Down-regulation of CIBZ, a Novel Substrate of Caspase-3, Induces Apoptosis*

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We previously identified and characterized a murine BTB domain-containing protein, CIBZ (ZBTB38 in human), that interacts with CtBP and binds to methylated CpGs. However, its physiological function remained unknown. As CtBP is reportedly involved in p53-independent programmed cell death, we examine here whether CIBZ is associated with apoptosis. We found that CIBZ was highly expressed in proliferating C2C12 cells but that its expression levels decreased upon induction of apoptosis by serum starvation. Knockdown of CIBZ by small interfering RNA in C2C12 cells induced apoptosis, as determined by an increase of annexin V/propidium iodide labeling, activation of caspase-3, and cleavage of poly(ADP-ribose) polymerase. CIBZ inhibition also activated caspase-7 and caspase-9, suggesting that CIBZ-associated apoptosis occurs through the mitochondrial pathway. Notably, knockdown of CIBZ in mouse embryonic fibroblast cells also activated caspase-3 and cleavage of poly(ADP-ribose) polymerase, indicating that CIBZ-associated apoptosis is mediated by a p53-independent pathway; however, because both common and distinct targets are regulated by CIBZ- and CtBP-associated apoptosis, we conclude that more than one pathway is involved. Finally, using mutagenesis and an in vitro caspase cleavage assay, we show that CIBZ is a novel substrate of caspase-3 and identify two caspase-3 recognition sites. These findings indicate, collectively, that CIBZ plays an important role by participating in the negative regulation of apoptosis in murine cells.

Apoptosis is a genetically controlled form of cell death that plays critical roles during development and tissue homeostasis by ensuring the removal of damaged or unnecessary cells (1). Activation of proteolytic enzymes called caspases is a key step in the apoptotic program. Once the initiator caspase, the best characterized of which is caspase-9, is activated by cellular stress signals, it processes and activates downstream effector caspases such as caspase-3 and caspase-7. For caspase-3, the 32-kDa inactive proenzyme is cleaved to 17- and 12-kDa fragments to form an active heterotetramer. This active form can specifically cleave its substrates at a DXXD motif to induce DNA fragmentation and morphological changes typical of cells undergoing apoptosis (1, 2). One of the major substrates of caspase-3 is poly(ADP-ribose) polymerase (PARP),3 and cleaved PARP and cleaved caspase-3 itself are regarded as signature markers of apoptosis (2, 3).

Murine C2C12 cells are a well established in vitro model system to study apoptosis as well as myogenesis in developing muscle because a significant fraction of C2C12 cells undergo apoptosis, cell cycle withdrawal, and differentiation when cultured with 2% horse serum-containing medium (referred to as differentiation medium (DM)) (4–7). Growing evidence indicates that the expression of many regulatory proteins is stimulated or suppressed during apoptosis induced by DM. The upregulated proteins include the cyclin-dependent kinase inhibitors p21 and p27 (4, 8) and the M-phase inducer phosphatase 3, a regulator of cell division (9). Prominent downregulated proteins include the paired box protein Pax-7 and heat shock protein HSP90-β (9). Given the complexity of apoptosis and the increasing numbers of regulatory molecules known to be involved, it is likely that more regulators remain to be discovered.

We previously identified the murine CtBP (C-terminal binding protein)-interacting BTB (broad complex/tramtrack/bric-a-brac) zinc finger protein CIBZ (also named ZENON in rat and ZBTB38 in human) (10–12), which can repress Gal4-driven thymidine kinase or SV40 promoters by functionally interacting with CtBP (13, 14) in a histone deacetylase-dependent manner. Like the BTB domain-containing transcription factor kaiso (15, 16), CIBZ/ZBTB38 lacks a signature methyl-CpG-binding domain but can bind methylated DNA through the conserved zinc fingers (12).4 Thus, CIBZ belongs to a novel class of methylated DNA-binding proteins. However, the physiological function of CIBZ remains largely unknown.

To determine the biological role of CIBZ, we focused on apoptosis because CtBP (17) and several BTB domain-containing zinc finger proteins such as PLZF (18) are involved in apoptosis. Several reports link CtBP with p53-independent cell death: (i) targeting of CtBP by ARF (alternate open reading frame) results in p53-independent apoptosis (19); (ii) HIPK2 (homeo domain-interacting protein kinase 2) mediates CtBP phosphorylation and degradation in UV-induced cell death (20); and (iii) UV-

3 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; DM, differentiation medium; siRNA, small interfering RNA; GM, growth medium; MEF, mouse embryonic fibroblast; RT, reverse transcription; GST, glutathione S-transferase; SP, spacer; FITC, fluorescein isothiocyanate; PI, propidium iodide.

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triggered active JNK1 (c-Jun N-terminal kinase) promotes apoptosis by phosphorylated and down-regulated CtBP in human lung cancer cells (21). These findings demonstrate that the CtBP protein plays important roles in regulating p53-independent programmed cell death, although the precise mechanism and signaling pathway by which it does so remain unclear.

We show here that CtBP is down-regulated during apoptosis induced by DM in C2C12 cells. Using a siRNA knockdown strategy, we find that CtBP plays an anti-apoptotic role in these cells. Our results suggest that CtBP-associated apoptosis is independent of p53. Moreover, our results support the existence of two overlapping but distinct pathways for CtBP- and CtBP-associated apoptosis. Finally, we present data indicating that CtBP is a caspase-3 substrate and identify two caspase-3 cleavage sites in CtBP.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—For N-terminally FLAG-tagged CtBP constructs, full-length CtBP cDNA was inserted into the BamHI and XhoI sites of pcDNA3 vector (Invitrogen) in-frame with two tandem repeats of the FLAG epitope coding sequence. For GST-CtBP deletion constructs, BTB (amino acids 1–158), RD2 (repression domain 2) (amino acids 158–339), zinc finger 1–5 (amino acids 335–358), spacer (SP; amino acids 539–1012), and zinc finger 6–10 (amino acids 967–1197) fragments were generated by PCR and inserted in-frame into vector pGEX-5X-2 (GE Healthcare). GST-CtBP SW2 (D618A/D910A) and FLAG-tagged CtBP (D618A/D910A) mutant constructs were made with the QuikChange site-directed mutagenesis kit (Stratagene). DNA sequences were verified using BigDye terminator and an automated sequencer (ABI PRISM310).

**Cells and Cell Conditions**—C2C12 mouse cells were cultured in growth medium (GM) comprising Dulbecco’s modified Eagle’s medium (Nacalai Tesque) supplemented with 15% fetal bovine serum, 2 mm L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen). p53−/− mouse embryonic fibroblast (MEF) cells, kindly provided by Dr. Richard Meehan (Medical Research Council Human Genetics Unit, Edinburgh, UK), were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum, 2 mm L-glutamine, 1% penicillin/streptomycin, and 1 mm sodium pyruvate (Invitrogen). To induce apoptosis in C2C12 cells, GM was replaced by DM (Dulbecco’s modified Eagle’s medium containing 2% horse serum, 2 mm L-glutamine, and 1% penicillin/streptomycin). 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mm L-glutamine, and 1% penicillin/streptomycin. Transfections were carried out with the CellPhect transfection kit (GE Healthcare) as described previously (22).

**Semiquantitative RT-PCR**—RT-PCR was conducted as described previously (10). Total RNA was extracted from cultured cells using a Sepasol®-RNA I Super (Nacalai Tesque) according to the manufacturer’s protocol. cDNA was synthesized from 1–5 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) and subsequently subjected to PCR using specific primers. The primers listed below were designed and confirmed as unique in the nonredundant NCBI Protein Database. Primers for the mouse genes were as follows: CtBP, 5’-CCGG ATCCCATAGATCAGACACTCTCCAT-3’ (forward) and 5’-CCGCGTCGAGCTGAGCTATGACAGAGGCCGAG-3’ (reverse); glyceraldehyde-3-phosphate dehydrogenase, 5’-CCATCACCATCTTCCAGGAG-3’ (forward) and 5’-CTCTGCTTCACCATTTCTTG-3’ (reverse); p53, 5’-ACT GCATGGACGATCGTGTG-3’ (forward) and 5’-GCCGATAG TTGCCCCGTTAAG-3’ (reverse); p21, 5’-ACGCGTTGCCAGA TTGACTTCCG-3’ (forward) and 5’-TCTGGCCTTGGAGT GTAGATA-3’ (reverse); and p27, 5’-CCGGGATCCAGATGTC AAGCTGAGAGTGTC-3’ (forward) and 5’-CCGGTCTCGAG TTCGACTTACGTCTGGCGTCGAAGGC-3’ (reverse). The reaction products were separated on a 2% agarose gel and visualized by staining with ethidium bromide. To confirm the identity of PCR products, single bands of the expected size were excised from the gels and sequenced.

**siRNA Suppression Assay**—C2C12 and p53−/− MEF cells were adjusted in 3.5- or 6-cm plates to 50–70% confluence in GM 1 day before transfection. The cells were transfected with 5 nm CtBP-specific Dicer substrate siRNA duplexes with or without Dicer substrate scrambled negative control (Integrated DNA Technologies), which does not target any sequence in human and mouse, using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. The siRNA-transfected cells were used 24–48 h after transfection for RT-PCR and Western blotting. Dicer substrate 27-mer siRNAs were synthesized by Integrated DNA Technologies. The CtBP siRNA-1 sequences were 5’-phospho-GCGGACCAUGUGGUA-AUUUGUdGdA-3’ (sense) and 5’-UCACAAAUUUCACC AUGUGGUCCGCU-3’ (antisense). The CtBP siRNA-2 sequences were 5’-phospho-GCGUAGAAACACCAUGUAA GAUGUGAdAdA-3’ (sense) and 5’-UUUCAAUUCUACUG GUUUCUUCAGC-3’ (antisense).

**Western Blotting**—Western blotting was performed as described previously (10). Briefly, cells were sonicated and lysed in lysis buffer. Proteins were separated by 8–15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with the following antibodies: anti-CtBP (10), anti-p21 (catalog no. sc-6246, Santa Cruz Biotechnology), anti-p27 (BD Biosciences) as described previously (23), with the actin gene as an internal control.

**Annexin V-FITC/Peroxidase Conjugated Apoptosis Assay**—The annexin V kit (MBL, Nagoya, Japan) was used according to the manufacturer’s protocol to detect phosphatidylserine translocation from the inner to the outer plasma membrane. Briefly, for each assay, cells were washed with phosphate-buffered saline, diluted in annexin V binding buffer containing annexin V and PI, and incubated for 15 min at room temperature in the dark. The cells were analyzed by fluorescence-activated cell sorting (BD Biosciences) as described previously (23), with the acquisition of a total 10,000 events/sample to ensure adequate data.
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In Vitro Caspase Cleavage and Immunoprecipitation Assays—GST fusion proteins were expressed in Escherichia coli BL21 and purified using glutathione-Sepharose 4B (GE Healthcare) as described previously (24). Purified GST fusion proteins were incubated with 290 ng of recombinant caspase-3 (Sigma) at 37 °C for 8–12 h in 30 μl of reaction buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 10 mM dithiothreitol, pH 7.5). The reactions were terminated by the addition of SDS-PAGE loading buffer and heating at 95 °C for 3 min. Samples were then subjected to 10% SDS-PAGE and subsequently stained by Coomassie Brilliant Blue. To assay immunoprecipitated CIBZ protein cleavage, FLAG-tagged wild-type CIBZ or FLAG-tagged CIBZ(D618A/D910A) constructs were transfected into 293T cells. After 48 h, whole cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) as described previously (10), followed by an in vitro caspase-3 cleavage assay as described above.

RESULTS

CIBZ Is Highly Expressed in Proliferating C2C12 Cells and Down-regulated during Serum Deprivation-induced Apoptosis—To determine whether CIBZ is associated with apoptosis, we first examined its expression in C2C12 cells by RT-PCR and Western blotting. CIBZ was highly expressed in proliferating C2C12 cells cultured in GM (Fig. 1) (data not shown). When GM was replaced with DM, apoptosis was induced within 24 h of serum starvation, as evidenced by the activation of caspase-3 (17/19 kDa) and cleavage of PARP (89 kDa), both of which are hallmarks of cell death. Interestingly, the CIBZ protein level decreased substantially within 8 h, becoming barely detectable at 24 h and undetectable at 48 h after the switch to DM. In contrast, expression of CIBZ mRNA, as monitored by semiquantitative PCR analysis, was relatively stable and had decreased only slightly at 48 h. This result suggests that CIBZ is associated with apoptosis and that CIBZ protein degradation accounts for the observed post-transcriptional regulation.

Reduction of CIBZ via RNA Interference Induces Apoptosis in C2C12 Cells—To test whether CIBZ inhibition is sufficient to induce apoptosis, we examined the effect of CIBZ reduction in C2C12 cells by siRNA using transient transfection of C2C12 cells with two Dicer substrate 27-mer siRNA duplexes targeting different regions of the CIBZ gene. siRNAs were used at very a low concentration (5 nM) to avoid unwanted off-target effects. To confirm the specificity of the CIBZ siRNA, we also monitored the levels of Bax and Noxa proteins, which have pro-apoptotic activities and are reported to be targets of apoptosis involving CtBP (17, 19). No effects on the expression of these proteins were observed when CIBZ was knocked down (data not shown), further suggesting that the reduction of the CIBZ level was an antisense-specific effect. As shown in Fig. 2A, CIBZ siRNA-1 and siRNA-2 led to effective down-regulation of CIBZ mRNA and protein levels in C2C12 cells, visualized by semiquantitative PCR and Western blotting, respectively. CIBZ knockdown by siRNA also resulted in caspase-9 activation and PARP cleavage, indicating that CIBZ inhibition induces apoptosis. Importantly, levels of activated caspase-9 and, to a lesser extent, caspase-7 also increased, sug-

![Figure 1. CIBZ protein is down-regulated during serum starvation-triggered apoptosis in C2C12 cells.](image1)

![Figure 2. Down-regulation of CIBZ by siRNA induces apoptosis in C2C12 cells.](image2)
suggesting that CIBZ-associated cell death occurs through the mitochondrion-mediated pathway. Additionally, reduction of CIBZ expression up-regulated the expression of p21 and caspase-3 proteins (Fig. 2A). This observation is in accordance with previous reports that serum deprivation-induced apoptosis stimulates the expression of these two cyclin-dependent kinase inhibitors in C2C12 cells (8, 9). Up-regulation of p21 and caspase-3 activation were both observed when CtBP (17, 21) or CIBZ was inhibited, suggesting the presence of a common pathway of apoptosis. Similar results were also obtained for two other 21-mer siRNA duplexes targeting different regions of the CIBZ gene (data not shown).

To confirm that CIBZ down-regulation induces apoptosis, binding of annexin V, another early indicator of apoptosis, was examined in C2C12 cells. Because annexin V binds to externalized phosphatidylserine on membranes of early apoptotic cells, we used flow cytometric analysis of cells stained with FITC-conjugated annexin V and the necrotic indicator propidium iodide, which binds to nucleic acids when the cell membrane is damaged. As shown in Fig. 2B, reduction of CIBZ by siRNA increased early apoptosis and late apoptosis/secondary necrosis, as determined by annexin V-positive/PI-negative and annexin V-FITC/PI-double positive cells, respectively, again indicating that CIBZ knockdown in C2C12 cells induces apoptosis.

**Reduction of CIBZ in p53−/− MEF Cells Induces Apoptosis**—p53 plays an important role in regulating apoptosis. To determine whether p53 participates in the process of CIBZ-associated apoptosis, we inhibited CIBZ in p53−/− MEF cells and monitored apoptosis. Both CIBZ siRNAs down-regulated the expression of CIBZ efficiently at the mRNA (data not shown) and protein (Fig. 3) levels in these cells; simultaneously, caspase-3 was activated, and PARP was cleaved. These results, together with the observation that CIBZ knockdown in C2C12 cells did not influence the expression of p53 (Fig. 2A), strongly suggest that CIBZ-associated apoptosis is independent of the p53-mediated pathway.

**CIBZ Is a Substrate of Caspase-3**—Inspection of the CIBZ protein sequence revealed five putative caspase recognition sites (DXXD): two in the N-terminal BTB domain and the other three in the spacer (Fig. 4, upper panel). Together with the finding that the down-regulation of CIBZ mediated apoptosis, this suggested that CIBZ might represent a novel caspase-3 substrate. To explore this possibility, CIBZ deletion mutants were fused to GST and subjected to an *in vitro* caspase-3 cleavage assay. As seen in Fig. 4 (lower panel), degradation and cleavage of GST-BTB, GST-RD2, GST-zinc finger 1–5, and GST-zinc finger 6–10 were not detected by treatment with recombinant caspase-3 at 37 °C for 8 h, conditions that are sufficient for complete digestion of GST-SP. Together, these results demonstrate that CIBZ is indeed a substrate of caspase-3 and that only the SP domain is susceptible to caspase-3 cleavage.

**Characterization of Caspase-3 Cleavage Sites in CIBZ**—We next wished to identify the caspase-3 cleavage sites on CIBZ-SP by means of an *in vitro* cleavage assay. As noted above, there are three DXXD motifs in the SP domain: DFQD618, DNFD667, and DSTD910. We speculated that DFQD618 and DSTD910 are cleavage sites for caspase-3 because the sizes of the expected products of cleavage at these sites matched the observed cleavage results (Fig. 4) (data not shown). To confirm this, we performed PCR-based site-directed mutagenesis: Asp618 and Asp910 were changed to alanine, referred to as D618A and D910A, respectively. We also constructed a double mutant in which Asp618 and Asp910 were both mutated to alanine (D618A/D910A). The results of *in vitro* assays showed that neither GST-SP(D618A) nor GST-SP(D910A) abrogated entirely the cleavage by recombinant caspase-3 (data not shown). By contrast, the GST-SP(D618A/D910A) mutation abolished the cleavage (Fig. 5A), indicating that...
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**FIGURE 5. Characterization of caspase-3 cleavage sites in CIBZ.** A, identification of caspase-3 cleavage sites in the SP domain is shown. A schematic view of the caspase-3 cleavage mutation D618A/D910A in which Asp618 and Asp910 were mutated to alanine (left panel) is shown. The GST-CIBZ(D618A/D910A) mutant was incubated in the absence or presence of recombinant caspase-3 for 12 h at 37 °C, and cleavage products were detected by SDS-PAGE and Coomassie Brilliant Blue staining (right panel). Asterisks denote nonspecific bands. B, the CIBZ(D618A/D910A) mutant overexpressed in 293T cells is resistant to degradation by recombinant caspase-3. A schematic view of the CIBZ(D618A/D910A) mutant construct (upper panel) is shown. 293T cells were transiently transfected with the FLAG-CIBZ(D618A/D910A) mutant (CIBZm) and wild-type CIBZ. At 48 h after transfection, total cell lysates were immunoprecipitated (IP) with anti-FLAG affinity gel and then incubated in vitro with recombinant caspase-3. The reaction products were separated by 10% SDS-PAGE and immunoblotted with anti-CIBZ antibody (lower panel). WB, Western blotting.

DFQD$^{618}$ and DSTD$^{910}$ are the essential sites for cleavage by caspase-3 *in vitro*. To further ascertain the effect of recombinant caspase-3 on mammalian expressed CIBZ(D618A/D910A), we constructed FLAG-tagged CIBZ(D618A/D910A) and transiently transfected it into 293T cells. Transfected cell lysates were immunoprecipitated with anti-FLAG affinity gel, subjected to an in vitro caspase-3 cleavage assay, and subsequently immunoblotted with anti-CIBZ antibody. As shown in Fig. 5B, protein degradation was effectively abrogated in FLAG-CIBZ(D618A/D910A) but not in wild-type CIBZ, confirming that DFQD$^{618}$ and DSTD$^{910}$ in the SP domain are the recognition sites used for caspase-3 cleavage.

**DISCUSSION**

The development of resistance to apoptosis is a hallmark of malignant cells, enabling them to survive despite apoptosis-inducing environmental signals. Mutation of p53 is common in tumor cells that can escape apoptosis, but the mechanism by which mutated p53 facilitates apoptotic escape remains elusive and controversial. The observation that knockdown of CIBZ induces cell death in p53$^{-/-}$ MEFs suggests that, in this case, cell death occurs through a p53-independent pathway and that CIBZ may play an important role by participating in this process. CIBZ down-regulation by siRNA in NIH3T3 fibroblasts does not induce apoptosis directly but sensitizes these cells to apoptosis triggered by UV light or actinomycin D (data not shown), suggesting that CIBZ inhibition-induced apoptosis is cell type-dependent. Together with the result that CIBZ knockdown induces apoptosis in C2C12 and p53$^{-/-}$ MEFs (Figs. 2A and 3), these findings suggest that CIBZ down-regulation may be a common precursor to apoptosis in murine cells.

Our previous report that CIBZ interacts with CtBP, a corepressor that also functions as a p53-independent anti-apoptotic factor, implied that common targets and/or a common pathway might exist for these two proteins. Indeed, activation of caspase-3 and up-regulation of p21 were both observed in CIBZ knockdown (Fig. 2A) and CtBP knock-out cells (17, 21), suggesting the further possibility that a common or overlapping pathway might exist and that p21 might act as a common target of CIBZ and CtBP. It is noteworthy that apoptotic signaling by these two proteins might also, on the other hand, follow distinct pathways: (i) knockdown/knock-out of CtBP results in up-regulation of several pro-apoptotic proteins such as Bak and Noxa (17), whereas such effects were not evident in CIBZ-depleted cells examined in the course of our present work (data not shown); (ii) it is not clear whether initiator caspases such as caspase-9 and effector caspases such as caspase-7 are also activated in CtBP-depleted cells; and (iii) up-regulation of p21 was observed only at the protein level in CIBZ knockdown cells (Fig. 2A), in contrast to CtBP knock-out cells, in which p21 mRNA was also up-regulated (17). The different cell lines used might account for the altered differential expression of apoptosis-associated genes caused by these two proteins.

Our results show that CIBZ inhibition resulted in the activation of caspase-9, -3, and -7 (Fig. 2A), but not caspase-8 (data not shown), indicating that CIBZ-associated apoptosis is mitochondrion-dependent. Considering that CIBZ functions as a transcriptional regulator (repressor or activator) when fused to the Gal4 DNA-binding domain (10) and can bind to methylated CpGs, we speculate that it might repress or activate expression of pro- or anti-apoptotic genes. However, we failed to observe fluctuation of several pro-apoptotic regulators (Bax, Bak, and Noxa) and anti-apoptotic regulators (Bcl-2, Mcl-1, and Bcl-xL) at both the mRNA and protein levels when CIBZ was inhibited in either C2C12 cells or p53$^{-/-}$ MEFs (data not shown). This suggests the complexity of CIBZ-associated apoptosis. Among regulators responsible for mitochondrion-mediated apoptosis, inhibitors of caspases are reported to block the activating cleavage of caspase-9, -3, and -7 (25), and the activities of these caspases are antagonized by the IAP inhibitors Smac/DIABLO and HtrA2/Omi (25, 26). Experiments are under way to examine expression of the above genes before and after CIBZ inhibition.

Our finding that CIBZ down-regulation by siRNA is sufficient to induce apoptosis in C2C12 and p53$^{-/-}$ MEFs suggests strongly that CIBZ functions as an anti-apoptotic regulator and that overexpressed CIBZ may prevent apoptosis induced by a
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Acknowledgments—We are grateful to Dr. Ian Smith for critical reading of the manuscript. We thank members of the Kawaichi laboratory for technical advice.

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May 23, 2008 • Volume 283 • Number 21

Journal of Biological Chemistry

Variety of triggers, in a similar fashion to Mirk (27). However, we found no evidence that ectopic CIBZ expression prevents serum-, UV light-, or staurosporine-induced apoptosis in several cell lines (data not shown). This is probably due to the rapid degradation of CIBZ protein under apoptosis induction (Fig. 1) (data not shown), which is likely to be a consequence of its post-transcriptional modification. This is reminiscent of the failure of ectopically expressed wild-type CtBP to prevent apoptosis, whereas its S422A mutant, which is resistant to phosphorylation, can effectively block cell death triggered by UV light (20, 28).

Cleavage of CIBZ by caspase-3 is unlikely to be the major cause either of its proteolysis or apoptotic cell death in C2C12 cells and p53−/− MEFs from the following evidences: (i) no CIBZ cleavage bands were detected, either by anti-CIBZ antibody against wild-type CIBZ or by anti-Myc antibody against N- or C-terminally Myc-tagged CIBZ, at a variety of time points (data not shown); and (ii) overexpression of CIBZ(D618A/D910A), a caspase-3-resistant mutant whose cleavage by active caspase-3 is completely blocked (Fig. 4A and B), was degraded as quickly as that of wild-type CIBZ under apoptosis induction and displayed no anti-apoptotic effect (data not shown). In fact, the proteasome inhibitor MG132 can efficiently prevent CIBZ degradation under apoptosis induction (data not shown), suggesting that CIBZ proteolysis depends mainly on the ubiquitin-proteasome pathway.

We have demonstrated both that CIBZ is a novel substrate for caspase-3 and that the recognition sites are located in the SP domain at DFQD618 and DSTD910. For many of the known caspase-3 substrates, the consequences of their cleavage have only been inferred by extrapolation from the function of the uncleaved protein (29). In the case of CIBZ, although the biological function of cleavage remains likewise to be proven, the existence of two cleavage sites, both located in the SP domain, is noteworthy. The SP domain also contains two putative bipartite nuclear localization signals, at amino acids 845–865 and 922–940, and the second cleavage site DSTD910 is situated between them. Experiments using the two mutated caspase-3 cleavage sites, in combination with mutated nuclear localization signal sequences, should illuminate the contributions of these motifs to the regulation of apoptosis, transcriptional activation, subcellular localization, DNA-binding activity, and protein-protein interactions.

In conclusion, our findings in this study are the first evidence that CIBZ has a physiological function, participating in the negative regulation of apoptosis in murine cells. To our knowledge, this is the first report that down-regulation of a methylated DNA-binding protein activates a member of the caspase family.