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Caspase-3-mediated cleavage of PICOT in apoptosis

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ABSTRACT

Mammalian protein kinase C-interacting cousin of thioredoxin (PICOT) is a multi-domain mono-thiol glutaredoxin that is involved in several signal transduction pathways and is necessary for cell growth and metastasis. Here, we demonstrate that PICOT is a cleavage substrate of the apoptosis-related protein caspase-3. *In vitro* cleavage assays indicated that PICOT was specifically cleaved by caspase-3. Similarly, endogenous PICOT was cleaved in cell death responses induced by staurosporine and etoposide. These phenomena were blocked in the presence of a pan-caspase inhibitor. Using site-directed mutagenesis, we identified two putative caspase-3 cleavage sequences in PICOT, DRLD(101)/G and EELD(226)/T. Interestingly, overexpression of either PICOT wild type or the D101A/D226A double point mutant accelerated etoposide-induced activation of caspase-3 whereas siRNA-mediated knockdown of PICOT blocked this phenomenon. Our data raise the possibility that the pro-apoptotic role of PICOT is actively regulated via caspase-3-mediated cleavage.

1. Introduction

Apoptosis is an active cell death process that is indispensable in development and homeostasis [1]. During the apoptotic process, several cysteine aspartate-specific proteases (caspases) cleave their respective substrates leading to cellular demise [2]. Typical morphologic features of apoptosis, such as DNA fragmentation [3], membrane blebbing [4,5], and nuclear condensation [6], occur by caspase-mediated cleavage of inhibitor of caspase-activated DNase (ICAD), Rho-associated coiled-coil protein kinase 1 (ROCK1), and lamins, respectively. To date, approximately 800 caspase substrates have been identified [7,8] through several screening methods including expression cloning [9], yeast two-hybrid method [10], differential two-dimensional gel electrophoresis (2-DE) methods [11–13], and recently developed gel-free proteomics techniques [14,15]. Regardless of the recent progress in identifying putative caspase substrates, it is still of great interest to identify further cell type-specific substrates and caspase substrates related to disease progression.

Mammalian PICOT/Txnl2/Grx3 is a member of the multi-domain mono-thiol glutaredoxin family [16]. PICOT is evolutionary conserved and essential during development. The protein consists of three domains including a thioredoxin-like domain and glutaredoxin domains 1 and 2 [16]. PICOT-deficient mice exhibit embryonic lethality and have a smaller body size and hemorrhaging in the head [17]. Recent reports showed that PICOT is involved in several signal transduction pathways [18]. In the course of identifying multiple endogenous caspase-3 substrates, we by chance noticed smaller sized PICOT bands in samples prepared from various tissues, including thymus, liver, pancreas, intestine, kidney, and hippocampus (Supplementary Fig. 1). Considering that tissue lysates were prepared in the presence of protease inhibitor cocktail, it is reasonable to assume that this cleavage of PICOT is not a consequence of non-specific proteolytic process during preparation but rather represents a physiological or pathophysiological phenomenon during maintenance of homeostasis. In the present study we investigated whether PICOT is cleaved by a set of proteases and found that PICOT was specifically cleaved by caspase-3. This phenomenon was also observed in cells following treatment with the prototypic apoptotic inducers etoposide or staurosporine. Using both *in vitro* and cell-based assays the two cleavage sites of PICOT by caspase-3 were mapped at DRLD(101)/G and EELD(226)/T. Intriguingly, we found that regulation of cellular PICOT by overexpression and knockdown strategies correlated with the production of active forms of caspase-3, a hallmark of apoptosis. Although

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we do not know the exact underlying mechanism, we propose a regulatory loop in which apoptosis-induced generation of active caspase-3 results in cleavage of critical functional domains of PICOT, directly affecting its pro-apoptotic function.

2. Materials and methods

2.1. Cell culture and drug treatment

MN9D cell line was a fusion product between embryonic mesencephalic dopaminergic neurons and N18TG neuroblastoma and cultivated as previously described [19]. HEK-293 and U-2 OS cells were purchased from ATCC (Manassas, VA, USA) and cultivated in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) in an atmosphere of 5% CO_2 at 37 °C. For drug treatment experiments, MN9D and U-2 OS cells were treated for 24 h with 1 μM staurosporine (STS, Sigma) in N2 serum-free medium [20] or 100 μM etoposide (ETO, Sigma) in DMEM. In some cases, cells were co-treated with 100 μM *N*-benzyl-oxycarbonyl-Val-Ala-Asp-fluormethylketone (Z-VAD-fmk; MP Biomedicals, Eschwege, Germany).

2.2. Immunoblot analysis

Following drug treatment, cells were washed with chilled PBS containing 2 mM EDTA and lysed on ice in RIPA buffer (50 mM Tris, pH 7.0, 2 mM EDTA, 1.0% Triton X-100) with protease inhibitor cocktail (Roche, Basel, Switzerland). Cell lysates were homogenized with a 1-ml syringe and a 26-gauge needle. Protein content was measured with the Bio-Rad protein assay kit. An equal amount of soluble proteins was separated on SDS–PAGE gels, blotted onto PVDF membranes, and processed for immunoblot analysis [19]. Anti-PICOT antibody (KM3710; 1:20,000) [21] recognizing the N-terminal region of PICOT was used as previously reported. Other primary antibodies used were anti-cleaved caspase-3 (Cell Signaling, Boston, MA, USA; 1:1000), HRP-conjugated anti-FLAG (Sigma; 1:5000), and anti-GAPDH (Millipore, Billerica, MA, USA; 1:3000) as a loading control. The HRP-conjugated secondary antibodies were goat anti-rabbit and anti-mouse IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA; 1:5000). Enhanced chemiluminescence (ECL; PerkinElmer Inc., Waltham, MA, USA) was used to detect specific bands. When appropriate, the relative band intensity was measured using Image J imaging software (NIH, Bethesda, MD).

2.3. Construction of vectors

The vector containing human PICOT cDNA was purchased from ImaGenes (IRAUp969D0141D; Berlin, Germany). Flag-tagged wild type PICOT was generated by a standard PCR method with the primers 5′-CTCTCGAGATGGACTATAAGGACGATGATGACAAGATGG CGGCGGGGGCGGCT-3′ and 5′-CCTCTAGATTAATTTTCTCCTCTCAG-3′ and sub-cloned into the pCI-Neo vector (Promega, Madison, WI, USA). pCI-Neo Vectors encoding D101A, D226A, and D101A/D226A PICOT point mutants were made with the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions with the following primer sets: 5′-GAAAATCGACCGATTAGCTGGTGCACAT GCCCCAG-3′ and 5′-CTGGGG CATGTGCACCAGCTAATCGGTC-GA TTTTC-3′ (D101A); 5′-AGAAGCATCTGAAGAACTAGCTACAATTT- GT CCCAAAGCTC-3′ and 5′-GAGCTTTGGGACAAATTGTAGCTAGTTCTTC AGATGCTTCT3′ (D226A). All constructs were confirmed by DNA sequencing.

2.4. Transfection and in vitro caspase cleavage assays

HEK-293 and U-2 OS cells were transfected with a predetermined amount of purified DNA using polyethyleneimine (PEI; Sigma) and Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) respectively according to the manufacturers' instructions. Fifty micrograms of lysate obtained from HEK-293 transfectants was incubated with 50 ng of recombinant caspase 3 (prepared by Dr. Il-Seon Park) in the presence or absence of 50 μM Z-VAD-fmk in 20 μl caspase reaction buffer (50 mM HEPES, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% glycerol, 10 mM dithiothreitol, pH 7.2) for 1.5 h at 37 °C [22]. We also tested caspases-2, -6, -7, and -8 (R&D systems, Minneapolis, MN, USA) and caspase-9 (Enzo Life Sciences, Plymouth Meeting, PA, USA). Reactions were stopped by addition of 5 μl of 5X sample buffer (250 mM Tris–HCl, 500 mM dithiothreitol, 10% SDS, 0.5% bromophenol blue, 50% glycerol, pH 6.8), following by boiling for 5 min. Reaction products were subjected to immunoblot analysis. For silencing of PICOT, AccuTarget™ Negative control siRNA and siRNA against PICOT were purchased from Bioneer (Daejeon, Korea). The sequences of siRNA duplexes against PICOT were 5′-CAUAGGAGGACUUGAUA-UA(dTdT)-3′ (sense) and 5′-UAUAUCAAGUCCUCCUAUG(dTdT)-3′ (antisense). Negative control siRNA and PICOT-targeting siRNA (100 nM) were transfected into U-2 OS cells at a confluency of approximately 50% for 48 h using Lipofectamine 2000 according to the manufacturer's instructions.

2.5. Statistics

Data were represented as mean ± SD. Significant differences were determined by Turkey test and one-way ANOVA. A value of $p < 0.01$ was considered statistically significant.

3. Results

Based on data shown in Supplementary Fig. 1 indicating the presence of smaller, cleaved forms of PICOT in various mouse tissues, we searched the CASBAH database for potential caspase substrates and found that PICOT is listed as an endogenous substrate with a cleavage site at DRLD(101)/G [7,15]. To examine whether PICOT is indeed a caspase substrate, lysates of HEK-293 cells that overexpressed N-terminal FLAG-tagged human PICOT were directly incubated with recombinant caspase-3 (50 ng) for 1.5 h in the presence or absence of the pan-caspase inhibitor Z-VAD-fmk. As shown in Fig. 1A, two smaller FLAG-positive bands were evident in the caspase-3-treated sample. This phenomenon was blocked in the presence of Z-VAD-fmk. To examine the specificity of this cleavage, lysate of HEK-293 cells that overexpressed N-terminal FLAG-tagged PICOT was incubated with recombinant caspase-2, -3, -6, -7, -8, -9, or -10 [23]. Caspase-mediated cleavage of PICOT occurred only in the presence of caspase-3 (Fig. 1B). We next investigated whether PICOT is also cleaved in cells following treatment with etoposide or staurosporine, prototypic apoptosis inducers. Immunoblot analysis demonstrated that treatment of MN9D cells with staurosporine led to the generation of two smaller bands coincident with caspase-3 activity (Fig. 2A; top and middle panels). These drug-induced fragments corresponded in size to those found in cell lysates following treatment with recombinant caspase-3. This phenomenon was blocked in cells that were co-treated with Z-VAD-fmk. To evaluate whether cleavage of PICOT is a general event in apoptosis, we also examined U-2 OS cells following treatment with etoposide. Again, smaller PICOT fragment was generated in etoposide-treated cells and this phenomenon was blocked in the presence of Z-VAD-fmk (Fig. 2B). In etoposide-treated cells, intensity of the cleaved band at the bottom was somewhat low and therefore its appearance was not evident in some blots (compare with two smaller bands in Fig. 3B).

Under normal circumstances, caspase-3 preferentially recognizes the tetra-peptide motif DXXD in its substrates and hydrolyzes the peptide bond after an aspartic acid residue

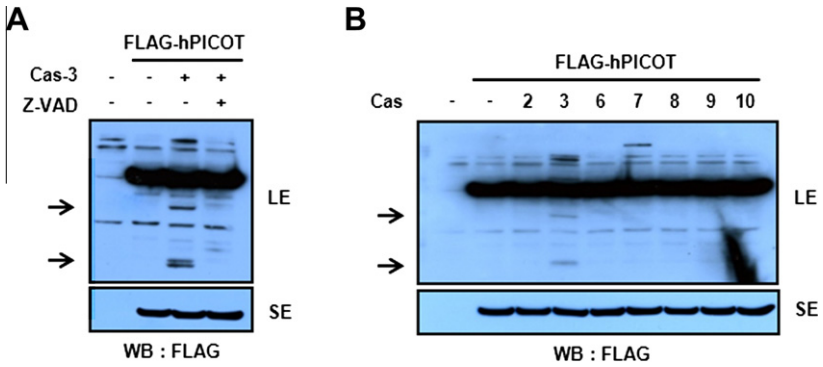

Fig. 1. PICOT cleavage assays. (A) Equal amounts of cellular protein (50 μg) obtained from HEK-293 cells overexpressing FLAG-tagged human PICOT were incubated for 1.5 h at 37 °C with or without recombinant caspase-3 (50 ng/reaction). When indicated, 100 μM Z-VAD-fmk was added to the reaction. Cellular lysates prepared from HEK-293 cells transfected with pCI-Neo vector were used as a control. After incubation, the reactants were separated on 15% SDS–PAGE and subjected to immunoblot analysis using HRP-conjugated anti-FLAG antibody. LE, long exposure; SE, short exposure. Arrows indicate PICOT fragments formed by caspase-3 mediated cleavage. (B) Equal amounts of cellular protein (50 μg) were incubated with 50 ng of each recombinant caspase: -2, -3, -6, -7, -8, -9, or -10. Reactants were analyzed by immunoblot analysis using HRP-conjugated anti-FLAG antibody.

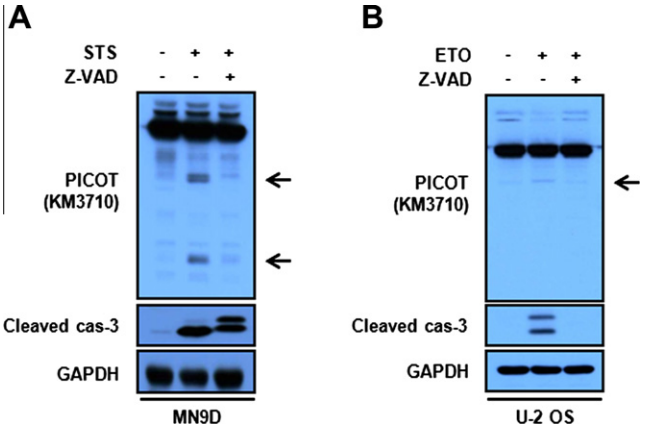

Fig. 2. PICOT cleavage in drug-induced apoptosis. (A) MN9D cells were treated for 24 h with 1 μM staurosporine (STS) in the presence or absence of Z-VAD-fmk. Cells were then processed for immunoblot analysis as described above. Arrows indicate two cleavage fragments of PICOT. (B) U-2 OS cells were treated for 24 h with or without 100 μM etoposide (ETO) in the presence or absence of 100 μM Z-VAD-fmk. Following drug treatment, cellular lysates were prepared and processed for immunoblot analysis using antibodies against PICOT (KM3710), cleaved caspase-3, and GAPDH as a loading control.

[24,25]. A search of CASBAH revealed that human PICOT contains only one DXXD sequence and is predicted to be cleaved by caspases at DRLD(101)/G (Supplementary Fig. 2A). Recently, Ohtsubo and colleagues showed that NRF2, a member of the NF-E2 family of transcription factors, is cleaved by caspase-3 at the unconventional tetra-peptide motifs TEVD/N and EELD/S [26]. As shown in Supplementary Fig. 2A, PICOT also contains the EELD(226)/T sequence. Therefore, we applied a site-directed mutagenesis approach to investigate these two putative caspase cleavage sequences of PICOT, and generated D101A and D226A point mutants and the D101A/D226A double point mutant. As shown in Fig. 3A, two smaller fragments were generated when wild type PICOT was incubated with recombinant caspase-3. However, when the D101A or D226A PICOT mutants were incubated with recombinant caspase-3, only the upper or the lower fragment was formed, respectively. Caspase-3-mediated generation of these two smaller fragments was not observed in the D101A/D226A double point mutant. We also confirmed that the smaller sized PICOT fragments were not generated following etoposide treatment of U-2 OS cells transfected with FLAG-tagged D101A/D226A PICOT double mutant (Fig. 3B). These data indicate that caspase-3 cleaves PICOT after these two aspartic acid residues.

Finally, we investigated the functional consequence of PICOT cleavage during drug-induced apoptosis. Transient U-2 OS transfectants with either overexpression or silencing of PICOT were treated with etoposide and caspase-3 cleavage was examined by immunoblotting. As shown in Fig. 4, etoposide-induced formation

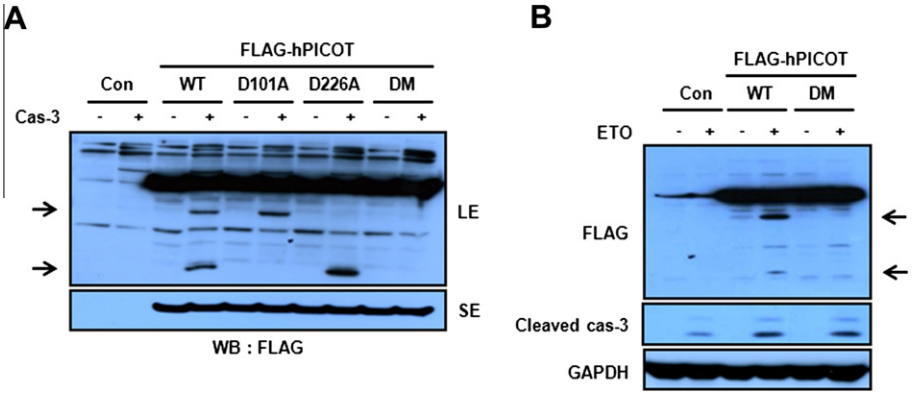

Fig. 3. Mapping of PICOT cleavage sites to DRLD(101) and EELD(226). (A) U-2 OS cells were transiently transfected with the indicated vectors and lysed in RIPA buffer. The pCI-Neo vector contained either wild type (WT) PICOT sequence, or PICOT carrying point mutations at D101A, D226A, or D101A/D226A (double mutant; DM). An empty pCI-Neo vector was used a control (Con). Equal amounts of protein (50 μg) were incubated for 1.5 h at 37 °C with or without 50 ng recombinant caspase-3. Reactants were processed for immunoblot analysis using HRP-conjugated anti-FLAG antibody. (B) U-2 OS cells transiently transfected with the indicated vector were treated with or without 100 μM etoposide for 24 h. Cells were then processed for immunoblot analysis as described above. Arrows indicate two cleavage fragments of PICOT.

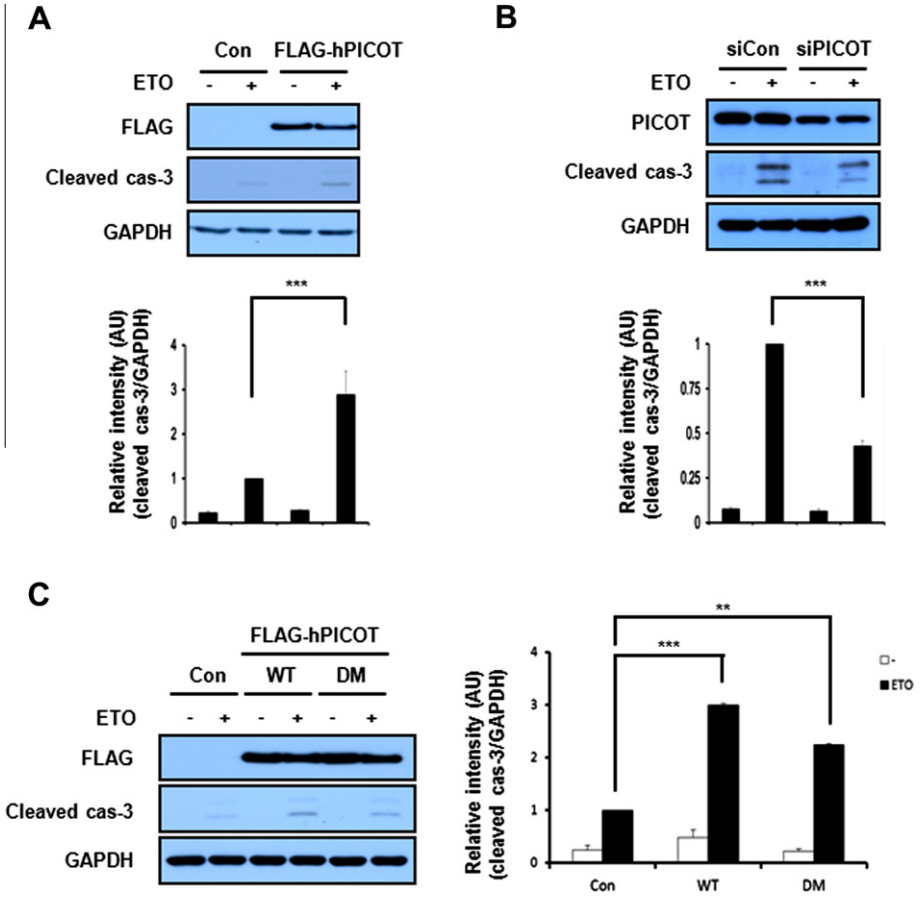

Fig. 4. The pro-apoptotic function of PICOT. U-2 OS cell were transfected with (A) pCI-Neo vectors containing FLAG-tagged WT PICOT, (B) siRNA sequence against PICOT, or (C) non-cleavable PICOT mutant. Following treatment with 100 μM etoposide for 24 h, cells were lysed in RIPA buffer and equal amounts of protein were processed for immunoblot analysis using anti-FLAG, anti-cleaved caspase-3, and anti-GAPDH antibodies. Empty pCI-Neo vector or siRNA non-targeting sequence was used as a control. Densitometric analysis was performed using Image J. The relative intensity of the two cleaved caspase-3 bands in each sample was normalized to the intensity of GAPDH in the corresponding samples. Each bar represents the mean ± SD from 2 to 3 independent experiments. Significant differences were determined by Turkey test and one-way ANOVA. $^{***}p < 0.001$; $^{**}p < 0.01$.

of cleaved caspase-3 was 2.5-fold higher in U-2 OS cells that overexpress wild type PICOT than in control cells (Fig. 4A). In contrast, generation of cleaved caspase-3 was 2-fold lower in U-2 OS cells with siRNA silencing of PICOT (Fig. 4B). Most importantly, the etoposide-induced generation of active forms of caspase-3 was also 2-fold higher in cells that overexpressed the PICOT double point mutant than in the control (Fig. 4C). The similar increase in caspase-3 cleavage in cells that overexpress wild type PICOT and those that overexpress the non-cleavable PICOT mutant suggests that caspase-3-mediated cleavage of PICOT may lead to loss of its pro-apoptotic function.

4. Discussion

To our knowledge, this is the first report demonstrating that PICOT is specifically cleaved by caspase-3 *in vitro* and in cell-based assays. We also determined the cleavage sequences of PICOT at DRLD(101)/G and EELD(226)/T. Our data demonstrating the presence of one or two smaller PICOT fragments in various tissues and cell types suggest that caspase-3-mediated cleavage of PICOT may be a universal event and might play an important role in physiology and pathophysiology. Qu et al. [27] previously reported an anti-apoptotic role of PICOT, showing that shRNA-mediated depletion of PICOT accelerated ROS-mediated apoptosis in breast cancer cells. In contrast, we found that overexpression of either wild type PICOT or the non-cleavable double point mutant accelerated etoposide-mediated activation of caspase-3 whereas introduction of siRNA against PICOT inhibited drug-induced activation of caspase-3, suggesting a pro-apoptotic function of PICOT, at least in U-2 OS cells. Although the exact nature of this inconsistency is not understood, it might be attributed to differences in apoptotic stimuli and/or cell types used. In support of this, previous studies have demonstrated that p53 plays a pro-survival role at a low level of apoptotic stimulus by transactivation of p21 and a pro-apoptotic role at a high level of apoptotic stimulus by transactivation of pro-apoptotic genes such as PUMA and NOXA [28]. Careful examination of the existence of anti-apoptotic and pro-apoptotic functions of PICOT in distinct contexts could reveal that it plays a dynamic role in cellular physiology.

Accumulating evidence supports the notion that PICOT is critically involved in various signal transduction pathways. For example, PICOT was first identified as a PKCθ interacting protein that inhibits c-Jun N-terminal kinase/AP-1 and NF-κB signaling via PKCθ inhibition [18]. PICOT also attenuates cardiac hypertrophy by disrupting the calcineurin-NFAT pathway by binding to MLP [17,29,30]. Grx3/4, the yeast homologues of PICOT, is involved in iron regulation, oxidative stress response, and actin dynamics [31–33]. In addition, recent reports showed that both yeast Grx3/4 and mammalian PICOT are iron–sulfur proteins [34–36]. Saito et al. [21] showed that PICOT binds to anamorsin, another iron–sulfur cluster-containing protein that is essential in cytosolic

iron–sulfur cluster biogenesis. Despite recent progress in understanding iron–sulfur cluster proteins, it is still largely unknown whether and how they directly affect the rate of cell death and survival. Furthermore, whether and how proteolytic cleavage of these proteins affects their biological functions has not been examined. As previously demonstrated [8], caspase-mediated cleavage of endogenous substrates can lead to either constitutive activity of one of the proteolytic fragments or loss of function of the substrate. Previous studies showed that transient overexpression of full length PICOT, but not its N-terminal or C-terminal fragment, in T cells inhibited the activation of c-Jun N-terminal kinase and AP-1 or NF-κB [18]. The PICOT C-terminal fragment containing the two glutaredoxin domains is necessary and sufficient for both binding to MLP and inhibition of calcineurin-NFAT signaling [30]. Interestingly, PICOT can form a dimeric Fe/S cluster through its two glutaredoxin domains [35]. Although more studies are required, our data, in conjunction with previous findings, suggest that caspase-3-mediated cleavage of PICOT at the N-terminal thioredoxin-like domain and in the C-terminal region between the two glutaredoxin domains may comprise one mechanism by which the pro-apoptotic function of PICOT may be regulated during the course of apoptotic cell death (Supplementary Fig. 2B).

We are puzzled by the fact that the amount of full length PICOT remained relatively unchanged even after caspase-3 activation and subsequent cleavage of PICOT. Johnson and colleagues have classified substrates of caspases into three groups based on the amount of remaining full-length protein at the late phase of apoptosis [8]. According to their classification PICOT is a group III substrate, of which the amount of full-length protein remains almost unchanged. Because PICOT exists as a homodimer and forms an iron–sulfur cluster, it is possible that only PICOT that is not bound to the iron–sulfur cluster is cleaved by caspase-3 during apoptosis. Alternatively, post-translational modifications may protect PICOT from caspase-3 mediated cleavage during apoptosis, as previously suggested [37,38]. Further investigation of PICOT with regard to its cleavage, modifications, and binding partners will provide an exciting opportunity to further understand its physiological and pathophysiological roles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.017.

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