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Modulation of Nucleobindin-1 and Nucleobindin-2 by Caspases

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Abstract

Nucleobindin-1 and nucleobindin-2 are multifunctional proteins that interact with Ca^{2+}, nucleic acids, and various regulatory proteins in different signaling pathways. So far, our understanding of the regulation of the biological functions of nucleobindins remains limited. In our proteome-wide selection for downstream caspase substrates, both nucleobindin-1 and nucleobindin-2 are found to be the downstream substrates of caspases. We report here the detailed analyses of the cleavage of nucleobindins by caspases. Significantly, the caspase cleavage sites are located exactly at one of the Ca^{2+}-binding EF-hand motifs. Our results suggest that the functions of nucleobindins could be modulated by caspase-mediated cleavage in apoptosis.

Keywords

Caspase substrates; nucleobindin-1; nucleobindin-2; EF-hand motif containing Ca^{2+}-binding proteins; cleavage of functional domains

INTRODUCTION

Nucleobindin is a class of EF-hand motif containing Ca^{2+}-binding protein that has multiple functions. Two nucleobindins have been identified so far, including NUCB1 (or CALNUC, NUC) and NUCB2 (or NEFA) [1,2]. These two proteins are highly homologous with 62% amino acid sequence identity, although they are encoded by two separate and unlinked gene loci. The most characteristic feature of NUCB1 and NUCB2 is the presence of multiple functional domains, including a signal peptide, a leucine/isoleucine rich region, a putative nuclear localization signal and a DNA-binding domain, two Ca^{2+}-binding EF-hand motifs, and a leucine zipper region (Figure 1A).

NUCB1 was first discovered to bind to the nucleosomal-laddered DNA in SLE-prone MRL/lpr mice during apoptosis [1,3]. Exogenous administration of the recombinant NUCB1 to MRL/n mice induced autoimmune phenomena and thymic apoptosis, indicating that NUCB1 plays an important role in inducing autoimmunity and apoptosis. Accumulating evidence suggests that NUCB1 performs multiple functions through its Ca^{2+}-binding, DNA-binding, and EF-hand motif mediated interactions with other proteins. It has been shown that NUCB1 is highly abundant in the Golgi region of a large variety of tissues and could play a key role in Ca^{2+} homeostasis in the cis-Golgi network and cisternae [2,4,5]. Despite its presence in Golgi, NUCB1 also possesses features of a transcription factor through the basic amino acid-rich and the leucine zipper regions [1]. In addition, NUCB1 interacts with multiple binding partners,

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including $G_{\alpha i3}$, $G_{\alpha i2}$, and cyclooxygenases [6,7]. NUCB2 was originally identified in the common ALL cell line KM3 [8]. Compared to NUCB1, NUCB2 is 40-residue shorter, but also present in Golgi, with the Golgi retention motif at the N-terminal leucine/isoleucine-rich region [9]. Despite their high similarity on the structures of the functional domains, NUCB1 and NUCB2 are divergent at the N-terminal region, the leucine-zipper, as well as the C-terminal sequences. NUCB2 was found to interact with necdin and ARTS-1, both through the EF-hand motifs [10,11]. Clinically, NUCB1 was implicated as a tumor-associated antigen that appeared to be overexpressed in many colon cancer tissues [12]. On the other hand, NUCB2 was implicated as an appetite suppressing protein that plays an important role in regulating feeding behavior in rats [13].

Despite the great importance of NUCB1 and NUCB2 in various signaling pathways, our understanding of the regulation of their biological functions remains limited. In this research letter, we show evidence that both NUCB1 and NUCB2 are cleaved by caspases and their functions mediated by the Ca^{2+}-binding EF-hand motifs could be modulated by caspase-catalyzed cleavage.

MATERIALS AND METHODS

Generation of radiolabeled NUCB1 and NUCB2 proteins

The DNA templates coding the fragments of NUCB1 and NUCB2 were isolated from our previous caspase cleavage selections [14]. The coding sequences for full-length NUCB1 and NUCB2 or their various fragments were PCR amplified from total RNAs of human or zebrafish using gene specific primers. The PCR products were purified and used as templates for a coupled *in vitro* transcription and translation (TNT) reaction in the presence of [^{35}S]-methionine. Expressed proteins were used for proteolytic cleavage assays.

In vitro proteolytic analysis of radiolabeled NUCB1 and NUCB2 by caspases

An aliquot of TNT reaction mixture was incubated in a caspase reaction buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% Glycerol, and 10 mM DTT) at 37 ° C for 0.5 to 12 h with 0.1 to 0.5 unit of a caspase of interest (Biovision, Mountain View, CA) or a caspase pre-inhibited with 10 μM of corresponding tetrapeptide inhibitor (Calbiochem, San Diego, CA). An aliquot of each reaction mixture was loaded onto a 10% or 15% SDS-PAGE gel for separation and the signals were detected by autoradiography.

In vitro proteolytic analysis of recombinant NUCB1 His×6-tagged at the N-terminus by caspases

To determine the cleavage of the N-terminal portion of NUCB1, 1 μg of a recombinant NUCB1 His×6-tagged at the N-terminus (GenWay Biotech, San Diego, CA) was subjected to cleavage assays by incubation with 0.5 unit of caspase-6 or -8 for 6 h. Alternatively, caspase-6 or -8 were pre-inhibited with Z-VEID-fmk or Z-IETD-fmk, respectively, for 30 min at 37 °C prior to the addition of recombinant NUCB1. Digestion reactions were resolved by SDS-PAGE gels, followed by probing with an anti-His×6 antibody (AnaSpec, San Jose, CA).

Ex vivo proteolytic analysis of NUCB1 by caspases

HeLa S3 cells were grown to stationary phase in DMEM media supplemented with 10% FBS. Cells were harvested and lysed as previously described [14]. Caspase-catalyzed proteolysis of endogenous NUCB1 was performed by the addition of 0.5 unit of a caspase of interest to the whole cell extract with 25 μg of total proteins. The reaction mixture was incubated at 37 °C at various time points in the presence or absence of a caspase inhibitor. Proteins in lysates were resolved by SDS-PAGE and blots were probed with anti-NUCB1 polyclonal antibody (Aviva

Systems Biology, San Diego, CA) that recognizes a region (EKKLLERLPEVEVPQHI) near the C-terminus of human NUCB1.

Cleavage of endogenous NUCB1 during apoptosis under physiological conditions

The *in vivo* cleavage experiments were performed by the addition of 2 μM or 10 μM CPT directly to MCF-7 or HeLa S3 cells, which were maintained in the appropriate media. For cleavage inhibition assays, cells were pre-treated with 50 μM of a tetrapeptide inhibitor for 3 h at 37 °C before a 12 h induction of apoptosis by 2 μM CPT. The inhibitors used were Z-VAD-fmk (pancaspase inhibitor), 5-[(S)-(+)-2-(Methoxymethyl)pyrrolidino] sulfonylisatin (caspase-3/7 inhibitor), Z-DEVD-fmk (caspase-3 inhibitor), Z-VEID-fmk (caspase-6 inhibitor), Z-IETD-fmk (caspase-8 inhibitor), Z-LEHD-fmk (caspase-9 inhibitor), and Z-AEVD-fmk (caspase-10 inhibitor). For time course experiments, MCF-7 or HeLa S3 cells were treated with CPT up to 36 h. Cells were lysed using a lysis buffer on ice for 1 h [15]. Crude extracts were cleared by centrifugation at 14,000 RPM for 20 min. Approximately 50 μg of lysate was loaded into each well, separated by SDS-PAGE, probed with C-terminal anti-NUCB1 and reprobed with anti-β-actin as a loading control.

RESULTS AND DISCUSSION

Caspase-mediated cleavage of the selected NUCB1 and NUCB2 at the fragment and full-length levels

To understand which proteins are specifically cleaved by a caspase of interest during apoptosis, we carried out proteome-wide selections for downstream substrates of different caspase family members [14]. Interestingly, NUCB1 was isolated from the caspase-6 cleavage selection, whereas NUCB2 was isolated from both the caspase-3 and caspase-8 cleavage selections.

We first examined whether the selected NUCB1 and NUCB2 fragments were indeed cleaved by various caspases using radiolabeled NUCB1 and NUCB2 fragments generated by TNT. As shown in Figures 1B and 1C, the selected NUCB1 and NUCB2 fragments were indeed cleaved by the caspases used in the selections, respectively, although the cleavage of the selected NUCB1 fragment was weak. Interestingly, both fragments were also cleaved by other caspases. The selected NUCB1 fragment could be cleaved by caspase-6 and -8, whereas the NUCB2 fragment could be cleaved by caspase-3, -6, and -8 (Figures 1 B and 1C). In both cases, the cleavage was completely abolished when the caspase was pre-inhibited with an inhibitor.

We analyzed the sequences of the selected NUCB1 and NUCB2 fragments and mapped their potential cleavage sites (Figure 1D). The NUCB1 fragment isolated from the selection was from E186 to N281, which includes a region (233-EELDGLDPNRFNPKTFFILHDINSDG-258) that contains two potential caspase cleavage sites (underlined). The observed cleavage by caspase-6 and -8 might occur at DGLDP or INSDG, although they are not the optimal cleavage sequences. The cleavage site on NUCB1 was further mapped by a combination of different approaches, including using a series of fragments that contain only one potential cleavage site. Our results indicate that only the fragment that contains DGLDP site ($FG_{174-460}$), but not any other fragments out of this region, was cleaved by caspase-6 and caspase-8 (Figure 1E and data not shown). Therefore, the cleavage most likely occurs at DGLDP. As for NUCB2, six and five NUCB2 fragments were isolated from the caspase-3 and caspase-8 cleavage selections, respectively. These 11 sequences have different lengths but all contain E175-L269 as the common region (Figures 1E). The same approaches were used to map the cleavage site at 237-DGLDP-241 (data not shown).

To examine the cleavage at the full-length level, we first tested the cleavage of endogenous NUCB1 by adding purified caspases to the HeLa S3 cell lysate. Figure 2A illustrates that NUCB1 was cleaved by caspase-3, -6, and -8, and slightly by caspase-7 and -9. Interestingly, a cleavage product with a molecular weight of approximately 27 kDa was detected. The observed proteolytic fragment was presumably from the C-terminal area, since the blot was probed using an anti-NUCB1 antibody that recognizes a region near its C-terminus. We also used radiolabeled full-length NUCB1 generated by TNT for caspase cleavage analysis (Figure 2B). The results were consistent with that when endogenous NUCB1 was used. Figures 2C and 2D illustrate that the cleavage of radiolabeled NUCB1 by caspases is fast, with a half-life of 3 h and 1 h by caspase-6 and -8, respectively.

Since we were not able to detect the expression of full-length NUCB2 by using the commercial anti-NUCB2 antibodies (Aviva Systems Biology and Abcam), full-length radiolabeled NUCB2 generated by TNT was used as substrate for proteolysis. As shown in Figure 2E, full-length NUCB2 was cleaved by caspase-3 and caspase-8 that were used in the selections, with a half-life around 0.5 h by caspase-8 (Figure 2F). The size of the proteolytic product is also consistent with a cleavage at the mapped site using the selected NUCB2 fragments. Intriguingly, full-length NUCB2 thus generated was also cleaved by caspases-6, -7, -9, and weakly by caspase-10. The cleavage patterns by different caspases were very similar, suggesting they have the same cleavage sites.

NUCB1 was specifically cleaved during apoptosis under physiological conditions

To examine the cleavage of nucleobindins under *in vivo* conditions, we induced programmed cell death in different cell lines by CPT. Western blot analysis, using anti-NUCB1 against the C-terminus, illustrates that NUCB1 was cleaved during apoptosis as observed by the significant decrease of the full-length in MCF-7 cells and the appearance of a cleavage fragment in HeLa S3 cells (Figure 3A). The decrease of NUCB1 in both MCF-7 and HeLa S3 cells was dependent on the concentration of CPT that was used to induce apoptosis. Figure 3B shows that the cleavage of NUCB1 during apoptosis in MCF-7 cells is efficient and has a half-life around 9 h following 2 μM CPT treatment. Interestingly, no cleavage product was observed in MCF-7 cells, suggesting that the cleavage products are further degraded *in vivo* and therefore not detectable by anti-NUCB1 that recognizes the C-terminus. In contrast, a proteolytic product was observed in HeLa S3 lysates (Figure 3A, right panel), presumably because different cell lines could behave differently after the induction of apoptosis by CPT.

The *in vivo* cleavage of NUCB1 was caspase-specific and completely inhibited by a caspase-8, caspase-9, or caspase-10 inhibitor (Figure 3C). Although our *in vitro* and *ex vivo* results indicate that NUCB1 was digested efficiently by caspase-3, -6 and -8 (Figures 2A and 2B), it is of great interest to examine which caspase plays a major role in cleaving NUCB1 during apoptosis under *in vivo* conditions. Since caspase-3 is not functional in MCF-7 cells [16], it is unlikely that caspase-3 is involved in the *in vivo* cleavage of NUCB1. Interestingly, NUCB1 digestion still occurred when the cells were pre-treated with a caspase-6 or caspase-3/-7 inhibitor, suggesting these caspases are less likely to play a major role in cleaving NUCB1. By combining the results of *in vitro* cleavage (Figures 2A and 2B) and of *in vivo* inhibition analyses (Figure 3C), the observed *in vivo* cleavage of NUCB1 in MCF-7 cells is likely mediated by caspase-8, although the role of other caspases could not be completely ruled out. It is worth mentioning that we were not able to detect the cleavage of NUCB1 in cells that were not subjected to an apoptotic stimuli, suggesting the cleavage of NUCB1 by the basal caspase activity is below the detection limit.

The conservation of the caspase cleavage sites on nucleobindins in different species

We analyzed the conservation of the putative caspase cleavage sites on both NUCB1 and NUCB2 in different species (Figure 4A). Compared to human NUCB1, the NUCB1 orthologs from bovine, rat, and mouse have amino acid sequence identities at 89%, 87%, and 87%, respectively. The cleavage-site containing E233-G258 region is identical among these four species. NUCB2 is also highly conserved among different mammalian species, with the identical DGLDP site. These results suggest that the cleavage of NUCB1 and NUCB2 by caspases is highly conserved in different species.

It appears that the mapped DXXD cleavage site is also conserved in other species, including in fish and fly. To test whether a nucleobindin protein from a non-mammalian species could be indeed cleaved by its cognate caspase, we synthesized the full-length zebrafish NUCB2 (Z-NUCB2, FL_{1-496}) and the putative cleavage site-containing fragment ($FG_{151-295}$) and used them as substrates for the recombinant zebrafish caspase-3 [17]. Figure 4B illustrates that both the full-length and the fragment of Z-NUCB2 were efficiently and specifically cleaved by zebrafish caspase-3. Interestingly, the cleavage was more efficient by zebrafish caspase-3 than by human caspase-3, suggesting that Z-NUCB2 might be a physiological caspase-3 substrate in zebrafish. However, the evolutionary conservation of the caspase cleavage sites on NUCBs does not necessarily mean that such conservation has got to do with a function. More investigation should be done to address the question.

Implication of the regulation of Ca^{2+}-signaling on NUCB1 and NUCB2 by caspase-mediated cleavage

As shown in Figure 1A, multiple functional domains are present on both NUCB1 and NUCB2 [1]. It appears that the multiple functions of NUCB1 and NUCB2 are partitioned at the first and the second halves of the proteins. The first half contains a signal peptide, a leucine/isoleucine rich region and a putative nuclear targeting signal. Therefore, this half of the nucleobindins is important for their subcellular localization. It has been reported that a NUCB2 mutant lacking the N-terminal leucine/isoleucine-rich region failed to be retained in the Golgi, whereas the deletion of all the other domains or regions had no effect [9]. The second half of nucleobindins contains two EF-hand motifs and a C-terminal leucine-zipper motif, which are critical in sensing the Ca^{2+} signal, interacting with a number of binding partners, and associating with DNA.

The caspase cleavage sites we identified on NUCB1 and NUCB2 are within the region of residues 235-255. In both nucleobindins, such cleavage sites are exactly located at the beginning of the first Ca^{2+}-binding EF-hand motif that is just downstream of the putative bipartite nuclear targeting signal. It has been demonstrated that the EF-hand motifs on NUCB1 and NUCB2 are very important for their binding with Ca^{2+} and also interaction with a number of other binding partners, including $G_{\alpha i3}$, $G_{\alpha i2}$, cyclooxygenases, necdin and ARTS-1. Therefore, it is likely that such functions could be affected by caspase-mediated cleavage. However, which specific functions of EF-hand motif are disrupted remains to be addressed.

The caspase-mediated cleavage will presumably truncate the nucleobindins into at least two pieces. Such cleavage will decouple the functional domains on the first and the second halves that are otherwise well integrated. After the cleavage, the second half is less likely to be retained in Golgi apparatus or translocated to nucleus, due to the removal of the N-terminal Golgi retention domain and the nuclear targeting signal. In light of this hypothesis, we traced the fate of the N-terminal portion of NUCB1 by using an N-terminal His×6-tagged NUCB1. It was found that the N-terminal tagged NUCB1 was rapidly degraded *in vivo*. Therefore, we addressed the question by incubating a recombinant NUCB1 His×6-tagged at the N-terminus with purified caspase-6 or -8 (Figure 3D). The result shows that a major fragment is produced

from such a cleavage, with a size consistent with a cleavage at the mapped site. Due to the rapid degradation of the N-terminal tagged NUCB1 in mammalian cells, the physiological role of the N-terminal region should be addressed using antibodies that specifically recognize the N-terminus of the endogenous protein or using caspase-resistant mutants. Future work will be directed to investigate the role of the N-terminus, the regulation of the biological functions of NUCB1 and NUCB2 by Ca^{2+} signals and by caspases under physiological conditions.

Acknowledgements

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ABBREVIATIONS

CPT
: camptothecin

NUCB1
: nucleobindin-1

NUCB2
: nucleobindin-2

TNT
: coupled *in vitro* transcription and translation

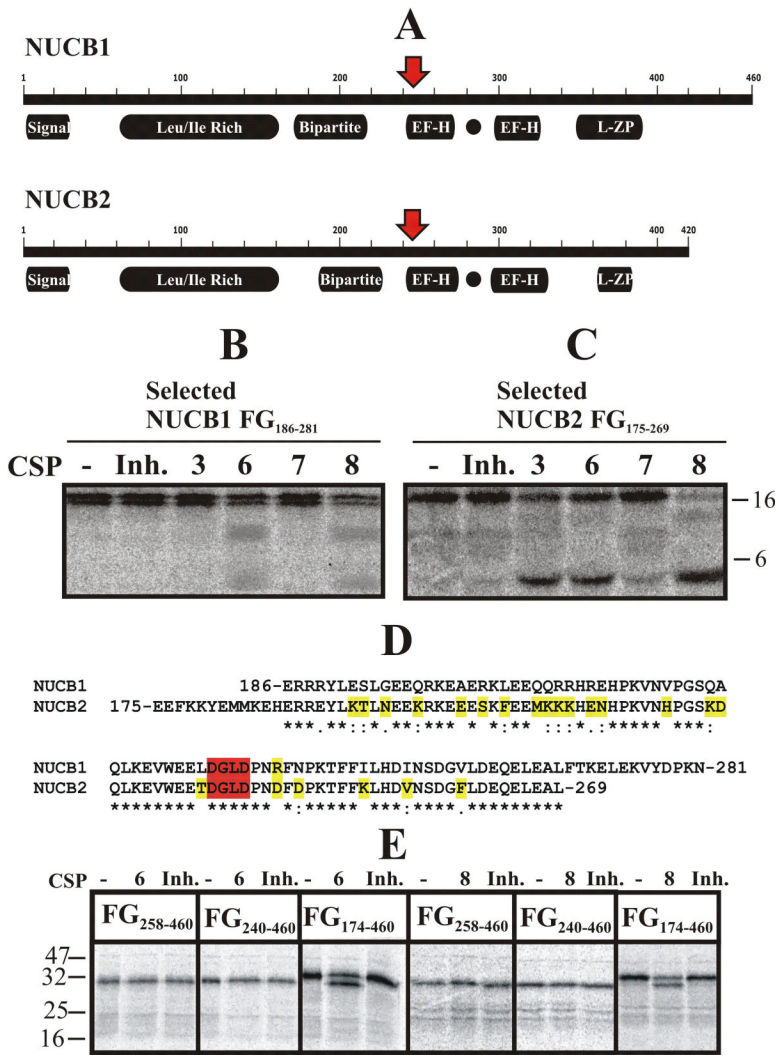

Figure 1.

Schematic representation of the functional domains on human NUCB1 and NUCB2 **(A)** and *in vitro* cleavage of the selected NUCB1 **(B)** and NUCB2 **(C)** fragments (FG) by caspase-3, -6, -7, and -8. The radiolabeled protein fragments were generated by TNT. EF-H: Ca^{2+}-binding EF-hand motif; L-ZP: leucine-zipper motif; I: caspase-3 inhibitor Ac-DEVD-fmk. The black dot between two EF-hand motifs represents an acidic region. The putative cleavage site is indicated by a red arrow. **(D):** The sequences of the NUCB1 and NUCB2 fragments isolated from different caspase cleavage selections. **(E):** Cleavage site mapping using radiolabeled NUCB1 fragments that cover different regions.

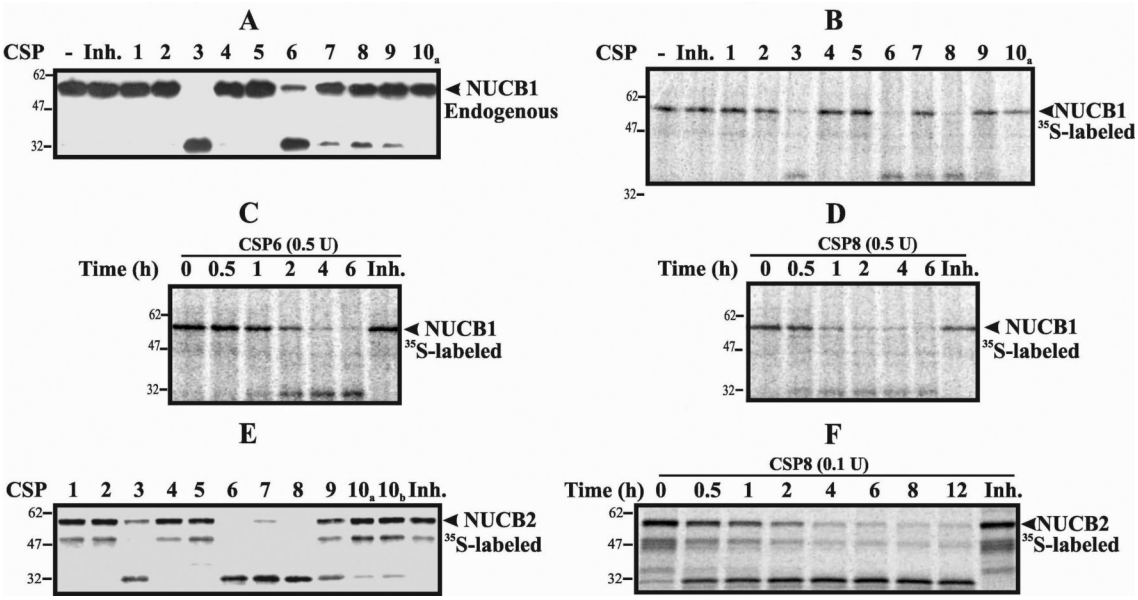

Figure 2.

The caspase cleavage specificity and time course of full-length nucleobindins. **(A):** *Ex vivo* caspase cleavage specificity of endogenous full-length NUCB1 from HeLa S3 lysate. **(B):** *In vitro* cleavage specificity of radiolabeled full-length NUCB1 generated by TNT. **(C):** *In vitro* cleavage time course of radiolabeled full-length NUCB1 by caspase-6. **(D):** *In vitro* cleavage time course of radiolabeled full-length NUCB1 by caspase-8. **(E):** *In vitro* caspase cleavage specificity of radiolabeled full-length NUCB2. **(F):** *In vitro* cleavage time course of radiolabeled full-length NUCB2. *Endogenous* NUCB1 was probed using an anti-NUCB1 antibody that recognizes its C-terminus. All radiolabeled NUCBs were detected through autoradiography. Negative controls without using any caspase or with a pre-inhibited caspase were labeled as “-” and “Inh”, respectively.

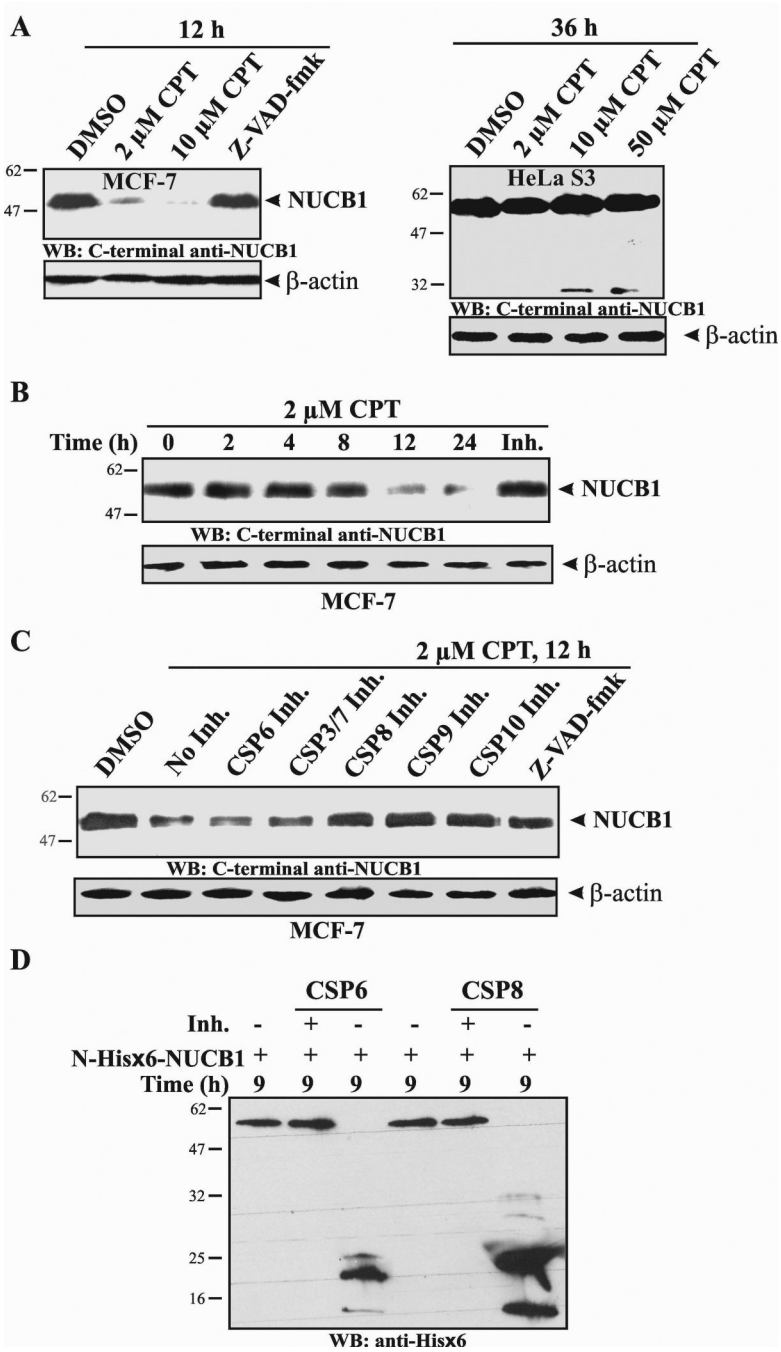

Figure 3.

In vivo analysis of NUCB1 cleavage in MCF-7 and HeLa S3 cells and *in vitro* N-terminal digestion tracking of recombinant NUCB1. **(A):** Cleavage of NUCB1 in MCF-7 (left panel) and HeLa S3 (right panel) cells after a 12 h or 36 h induction with various concentrations of CPT. **(B):** Western blot analysis of the time-dependent cleavage of NUCB1 after the induction of apoptosis by 2 μM CPT in MCF-7 cells. **(C):** *In vivo* inhibition assay using different caspase inhibitors. Cells were pre-treated with 50 μM of various inhibitors prior to the induction of apoptosis with 2 μM of CPT (bottom panel). The inhibitors used were Z-VAD-fmk, 5-[(S)-(+)-2-(Methoxymethyl)pyrrolidino] sulfonylisatin, Z-VEID-fmk, Z-IETD-fmk, Z-LEHD-fmk, and Z-AEVD-fmk for pancaspase, caspase-3/-7, caspase-6, caspase-8, caspase-9, and

caspase-10, respectively. NUCB1 was probed using an anti-NUCB1 antibody that recognizes its C-terminus. **(D):** Tracking the cleavage of the N-terminal portion of NUCB1. N-His×6-NUCB1 was digested with caspase-6 or –8 or caspases pre-inhibited with the appropriate inhibitor. Blots were probed with an anti-His×6 antibody.

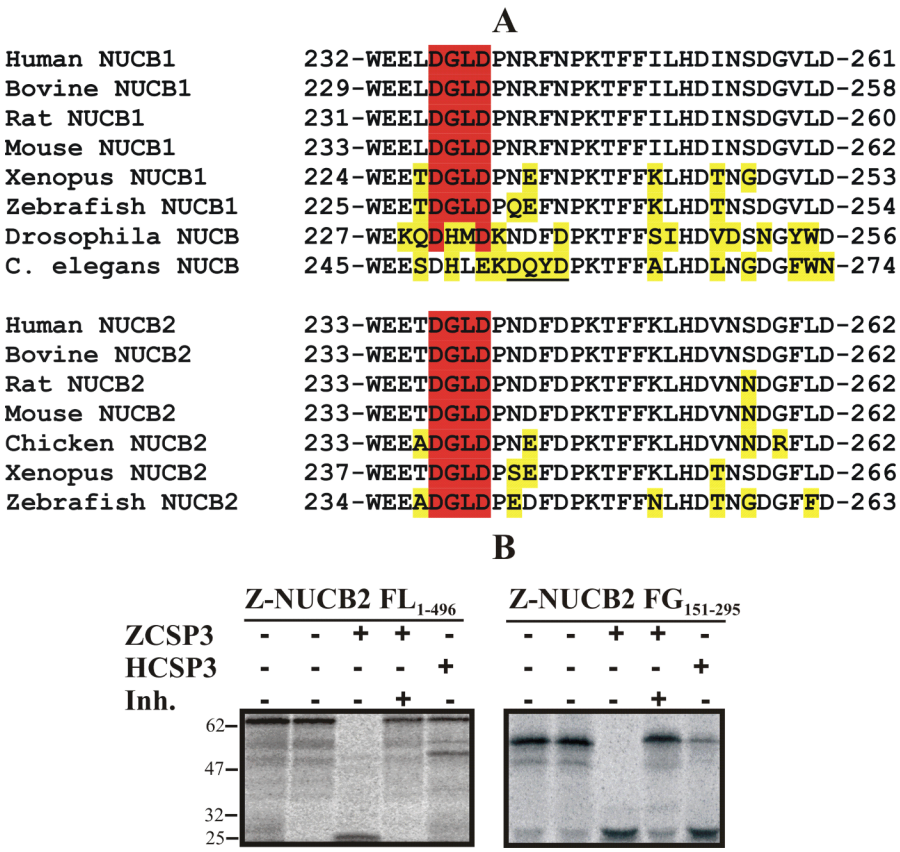

Figure 4.

(A): Alignment of the selected, cleavage site-containing human NUCB1 and NUCB2 fragments with the corresponding regions on their orthologs from other species. The conserved caspase recognition sites are highlighted in red. Non-conserved residues are marked in yellow. The putative DQYD cleavage site on *C. elegans* NUCB is underlined. **(B):** *In vitro* cleavage of zebrafish NUCB2 full-length or putative cleavage site-containing fragment by zebrafish caspase-3 (ZCASP3) and by human caspase-3 (HCASP3). Ac-DEVD-CHO was utilized as an inhibitor of ZCASP3.