un-stressed cells (33), whereas depletion of B56s destabilizes p53. It is likely that B56s and B56γ regulate p53 through distinct mechanisms. Interestingly, existing evidence suggests that B56 family members can destabilize p53 by controlling the binding between p53 and MDM2. In this mechanism, Cyclin G, a transcriptional target of p53 (65, 66), recruits B56-containing PP2As to MDM2 and increases the binding affinity between MDM2 and p53 by dephosphorylating MDM2 at Thr-216 and Ser-166 (34–36). A number of B56 family members have been shown to interact with cyclin G (34, 67). Therefore, it is tempting to speculate that B56s controls the stability of p53 through the Cyclin G/MDM2-dependent mechanism.

In addition to its role in regulating apoptosis, B56s undergoes caspase-dependent proteolytic cleavage. This cleavage occurs on the carboxyl side of a conserved DKXD motif and is mediated by caspase-3. It appears that cleavage of B56s has an impact on the stability of B56s protein. Interestingly, cleavage of endogenous B56s cannot be detected under normal conditions, but occurs massively upon activation of the p53-dependent apoptotic pathway. This makes it likely that cleavage of B56s only happens during apoptosis. Cleavage of B56s, thus, serves as a molecular marker for the activation of caspase-3 and p53-dependent apoptosis. However, it is important to note that we cannot rule out the possibility that cleavage of B56s occurs under physiological conditions. Although executioner caspases, including caspase-3, remain inactive in most cases, caspases have apoptosis-independent functions (68, 69). In addition to function in the apoptotic pathway, caspase-3 is involved in cell proliferation and differentiation, for example, skeletal muscle differentiation (70). Activation of caspase-3 in non-apoptotic cells has been documented in a number of studies (71–75). Thus, it is possible that the level of e-cle in normal cells is simply below the limit of detection. In this scenario,
caspase-dependent cleavage may provide an effective way to regulate the turnover rate of B56ε. It is also worth mentioning that the N terminus of B56ε contains multiple conserved Ser/Thr residues. Among these, phosphorylation on Thr-7 has been documented (76). Although the biological significance of Thr-7 phosphorylation remains unclear currently, it is possible that phosphorylation on these N-terminal Ser/Thr residues has an important impact on the function of B56ε. By removing the N-terminal Ser/Thr-rich region, caspase-dependent cleavage results in a form of B56ε that is no longer regulated by the N-terminal phosphorylation. It is likely that caspase-dependent cleavage works as a fine-tuning mechanism to regulate B56ε-dependent protein dephosphorylation.

Of note, results presented here are reminiscent of studies of synphilin-1 and DJ-1 from da Costa’s group. In 2006, Gaime et al. (77) reported that synphilin-1, an anti-apoptotic protein that inhibits the expression of p53, is a substrate of caspase-3. The C-terminal fragment of synphilin-1, generated by caspase-dependent cleavage, is responsible for the anti-apoptotic function of synphilin-1. The uncleavable mutant form of synphilin-1 exhibits a markedly reduced anti-apoptotic activity (77). In the case of DJ-1, caspase 6-dependent cleavage generates a C-terminal fragment of DJ-1, which blocks the p53 pathway and inhibits apoptosis. D149A, an early-onset Parkinson disease-associated mutation of DJ-1, renders DJ-1 resistant to caspase cleavage and abolishes its anti-apoptotic function (78). This suggests that caspase-dependent proteolysis is an important regulatory mechanism to modify protein functions.

Previously, we identified an alternative translation isoform of B56ε (44). In the present work, we report that B56ε undergoes caspase-dependent cleavage. To gain further insight into the biological significance of post-transcriptional regulation of B56ε, we measured the ability of these isoforms to form a complex with PP2Ac. Strikingly, we were unable to detect interaction between B56ε-s and PP2Ac, even though binding between PP2Ac and two other forms of B56ε was observed under the same assay conditions. This suggests that B56ε-s are deficient in forming a functional PP2A holoenzyme. Alternatively, B56ε-s may interact with the catalytic subunit with a significantly reduced affinity. B56ε-s is required for the Wnt pathway during development (44). What is the mechanism by which B56ε-s regulates Wnt signaling? An attractive working hypothesis is that B56ε-s functions as a dominant-negative form and regulates Wnt signaling by sequestering substrate(s) of endogenous B56ε-containing PP2As. Interestingly, although B56ε-s lacks the N-terminal 76 amino acid residues of B56ε, it contains all residues required for interacting with PP2Aa and PP2Ac (10, 11). Lack of binding between B56ε-s and PP2Ac, thus, raises the possibility that the N terminus of B56ε, which does not directly interact with other subunits of PP2A, plays a regulatory role during PP2A holoenzyme assembly.

In summary, the present study demonstrates that B56ε suppresses p53-independent apoptosis during neural development and induces p53-dependent apoptosis through stabilizing p53. B56ε undergoes caspase-dependent cleavage, leading to an unstable cleavage product. Further study is needed to identify molecular mechanisms by which B56ε regulates apoptosis.

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