Dentatorubral Pallidoluysian Atrophy (DRPLA) Protein Is Cleaved by Caspase-3 during Apoptosis

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Dentatorubral pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder. It is associated with an abnormal CAG repeat expansion resulting in formation of a protein with an elongated polyglutamine stretch. However, neither the physiological roles of the DRPLA gene product nor molecular mechanisms of its pathogenesis have yet been elucidated. Here we report that the DRPLA protein is cleaved at a site near the N terminus during apoptosis induced by VP-16, staurosporine, or glucocorticoid. Moreover, the in vitro translated DRPLA protein is cleaved by recombinant caspase-3, a member of the cysteine protease family, which is thought to be a main executor of apoptosis. Using mutant DRPLA proteins, the cleavage site was identified as 106DSLDG110. The cleavage, however, was not modulated by the length of the polyglutamine stretch. These findings suggest that the DRPLA protein is one of the physiological substrates of caspase-3, and its cleavage may result in structural and biochemical alterations associated with apoptosis.

Apoptosis is an indispensable phenomenon for normal development and maintenance of homeostasis in multicellular organisms. Although the signal transduction of apoptotic stimuli remains unclear, accumulating evidence indicates that various types of apoptotic signals ultimately converge on the activation of the same machinery. The components of this machinery are cysteine proteases belonging to a caspase family (1, 2). So far, about 10 members have been identified including interleukin-1β-converting enzyme (ICE or caspase-1) (3) and caspase-3 (CPP32/Yama/Apopain) (4–6). Each of these enzymes is synthesized as a proenzyme, and then proteolytically activated. Like systems in blood clotting and complement activation, the protease cascade is known to exist where one protease activation can lead to processing of the same or other members of the caspase family. Poly(ADP-ribose) polymerase (PARP) is identified for the first time as a substrate of the caspase family (5). Since the function of PARP is associated with genome maintenance and DNA repair, the cleavage of PARP may play an important role during apoptosis. However, it is unlikely that its cleavage is essential for apoptosis since PARP-deficient mice do not show any deregulation of apoptosis (7). Therefore, there must be other substrates that are critical for apoptosis, or more likely, the coordinated degradation of several key proteins is necessary for the execution of apoptosis. At least 10 proteins are known to be cleaved during apoptosis, including nuclear lamins, actin, a 70-kDa protein component of the U1-ribonucleoprotein (U1–70 kDa), DNA-dependent protein kinase, and DNA fragmentation factor (8–12). To elucidate the final step of apoptosis, other candidates of the “death substrates” cleaved during apoptosis need to be identified.

Dentatorubral pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder characterized by progressive dementia, myoclonic epilepsy, cerebellar ataxia, and choreoathetotic movement. Pathological findings of the brain were represented as combined degeneration of dentatofugal and pallidothalamic pathways. We and others previously found expansion of unstable CAG repeats of the DRPLA gene in the patients (13, 14). Although the physiological functions of the DRPLA gene product or the pathogenesis of DRPLA are still unclear, it is tempting to speculate that they are related to apoptosis for a number of reasons. First, neuronal death is reported to be apoptotic in other disorders such as Huntington disease or Machado-Joseph disease, which are also caused by expansion of CAG repeats (15, 16). Second, mice deficient for the Huntington disease gene show increased apoptosis and embryonic lethality (17). Here we report that the DRPLA protein is proteolytically cleaved during apoptosis by caspase-3.

EXPERIMENTAL PROCEDURES

Cell Culture—Human teratocarcinoma cell line NT2 (Ntera2/D1, Stratagene) (18) and human pre-B cell leukemia cell line 697 (19) were maintained in Dulbecco’s modified Eagle’s medium and RPMI 1640, respectively, supplemented with 10% heat-inactivated fetal calf serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO2.

Western Blotting—Immunoblot assays were carried out as described previously (20). In brief, resolved proteins (30 μg) in SDS-PAGE gels were transferred to a nitrocellulose membrane (Schleicher & Schuell) by electrot blotting. Immunoblotting was performed using a 1:750 dilution of the primary anti-DPRA antibody followed by a 1:5,000 dilution of horse-radish peroxidase-conjugated goat anti-rabbit IgG (Sigma) as a second antibody, and the proteins were visualized by ECL (Amersham Corp.). A Vectorstain ABC kit (Vector Laboratories Inc.) and 3-amino-9-ethyl carbazole were also used for some of the developments. Rabbit anti-DRPLA antisera were raised against GST-DRPLA fusion protein (see below). Prior to immunoblotting, the antisera were affinity-purified by using GST and protein A columns. To detect PARP, a 1:2,000 dilution of anti-PARP monoclonal antibody (Biomol) and a 1:1,000 dilution of rabbit horseradish peroxidase-conjugated anti-mouse immunoglobulins (DAKO) were used for the primary and secondary antibodies, respectively.
Plasmid Construction—To produce the GST-DRPLA fusion protein, a Pst-I-PstI fragment of DRPLA cDNA (nucleotides 2259–3158, residues 676–973) was blunt-ended using T4 DNA polymerase and subcloned into the SmaI site of pGEX-5X (Pharmacia). pMY1240 and pMY1247, which contained full-length DRPLA cDNA with 14- and 71-repeat CAG, respectively, were subcloned into a pBluescript SK vector.2 To make a series of C-terminal deletion mutants of the DRPLA protein, pMY1240 was digested using the Kpn-I-sequencing deletion kit (Takara Shuzo, Japan) after digestion with EcoRi and KpnI. To disrupt each of the two potential caspase-3 cleavage sites (290DPRD123 and 290DPKD123), a polymerase chain reaction overlap extension technique was used (25). The following amplification primers were used: Pu DNA polymerase (Stratagene) and pMY1240 as a template: 5′-GGAGT-GAGGAGATCTCAGAGAGTG-3′ (BglII site underlined); 5′-GGTTGGT-TCAAAGCTAGCTTGCTG-3′ (Nhel site underlined); 5′-GATCTT-GAATTCGTTGGCCGGCCGGG-3′ (mutations underlined); 5′-CGGGG-GCGAACTATTCAGATC-3′ (mutations underlined); 5′-GTCGAT-AGCCCCCTAGGGCCGTCGT-3′ (mutations underlined). The final polymerase chain reaction products were gel-purified, digested with BglII and Nhel, and subcloned into BglII and Nhel sites of pMY1240 to produce the plasmids pNSLA and pAPRA (mutations underlined).

Analysis of Caspase-3 Activities—Cell extracts were prepared by resuspending the cell pellet in 100 μl of lysis buffer (50 mM Tris, pH 7.5, 0.5% Nonidet, 10 μl of cell extract, 150 mM NaCl). 30 μl of cell extract were mixed with 100 μl of reaction buffer (20 mM HEPES, pH 7.5, 0.1% SDS, 5 mM dithiothreitol) and 50 μl of 100 mM Ac-DEVD-MCA (Peptide Institute, Inc., Japan) which are specific inhibitors of caspase-3. After incubation at 37 °C for 2 h, the standard curve was prepared using solutions of 7-amino-4-methylcoumarin at various concentrations in the reaction buffer. The fluorescence of the cleaved substrates was measured using a spectrofluorometer set at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. One unit corresponds to the activity that cleaves 1 nmol of Ac-DEVD-MCA at 37 °C in 2 h.

Cleavage of in Vitro Translated DRPLA Protein—The in vitro translated proteins were prepared using the TNT coupled reticulocyte lysate systems (Promega) and radiolabeled with [35S]methionine. Two μl of in vitro translation reaction mixture were incubated in cleavage buffer (50 mM PIPES/KOH (pH 6.5), 2 mM EDTA, 0.1% (w/v) Chaps, 5 mM dithiothreitol) along with either apoptotic cell extracts (about 0.5 unit) or recombinant caspase-3 (20 μl final volume). Apoptotic cell extracts were prepared from 697 cells treated with 10 M dexamethasone for 24 h (23). Recombinant caspase-3 was kindly provided by M. Enari and S. Nagata (24). For some of the reactions, we added various concentrations of Ac-YVAD-CHO or Ac-DEVD-CHO (Peptide Institute, Inc., Japan) which are specific inhibitors of caspase-1- or caspase-3-like proteases, respectively. The mixture was incubated at 37 °C for 1 h and subjected to 6% SDS-PAGE after boiling at 95 °C for 5 min with Laemmli buffer. After electrophoresis, the gels were dried and autoradiographed. Quantitative evaluation of DRPLA cleavage was performed using a BAS2000 Bio-Imaging analyzer (Fuji film).

RESULTS

DRPLA Protein Is Cleaved during Apoptosis—We first confirmed the specificity of the antibody used in this study that had been raised against GST-DRPLA fusion protein. As shown in Fig. 1A, the antibody reacted specifically with in vitro translated DRPLA proteins but not with an irrelevant protein, p53. As described previously (25), the molecular masses of the DRPLA proteins containing 14 and 17 repeats of glutamine were 160 and 170 kDa, respectively, which were much larger than the predicted sizes based on their amino acid compositions.

p53 protein were subjected to Western blotting using an anti-DRPLA antibody. Closed and open triangles indicate DRPLA gene products from pMY1247 and pMY1240, respectively. B and C, NT2 cells were treated with 10 M dexamethasone for 24 h. Cell lysates were obtained at various time points and subjected to Western blotting using anti-DRPLA (B) and anti-PARP (C) antibodies. Undeaved and cleaved proteins are indicated by closed and open triangles, respectively. D and E, 697 cells were incubated with 10 M dexamethasone (DEX) and followed by the same procedures as B and C.

2 K. Nakamura, manuscript in preparation.
The cleavage is mediated by caspase-3-like proteases. A, in vitro translated DRPLA protein derived from pMY1240 (Fig. 1) was incubated with either control (C) or apoptotic (A) 697 cell extracts and with or without varying concentrations of the caspase inhibitors, Ac-YVAD-CHO or Ac-DEVD-CHO. Reaction mixtures were then subjected to 6% SDS-PAGE, followed by autoradiography. Uncleaved and cleaved proteins are indicated by closed and open triangles, respectively. B, inhibition of pMY1240 cleavage in apoptotic extracts by Ac-YVAD-CHO or Ac-DEVD-CHO, whereas the inhibition was negligible with the latter (Fig. 2B). These data indicate that the cleavage is mediated by a caspase-3-like protease but not by a caspase-1-like protease.

To investigate the localization of the cleavage site, a series of C-terminal deletion mutants (Fig. 3) as well as full-length wild type cDNA were in vitro translated using T3 RNA polymerase. The open reading frames are shown in boxes. The regions encoding a putative nuclear localization signal and polyglutamines are hatched and filled, respectively. The numbers below the boxes represent the position of the amino acid counted from the initiation methionine of the DRPLA protein. Uppercase letters following the boxes represent amino acids derived from the vector sequence before the stop codon.

Cleavage Site Is 106DSLDG110—To identify the exact cleavage site of the DRPLA protein, we searched for a DXD motif, which is the consensus cleavage site for caspase-3 (27). Three caspase activity after treatment with dexamethasone was described previously (23). As shown in Fig. 2A, we observed an additional band of 145 kDa when incubated with apoptotic lysate but not with nonapoptotic 697 lysate (lanes 1 and 2). The DRPLA Protein Translated in Vitro Is Cleaved by Recombinant Caspase-3—To address the question if the responsible protease is caspase-3 itself or another protease(s) resembling caspase-3, we used recombinant caspase-3 prepared in Escherichia coli. As shown in Fig. 5A, the pattern of cleavage observed with comparable units of recombinant caspase-3 was the same as that for the apoptotic lysate. These results show that the DRPLA protein is a novel substrate of at least caspase-3.

Cleavage Site Is 106DSLDG110—To identify the exact cleavage site of the DRPLA protein, we searched for a DXD motif, which is the consensus cleavage site for caspase-3 (27). Three
such motifs were found in the DRPLA protein, two of them were located near the N terminus (106DSLD109 and 120DPRD123). Two plasmids, pNSLA and pAPRA, were generated to produce mutant DRPLA proteins in which either 106DSLD109 or 120DPRD123 was disrupted, respectively (Fig. 3). The mutant DRPLA protein NSLA was not cleaved by caspase-3, while APRA showed the same cleavage pattern as the wild type protein (Fig. 5A, lanes 7 and 8). Therefore, we conclude that the cleavage site of the DRPLA protein is 106DSLDG110. A faint cleaved product of NSLA may represent an alternative cleavage at 120DPRD123 when 106DSLD109 was disrupted (lane 8).

Polyglutamine Tract Does Not Modulate Caspase-3 Cleavage—Since patients with DRPLA are associated with expansion of unstable CAG repeats of the DRPLA gene, we next investigated whether the length of the polyglutamine tract affected the protein cleavage by caspase-3. Plasmids pMY1240 and pMY1247 which had 14 or 71 CAG repeats, respectively, were used for the analysis. In vitro translated proteins with different sizes of the polyglutamine tract were similarly cleaved by varying concentrations of recombinant caspase-3, indicating that the size of the CAG repeat does not modulate DRPLA cleavage by caspase-3 (Fig. 5, A and B).

**DISCUSSION**

Activation of a group of cysteine proteases recently termed caspases is commonly observed during apoptosis triggered by a variety of stimuli (1, 2). The importance of the caspase family for the execution of apoptosis is underscored by several findings. First, CED-3, a caspase homolog of *Caenorhabditis elegans*, is required for programmed cell death during development of the nematode (3). Second, protease inhibitors specific for caspases have the ability to inhibit apoptosis (28). Third, mice deficient for a caspase such as caspase-1 or -3 show abnormal organ development or deregulations of apoptosis induced by some but not all kinds of signals (29, 30). However, how the activation of these proteases can lead to the characteristic changes of apoptosis is totally unknown. Although more than 10 proteins are known to be cleaved during apoptosis, it has not yet been demonstrated that cleavage of any one of them is essential for apoptosis. Therefore, the identification of new substrates as well as novel caspases should provide an insight into the mechanism of apoptosis.

In this report, we demonstrated that the DRPLA protein is cleaved by caspase-3 during apoptosis. It is cleaved in vivo with a very similar kinetics to PARP, one of the known substrates for caspase-3. To our knowledge, the DRPLA protein is the ninth substrate of caspase-3 demonstrated experimentally to date. The cleavage site was identified as 106DSLDG110, which is

**FIG. 4.** DRPLA protein is cleaved at a site near N terminus. In vitro translated proteins derived from a series of truncated DRPLA cDNAs depicted in Fig. 3 were incubated with either control (C) or apoptotic (A) 697 cell extracts and then subjected to SDS-PAGE followed by autoradiography.

**FIG. 5.** Cleavage of the DRPLA protein by recombinant caspase-3 is not modulated by the length of polyglutamine tract. A and B, an equivalent amount of *in vitro* translated full-length DRPLA protein derived from pMY1240 (with 14 CAG repeats) and pMY1247 (with 71 CAG repeats), and mutant proteins derived from pNSLA and pAPRA (Fig. 1) were incubated with varying amounts of recombinant caspase-3 (rCaspase-3) at 37 °C for 1 h and then subjected to SDS-PAGE followed by autoradiography. Uncleaved and cleaved proteins were indicated by closed and open triangles, respectively. C, a graph of DRPLA cleavage by recombinant caspase-3 shows that the rate of cleavage is not modulated by the length of polyglutamine tract. Cleavage was quantified by an imaging analyzer. The experiment was performed twice with similar results.
similar to the consensus sequence for the caspase-3 cleavage site, D^XXD^P (27). It should be noted that the DRPLA protein was cleaved at a physiological concentration of caspase-3.Excess amounts of caspase-3 could cleave proteins with less stringent requirements for the recognition sequence (data not shown). Obviously it is still possible that other members of the growing caspase family, either known or unknown, can also cleave this protein.

Since the biological function of DRPLA is still unknown, it is elusive how important role the proteolysis of this protein plays during apoptosis. Moreover, the proposed roles for the cleavage of any substrates reported previously remain largely hypothetical. Interestingly, some substrates of caspases are nuclear repair proteins such as PARP, DNA-dependent protein kinase, or U1–70 kDa whose cleavage may result in the DNA degradation characteristic of apoptosis (7, 10, 11). The cleavage of these proteins separates key functional domains of the molecule. In the light of these findings, it is intriguing that a bipartite nuclear localization signal (31) at the N terminus of the DRPLA protein (Fig. 3, 16RK KEAPGPREEL RSRRG) is removed by proteolysis during apoptosis, possibly resulting in a loss of function. Although we previously detected the DRPLA gene product in the cytoplasm using immunohistochemistry (25), we have also shown that it is present. However, we have also shown that the cleavage of this protein may result in the cytoplasm after apoptotic cleavage. Aversely, like DNA fragmentation factor (12), it is also likely that the cleaved DRPLA protein can induce apoptosis. In this scenario, instead of the protective effect of the uncleaved protein, the cleaved fragments would be toxic to the cells or have a dominant negative effect.

Some of the other known substrates for caspases such as nuclear lamins, actin, fodrin, the actin-associated protein, or U1–70 kDa whose cleavage may result in the DNA degradation characteristic of apoptosis (12) are cleaved at a physiological concentration of caspase-3. Excess amounts of caspase-3 could cleave proteins with less stringent requirements for the recognition sequence (data not shown). Obviously it is still possible that other members of the growing caspase family, either known or unknown, can also cleave this protein.

Cleavage of DRPLA Protein by Caspase-3

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