Characterization of the Rat DNA Fragmentation Factor 35/Inhibitor of Caspase-activated DNase (Short Form)

THE ENDOWED INHIBITOR OF CASPASE-DEPENDENT DNA FRAGMENTATION IN NEURONAL APOPTOSIS

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Nuclear changes, including internucleosomal DNA fragmentation, are classical manifestations of apoptosis for which the biochemical mechanisms have not been fully elucidated, particularly in neuronal cells. We have cloned the rat DNA fragmentation factor 35/inhibitor of caspase-activated DNase (short form) (DFF35/ICADS) and found it to be the predominant form of ICAD present in rodent brain cells as well as in many other types of cells. DFF35/ICADS forms a functional complex with DFF40/caspase-activated DNase (CAD) in the nucleus, and when its caspase-resistant mutant is over-expressed, it inhibits the nuclelease activity, internucleosomal DNA fragmentation, and nuclear fragmentation but not the shrinkage and condensation of the nucleus, in neuron-differentiated PC12 cells in response to apoptosis inducers. DFF40/CAD is found to be localized mainly in the nucleus, and during neuronal apoptosis, there is no evidence of further nuclear translocation of this molecule. It is further suggested that inactivation of DFF40/CAD-bound DFF35 and subsequent activation of DFF40/CAD during apoptosis of neuronal cells may not occur in the cytosol but rather in the nucleus through a novel mechanism that requires nuclear translocation of caspsases. These results establish that DFF35/ICADS is the endogenous inhibitor of DFF40/CAD and caspase-dependent apoptotic DNA fragmentation in neurons.

Apoptosis is a genetically controlled cell suicide program requiring the activation of caspases (1–5). Inappropriate activation of the apoptosis machinery in neurons has been implicated in the pathogenesis of a number of neurological disorders such as stroke, head trauma, and neurodegenerative diseases (6–11). There is strong evidence that caspase activation and subsequent proteolytic degradation of cellular substrates play a central role in the execution of neuronal death in ischemic and traumatic brain injury (12–14). Caspases cleave various cellular substrates, which leads to the morphological and biochemical changes in apoptosis (15, 16). Caspase-mediated nuclear alterations, such as chromatin condensation and internucleosomal cleavage of DNA, have been considered the hallmarks of apoptosis and are regular findings in neuronal apoptosis. However, the molecular mechanisms regarding the regulation and execution of these nuclear events, particularly in the neuronal context, have not been fully elucidated.

Recent studies using non-neuronal cells have indicated that apoptotic DNA fragmentation and associated nuclear changes are largely due to the action of a 40-kDa nuclease termed DNA fragmentation factor 40 (DFF40)1 (17–19), also known as caspase-activated deoxyribonuclease (CAD) for its mouse counterpart (20, 21). Inactive DFF40/CAD in non-apoptotic cells is a heterodimeric complex with its natural inhibitor DFF45 (17), also known as ICAD (21). Further studies in murine cells have found that transcription of the ICAD gene results in not only the previously reported 45-kDa protein (ICADl or DFF45) but also a 35-kDa protein (ICADs or DFF35) due to alternative splicing (20, 22). Whereas both DFF45 and DFF35 can bind to DFF40/CAD and share an identical domain (amino acid residues 101–180) (22), only DFF45 was reported to be functional. DFF45 seems to be important for the proper folding of the newly synthesized DFF40/CAD, and when binding to the latter, it also inhibits the DNA nuclease activity of DFF40/CAD (21–24). Although the role of DFF35/ICADS is not clear, it appears to possess a stronger ability to bind to DFF40/CAD in vitro than DFF45/ICADL (22), which suggests that DFF35/ICADS participates in the regulation of DFF40/CAD activity in vivo as well.

The activation of CAD involves the cleavage of ICAD, which results in the relief of the inhibition of CAD. ICAD is specifically cleaved by caspase-3 or -7 (25). ICAD with mutated caspase cleavage sites possess the ability to suppress DNA fragmentation during apoptosis (20). It has been postulated that caspase-3-mediated ICAD cleavage occurs in the cytosol and that CAD is translocated to the nucleus after it is released from the complex, where it exercises its nuclease activity (21). This hypothesis, based on the studies of lymphoid cells, however, has not been fully tested in vivo and in other types of cells.

1 The abbreviations used are: DFF, DNA fragmentation factor; CAD, caspase-activated deoxyribonuclease; ICAD, inhibitor of caspase-activated DNase; ICADl, ICAD (long form); ICADs, ICAD (short form); RACE, rapid amplification of cDNA ends; bp, base pair(s); PCR, polymerase chain reaction; PI, propidium iodide; rDFF, rat DFF; wt, wild type; dm, double mutant; STS, staurosporin.

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To further elucidate the precise mechanisms by which caspases promote neuronal apoptosis, we have investigated the nuclear apoptotic events mediated by caspases in neuronal cells. Here we report the cloning and characterization of a DFF35/ICADΔ7 counterpart in the rat, the species that is extensively used for modeling neurological diseases. We found that DFF35/ICADΔ7, but not DFF45/ICADΔ3, is the predominant form of ICAD expressed in the rodent central nervous system and many other organ systems. DFF35/ICADΔ9 binds to DFF40/CAD and is fully functional. It suppresses DFF40/CAD activity and internucleosomal DNA fragmentation in caspase-3-dependent neuronal apoptosis. Furthermore, we found that the DFF35/DFF40 complexes are mainly localized in the nucleus of neuronal cells and that the activation of DFF40/CAD is a nuclear process that requires nuclear translocation of caspase-3. These results identify DFF35/ICADΔ9 as the predominant endogenous inhibitor of DFF40/CAD in neurons and demonstrate a novel mechanism by which DFF40/CAD is activated during apoptosis.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and Site-directed Mutagenesis of ICAD—To clone the rat homologues of DFF, a rat brain cDNA library was constructed using the Marathon™ cDNA amplification kit (CLONTECH) as described previously (14). The double-strand cDNA was purified by phenol/chloroform/isooamyl alcohol and chloroform extraction. The Marathon™ cDNA adaptor was ligated to both ends of the double-strand cDNA using a T4 DNA ligase and then subjected to rapid amplification of cDNA 5′ and 3′ ends (5′- and 3′-RACE). A 321-bp cDNA fragment encoding the rat homologue of DFF/ICAD was obtained by reverse transcriptase-PCR using primers based on the conserved sequences in human DFF45 and mouse ICAD: 5′-cta cct ctg ctc ttc-3′ and 5′-ctc tct gct gac aag cac-3′. Based on the sequence of this cDNA fragment, the RACE primers were synthesized: 5′-gtc tct ctc tgg tca aca ccc (for 5′-RACE), 5′-tga cta ctt ctc cct ctc ttc-3′ (for 3′-RACE), and 5′-ctc tac taa tca gac tca cta tag ggc-3′ (adaptor primer). The adapter-ligated double-strand cDNA served as templates for RACE. The 5′-RACE- and 3′-RACE-amplified fragments were subcloned into pSPORT1 vector (Life Technologies, Inc.) and PGEM™-T easy vector (Promega), respectively. The cDNAs were sequenced on both strands. The full-length cDNA was then obtained using PCR, based on the obtained 5′- and 3′-end sequences. The cDNA containing the open reading frame of the long isoform of mouse ICAD (ICADΔ7) was obtained using PCR from a mouse spleen cDNA library. The primers used were 5′-agt gag ctg ccc gga gag ggc cgc-3′ and 5′-ctc cct gga gca tgt gtt tgg gtc-3′.

D117E and D224E double mutants of rat ICAD and mouse ICAD were generated by site-directed mutagenesis using the Gene Editor™ system from Stratagene. The mutations were introduced into the PGEX-2T vector. The mutated cDNA plasmids were transfected into PC12 cells with the assistance of LipofectAMINE reagent (Life Technologies, Inc.).

**Generation of GST Fusion Proteins**—Wild type and mutant rat ICAD and mouse ICADΔ7 cDNAs were amplified and fused into the GST gene in PGEX-2T (Amersham Pharmacia Biotech). The GST fusion proteins were expressed in Escherichia coli BL21 cells and absorbed to a glutathione-Sepharose 4B column. The fusion proteins were then cleaved by thrombin in 16 h at room temperature to remove the GST portion. The elute was collected by centrifugation at 500 × g for 5 min at 4 °C. The protein was electrophoresed onto a 15% SDS-polyacrylamide gel and subjected to Coomasie Blue staining.

**Northern Blot Analysis**—Polyadenylated RNA was isolated from various rat tissues using the polyATrac™ System 1000 (Promega), electrophoresed on a 1.2% agarose formaldehyde gel, and blotted onto a Zeta-probe GT blotting membrane (Bio-Rad). After prehybridization for 2 h at 42 °C, the membrane was hybridized with the 32P-labeled rat ICAD cDNA probe for 24 h at 42 °C. Autoradiography was done at −80 °C overnight with an intensifying screen.

**Immunoprecipitation and Western Blot Analysis**—Protein extraction from whole cells and cytosolic or nuclear fractions was performed using standard procedures. Immunoprecipitation using an anti-DFF40/CAD polyclonal antibody (Caymen Chemical). ICAD was detected by Western blotting using an affinity-purified polyclonal antibody (designated p35) recognizing the internal peptide of both rat ICAD and mouse ICADΔ7 (WKNVARKQKDLDSS1) or a polyclonal antibody against the C-terminal peptide of mouse ICADΔ7 (Caymen Chemical), which only recognizes ICADΔ7 (designated p45). Immunoblot was performed using the horseradish peroxidase-conjugated goat anti-rabbit antibody and developed using the enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

**Induction of Apoptosis in Neuron-differentiated PC12 Cells**—PC12 cells were plated onto collagen-coated dishes and maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 7.5% heat-inactivated horse serum and 7.5% fetal bovine serum (Life Technologies, Inc.). When cells reached 80% confluence, cells were re-plated at a density of 2 × 10^4 cells/cm^2 for nerve growth factor differentiation by supplementing Dulbecco’s modified Eagle’s medium with 1% serum and 100 ng/ml 2.5-S nerve growth factor (Collaborative Biochemicals Inc.) and incubating at 37 °C for 6 days.

Apoptosis was induced in neuron-differentiated PC12 cells by incubating the cells at the indicated concentrations with staurosporin or etoposide (Biomol). Cell death was examined using phase-contrast microscopy and quantified using propidium iodide (PI) staining. At the conclusion of the experiments, PI (Sigma) was added directly to the culture medium to a final concentration of 50 μg/ml, and the cultures were subjected to examination under a fluorescent microscope. The percentage of PI-stained cells was determined by counting at least 3000 cells under each experimental condition; the nuclear morphology of PI-stained cells was evaluated. Nuclear morphology was also examined in 2% paraformaldehyde-fixed cells using Hoechst 33342 staining.

**In Vitro DNA Fragmentation and Apoptosis Assay**—PC12 cells were grown and collected, and the nuclei were isolated in a hypotonic buffer as described previously (26, 27). For the isolation of brain cell nuclei, the cortices were dissected from rat brain, rinsed with ice-cold phosphate-buffered saline, and homogenized in the hypotonic lysis buffer.

The in vitro DNA fragmentation assay was performed as described (27). Neuronal nuclei (1 × 10^6 per reaction) were incubated in 250 μg of S-100 fraction prepared from PC12 cells for 3 h at 37 °C with the addition of recombinant active caspase-3 (30 ng). DNA fragmentation was determined in extracted genomic DNA using 2% agarose gel electrophoresis followed by ethidium bromide staining. To test the ability of recombiant rat ICAD, and mouse ICADΔ7, to inhibit DNA fragmentation, the thieno-cleaved proteins, treated with or without caspase-3, were added to the above in vitro assay system.

**Western Blot Analysis**—Western blot analysis was performed by incubating neuronal nuclei (1 × 10^6 per reaction) for 2 h at 37 °C with a crude cytoplasm fraction (250 μg) prepared from PC12 cells treated with the indicated apoptosis inducers. The nuclei were stained with PI, and the morphology was evaluated under a fluorescent microscope equipped with an image analysis system. Recombinant rat ICAD was added at the beginning of the reactions to test its ability to inhibit neuronal apoptosis.

**Stable Transfection of Rat ICAD in PC12 Cells**—cDNA containing the D117E and D224E double mutations (rat ICADΔ7) was amplified using the primers 5′-gcc gcc acc acc gat gct ttg tca gca gcc-3′ and 5′-ctc ttg gcc ccc ctc tta atc ccc-3′ and subcloned into the multiple cloning site of the pcDNA3.1 expression vector containing the CMV promoter (Invitrogen). The empty vector or the vector containing rat ICADΔ7 was transfected into PC12 cells with the assistance of LipofectAMINE reagent (Life Technologies, Inc.). Forty-eight h following the transfection, the cells were split at a ratio of 1:3, and on the next day, G418 (Life Technologies, Inc.) was added at a concentration of 450 ng/ml. Cells were kept on the G418 for 1 month to ensure selection of a stable cell line. To confirm the expression of caspase-resistant rat ICAD in transfected PC12 cells, cells were grown to reach confluence, collected, and subjected to protein extraction. Protein (50 μg) was incubated with 10 ng of active caspase-3 for 2 h at 37 °C, and the reaction mixture was subjected to immunoblotting using the p35 antibody.

**RESULTS**

**Cloning of Rat DFF cDNA**—In a search for a rat brain homologue of DFF/ICAD, we performed 5′- and 3′-RACE based on a 321-bp cDNA fragment obtained by reverse transcriptase-PCR using primers based on the conserved sequences in human and mouse DFF45 (see “Experimental Procedures”). The RACE procedures generated two cDNA fragments of approximately 600 and 900 bp. Based on the sequences of these RACE products, a full-length cDNA (1186 bp) was subsequently obtained using reverse transcriptase-PCR. This cDNA fragment contains an open reading frame of 798 bp that encodes a protein of 265 amino acids (Fig. 1a). A stop code was found in the 5′-untranslated region upstream of the first methionine in frame, and a conserved Kozak sequence was also identified around this}
FIG. 1. Cloning of rat DFF35. a, nucleotide sequence and deduced amino acid sequence of rat DFF35 (GenBank™ accession number AF136601). The predicted motifs for caspase cleavage are underlined. b, comparison of amino acid sequences among rat (r), mouse (m) (GenBank™ accession number NM010044), and human (h) (GenBank™ accession number AF087573) DFF35. Identical amino acids are presented as dashes. The caspase cleavage motifs are bold. c, SDS-polyacrylamide gel electrophoresis analysis of extract of in vitro translation assay product from rat DFF35 cDNA. The addition of active caspase-3 results in degradation of the product from the wild type but not the mutant DFF35 cDNA. d, Northern blot analysis of DFF35 mRNA in various rat tissues and various brain regions. The transcription species resulting from the hybridization is ~1.2 kilobases.
Tissue distributions of endogenous DFF35 protein. a, verification of the anti-DFF antibodies using purified recombinant DFF proteins. Lanes 1–3, Coomassie Blue staining of recombinant mouse DFF45 (lane 2), rat DFF35 (lane 3), and size markers (lane 1). Although the p35 antibody detects both DFF45 (lane 4) and DFF35 (lane 5), the p45 antibody recognizes only the DFF45 protein (lane 6). b, Western blot analysis of DFF/ICAD protein in the rat central nervous system and human brain tissues using the p35 antibody (top) and the p45 antibody (bottom), respectively. Purified mouse (m) DFF45 and rat (r) DFF35 proteins serve as controls. c, Western blot analysis of DFF/ICAD protein in various rat (top) and mouse (bottom) tissues using the p35 antibody. Purified mouse DFF45 and rat DFF35 proteins serve as controls. d, immunoprecipitation (IP) of the ICAD-CAD complex in rat brain cell extracts using anti-CAD antibody. The complex contains DFF35 (top, lane 3), which can be cleaved by caspase-3 (top, lane 4), but it does not contain DFF45 (bottom). Normal rabbit IgG (N IgG) and brain cell extracts (Lysate) serve as negative and positive controls, respectively.

The Endogenous DFF/ICAD in Rodent Tissues Is Mainly the Short Form—The cloning data and the Northern blots suggest that DFF/ICAD might be transcribed mainly as the short form in rat tissues. To confirm whether this is true at the protein level, we performed Western blots using two different antibodies that differentiate between DFF45/ICADL and DFF35/ICADs. The p35 antibody was raised against a peptide sequence that is identical between the short and long forms of DFF/ICAD; the p45 antibody was designed to recognize only the long form. For each Western blot performed, purified recombinant rat DFF35/ICADs (35 kDa) and mouse DFF45/ICADs (45 kDa) proteins were used as size markers (Fig. 2a). As shown in Fig. 2, b and c, DFF/ICAD immunoreactivity was readily detectable in all rat or mouse tissues except the liver, which contained little DFF/ICAD. In most tissues, the size of endogenous DFF/ICAD protein was consistent with the DFF35/ICADs form but not the DFF45/ICADs form. However, it appeared that in the skeletal muscle, spleen, and heart of the rat and spleen and heart of the mouse, DFF/ICAD was present in both short and long forms. In the central nervous system of rats, mice, and humans, DFF/ICAD was exclusively detected in the DFF35/ICADs form.

Expression of DFF/ICAD protein in rat neuronal cells was also examined in primary cultures of cortical, hippocampal, and cerebellum granular neurons as well as in neuron-differentiated PC12 cells. In all cells, only the DFF35/ICADs form was detected (data not shown).

To determine which form of DFF/ICAD binds to DFF40/CAD in cells, whole cell protein extracts prepared from either rat or mouse brain were immunoprecipitated with the anti-CAD/ DFF40 antibody and then subjected to Western blot analysis using the p35 and p45 antibodies, respectively. As shown in Fig. 2d, only the DFF35/ICADs form was detected in the immunoprecipitates. These data suggest that, in rodent tissues, particularly in the central nervous system, DFF35/ICADs is the predominant form of ICAD that forms a stable complex with DFF40/CAD.

DFF35/ICADs Is Functional in the Cell-free Apoptosis System—To determine whether DFF35/ICADs possesses the abil-
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**DFF and Neuronal Apoptosis**

The presence of active caspase-3 (Fig. 3b) was nearly completely degraded within 2 h of incubation in the presence of active caspase-3 (Fig. 3c). The exogenous DFF35dm was highly resistant to caspase-3 activity even after prolonged incubation (Fig. 3c). The results of DFF35/ICAD<sub>Δ</sub> degradation thus correlate with the temporal profile of the in vitro inhibitory effect of DFF35<sub>Δ</sub> and DFF35<sub>Δm</sub> against caspase-3-induced DFF40/CAD activity as shown in Fig. 3b. Furthermore, DFF35<sub>Δm</sub> was found to be co-immunoprecipitated with DFF40/CAD in the reaction mixture (Fig. 3d), which is consistent with the notion that exogenous DFF35/ICAD<sub>Δ</sub> inhibits DNase activity via binding to DFF40/CAD.

DFF35/ICAD<sub>Δ</sub> Inhibits DNA Fragmentation and Nuclear Changes in Neuronal Apoptosis—To further determine the functional role of rat DFF35/ICAD<sub>Δ</sub> in vivo, transfection studies were performed in neuron-differentiated PC12 cells. PC12 cells are clonal cells derived from rat adrenal pheochromocytoma that, upon differentiation via nerve growth factor, extend neuronal processes and exhibit a neuronal phenotype. As shown in Fig. 4a, whereas the normally expressed endogenous DFF35/ICAD<sub>Δ</sub> was caspase-3-sensitive, PC12 cells stably transfected with the DFF35<sub>Δm</sub> DNA, but not the empty vector, expressed the caspase-3-resistant DFF35/ICAD<sub>Δ</sub> protein. The DFF35<sub>Δm</sub>-transfected cells contained little DFF35/ICAD<sub>Δ</sub> protein that is sensitive to caspase-3 cleavage, suggesting that the endogenous DFF35/ICAD<sub>Δ</sub> protein has largely been replaced by DFF35<sub>Δm</sub>. However, this transfection affects neither the cellular levels of DFF40/CAD, caspase-3, or caspase-7 (Fig. 4b) nor the differentiation process of PC12 cells (data not shown).

Many lines of agents can induce apoptosis in neuron-differentiated PC12 cells, displaying nuclear changes characteristic of apoptosis. In this study, we tested the effect of two potent apoptosis inducers, staurosporin (STS) and etoposide (VP-16), in non-transfected (wild type) PC12 cells and cells that were transfected with DFF35<sub>Δm</sub> or the empty vector. STS or VP-16 induced apoptosis in wild type cells in a dose-dependent manner as revealed by PI staining (Fig. 4c), and this cell death was clearly mediated by caspase-3 and caspase-7. The caspase-3/7 inhibitor N-benzyloxy carbonyl-Asp-Glu-Val-Asp-fluoro-methylketone offered significant protection against the cell death; the STS- or VP-16-induced cells contained markedly increased proteolytic activity for the caspase-3/7 substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Fig. 4c). Coincident with the activation of caspase-3, endogenous DFF35/ICAD<sub>Δ</sub> was degraded in a time-dependent manner in wild type cells and in cells transfected with the empty vector. In contrast, little or no DFF35/ICAD<sub>Δ</sub> cleavage was detectable in DFF35<sub>Δm</sub>-transfected cells induced by either inducer (Fig. 4d). Correspondingly, transfection of DFF35<sub>Δm</sub> completely blocked internucleosomal DNA fragmentation induced by STS or VP-16 (Fig. 4d). This effect by DFF35<sub>Δm</sub> was not due to the reduction of the levels of caspase-3 activation, because the STS- or VP-16-induced caspase-3 proteolytic process, indicative of caspase-3 activation, was not different between DFF35<sub>Δm</sub>-transfected cells and empty vector-transfected cells or wild type cells (Fig. 4e). These results thus support a role of DFF35/ICAD<sub>Δ</sub> as the potent endogenous antagonist of DFF40/CAD activity in neurons.

To further determine whether blockage of DNA fragmentation by DFF35<sub>Δm</sub> expression rescues cells from death, we treated wild type cells and cells transfected with DFF35<sub>Δm</sub> with STS or VP-16. Expression of DFF35<sub>Δm</sub> significantly delayed, but failed to prevent, cell death by either inducer (Fig. 4f), consistent with previous findings that DNA fragmentation in cells deficient in DFF/ICAD does not determine cellular fate because these cells still die in response to apoptotic stimuli (22).

Transfection of DFF35<sub>Δm</sub> into PC12 cells had a dramatic

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**Fig. 3. In vitro effects of rat DFF35 recombinant protein.** a, Coomassie Blue analysis of rat DFF35 and mouse DFF45 recombinant proteins. The D117E/D224E double mutants but not the wild type DFF protein are resistant to active caspase-3 (lanes 6–7). Lane 1, the size marker. b, inhibitory effect of DFF proteins on caspase-induced DNA fragmentation in isolated brain cell nuclei. Panel A, Incubation duration-dependent DNA fragmentation. Panel B, DFF35 inhibits DNA fragmentation in a concentration-dependent manner (lanes 1–4, 0, 0.1, 0.3, and 1 µg/ml, respectively). Panel C, DFF35 is equally effective as DFF45 in inhibiting DNA fragmentation. The mutant DFF proteins (dm) continue to be effective even after prolonged incubation (lanes 5–8). c, Western blot analysis of protein processing for rat DFF35 in the cell-free apoptosis system. Both wild type (wt) and D117E/D224E double mutant (dm) rat DFF35 ICAD<sub>Δ</sub> and mouse DFF45 ICAD<sub>Δ</sub> were produced and subsequently purified. The wild type DFF/ICAD but not the cleavage site mutant could be cleaved by active caspase-3, generating peptides at anticipated sizes (Fig. 3a). This result confirmed the validity of the caspase cleavage sites in the DFF35/ICAD<sub>Δ</sub> and DFF45/ICAD<sub>Δ</sub> proteins.

Incubation of isolated brain cell nuclei in caspase-3-activated S-100 protein fraction induced internucleosomal DNA fragmentation in a time-dependent manner (Fig. 3b, panel A). The addition of DFF35<sub>Δm</sub> to the reaction mixture prior to the reaction resulted in a dose-dependent inhibition of DFF40/CAD activity (Fig. 3b, panel B). The DFF35<sub>Δm</sub> and DFF45<sub>Δm</sub> proteins were equally effective in inhibiting CAD activity at the concentration tested (1 µg/ml) when the reaction time was 4 h (Fig. 3b, panel C). When the reaction duration was extended to 8 h, however, both DFF35<sub>Δm</sub> and DFF45<sub>Δm</sub> completely lost their inhibitory effect (Fig. 3b, panel C). In contrast, DFF35<sub>Δm</sub> and DFF45<sub>Δm</sub> were able to retain their inhibitory ability even after a prolonged incubation (8–16 h).

Immunoblot revealed that the endogenous DFF35/ICAD<sub>Δ</sub> was nearly completely degraded within 2 h of incubation in the presence of active caspase-3 (Fig. 3c). The exogenous DFF35<sub>Δm</sub> at the concentration of 1 µg/ml tolerated caspase-3 for up to 4 h, whereas DFF35<sub>Δm</sub> was highly resistant to caspase-3 activity even after prolonged incubation (Fig. 3c). The results of DFF35/ICAD<sub>Δ</sub> degradation thus correlate with the temporal profile of the in vitro inhibitory effect of DFF35<sub>Δm</sub> and DFF35<sub>Δm</sub> against caspase-3-induced DFF40/CAD activity as shown in Fig. 3b. Furthermore, DFF35<sub>Δm</sub> was found to be co-immunoprecipitated with DFF40/CAD in the reaction mixture (Fig. 3d), which is consistent with the notion that exogenous DFF35/ICAD<sub>Δ</sub> inhibits DNase activity via binding to DFF40/CAD.
effect on the morphological changes of the nucleus during apoptosis. Incubation of the wild type cells or the cells transfected with the empty vector, results in the production of caspase-resistant DFF35 protein.  

**Fig. 4. In vivo effects of caspase-resistant DFF35 in neuronal apoptosis.**  

*a*, Western blot analysis of DFF35 in neuron-differentiated PC12 cells without transfection or transfected with DFF35dm or the empty vector. Note that transfection of DFF35dm cDNA, but not the empty vector, results in the production of caspase-resistant DFF35 protein.  

*b*, transfection of DFF35dm in PC12 cells does not alter the cellular levels of CAD/DFF40, caspase-3, or caspase-7.  

*c*, caspase-dependent neuronal apoptosis induced by STS or VP-16 in PC12 cells. Data are expressed as the mean ± S.E. from three independent experiments. *, p < 0.05; **, p < 0.01 versus cells without z-DEVD-fmk treatment (analysis of variance and post hoc Scheffe’s tests).  

*d*, transfection of DFF35dm in PC12 cells inhibits STS- or VP-16-induced cleavage of DFF35 (left) and internucleosomal DNA fragmentation (right).  

*e*, transfection of DFF35dm does not alter the levels of caspase-3 activation induced by STS or VP-16.  

*f*, transfection of DFF35dm in PC12 cells inhibits STS- or VP-16-induced cleavage of DFF35 and internucleosomal DNA fragmentation.
immunoreactivity was also seen in the nuclear fraction of untreated neuron-differentiated PC12 cells (Fig. 6a), cultured primary cortical neurons, cerebellum granular cells, HeLa cells, and other rat tissues such as kidney, spleen, and heart (data not shown). Interestingly, Samejima and Earnshaw (28) recently showed that a GFP-CAD fusion protein is located in the nucleus rather than in the cytosol of transfected cells. Taken together, these observations raise the possibility that the process for DFF40/CAD activation during apoptosis may take place in the nucleus instead of in the cytosol.

To address this issue, we induced apoptosis in differentiated PC12 cells using STS and subsequently examined the temporal profiles of DFF40/CAD and DFF35/ICADs alterations in the cytosolic and nuclear fractions. STS at the concentration of 1.2 μM induced marked activation of caspase-3 within the first hour of incubation, and more than 85% of cells underwent apoptosis within 6 h. Degradation of cytosolic DFF35/ICADs began to be detectable in the first hour of incubation and reached completion between 2 and 4 h (Fig. 6b). Nuclear DFF35/ICADs, on the other hand, was intact within the first hour but was completely cleaved within 4–6 h following STS treatment. The presence of DFF35/ICADs cleavage products in the nucleus was probably not the consequence of translocation of cytosolic DFF35/ICADs into the nucleus, because DFF35/ICADs lacks a nuclear transmembrane domain. Furthermore, the levels of DFF40/CAD in the nuclear fraction remained unchanged during the course of apoptosis, suggesting that nuclear translocation of DFF40/CAD probably did not occur (Fig. 6c). These results strongly supported our hypothesis that DFF40/CAD can be activated within the nucleus during apoptosis.

Degradation of DFF35/ICADs in both cytosolic and nuclear fractions during STS-induced neuronal apoptosis was dependent on caspase-3 activity. However, caspase-3 normally is not present in the nucleus. Hence, it is plausible that degradation of nuclear DFF35/ICADs and DFF40/CAD activation may require nuclear translocation of active caspase-3. To address this issue, caspase-3 immunoreactivity was examined in the nuclear and cytosolic fractions before and after STS treatment in PC12 cells. Neither the proenzyme nor the active form of caspase-3 was detectable in the nucleus of untreated PC12 cells. Beginning 2 h after STS treatment and thereafter, caspase-3 (mainly the 17-kDa active peptide) was readily detected in the nuclear fraction (Fig. 6d).

The Role of the Cytosol in the Activation of DFF40/CAD—

The active recombinant caspase-3 (p17) alone was not sufficient to induce DNA fragmentation in isolated nuclei (Fig. 7a), suggesting that certain cytosolic components may be indirectly involved in DFF40/CAD activation. To address the potential role of the cytosol in the activation of nuclear DFF40/CAD during apoptosis, we performed the cell-free DNA fragmentation assay using the S-100 protein fraction prepared from un-
In the cytosolic (C) and nuclear (N) fractions prepared from normal brain cells, primary cortical neuron cultures, and neuron-differentiated PC12 cells (left panel). Poly(ADP-ribose) polymerase (PARP) and α-tubulin Western blots serve to confirm the validity of the subcellular fractionation procedure. As determined using immunoprecipitation, the DFF35/CAD complex is mainly localized in the nucleus in PC12 and brain cells (right panel). IgG, normal rabbit IgG; IP, immunoprecipitation. b, time course of DFF35 cleavage in the cytosolic or nuclear fraction of neuron-differentiated PC12 cells induced by STS. c, detection of CAD/DFF40 in the PC12 cell nuclear fraction. The level is unchanged before and during STS-induced apoptosis. The proliferating cell nuclear antigen (PCNA) Western blot serves as a nuclear protein sample-loading control. d, activation and nuclear translocation of caspase-3 in PC12 cells induced by STS. The 17-kDa band represents the active form of caspase-3. z-VAD (M), N-benzyloxycarbonyl-Val-Ala-Asp-fluoro-methylketone.

One major finding of this study is that the short form of ICAD, DFF35/ICAD_b, is the predominant DFF40/CAD inhibitor expressed in various rodent tissues. Significantly, DFF35/ICAD_b is the exclusive form of ICAD found in the rat and mouse central nervous system and several other systems (Fig. 2). Furthermore, DFF35/ICAD_b, but not DFF45/ICAD_L, was co-immunoprecipitated with DFF40/CAD in rat brain cell extracts, indicating that DFF35/ICAD_b is the endogenous inhibitory subunit of the DFF complex. Our results contradict the recent report (30) in which DFF40/CAD was found to be predominantly associated with DFF45/ICAD_L in mouse WR19L and human Jurkat T lymphoma cells. This discrepancy can be explained, at least in part, by the alternative expression of the two forms of ICAD in different systems. Whereas DFF35/ICAD_b and DFF45/ICAD_L are present at equivalent levels in these cell lines (30), little or no DFF45/ICAD_L is detectable in the rodent central nervous system.

It has been shown in the reticulocyte transcription/translation system and bacteria protein expression system that forming a complex with DFF45/ICAD_L, presumably to allow appropriate protein folding, is a prerequisite for DFF40/CAD to acquire its DNase activity (19, 23, 24, 30, 31). In contrast to DFF45/ICAD_L, DFF35/ICAD_b seems to be unable to facilitate such conformational changes of DFF40/CAD (30). Hence, it was thought that DFF40/CAD that binds to DFF35/ICAD_b or its counterpart would not respond to caspase-3 and exhibit DNase activity. However, our results are inconsistent with this hypothesis. Brain cell extracts that contain almost exclusively the DFF35/ICAD_b counterpart as the heterodimeric partner of DFF40/CAD displayed a potent DNase activity in the presence of active caspase-3 (Fig. 3). In addition, the purified rat DFF35/ICAD_b, and/or DFF35/ICAD_b, consistent with this regulation, we have identified a counterpart of DFF35/ICAD_b in rat brain that binds to DFF40/CAD and suppresses apoptotic DNA fragmentation.

DISCUSSION

Subsequent to the identification of the DFF complex as the major regulator of apoptotic DNA degradation in HeLa cells (17), homologues of DFF with comparable functions had been discovered in humans, mice, and Drosophila melanogaster (18, 20, 21, 29). Hence, DFF-mediated nuclear destruction constitutes an evolutionarily conserved mechanism that plays a pivotal role in the execution of apoptotic cell death. An important functional property of DFF is that the DNase activity displayed by DFF is negatively regulated by its inhibitory subunits, DFF45/ICAD_L and/or DFF35/ICAD_b. Consistent with this regulation, we have identified a counterpart of DFF35/ICAD_b in rat brain that binds to DFF40/CAD and suppresses apoptotic DNA fragmentation.
ICAD<sub>3</sub> recombinant protein was able to bind to DFF40/CAD and inhibit caspase-3-mediated internucleosomal DNA fragmentation. The in vitro inhibitory effect of rat DFF35/ICAD<sub>3</sub> was at least as potent as that of DFF45/ICAD<sub>L</sub> (Fig. 3). Moreover, during apoptosis in neuronal PC12 cells where DFF40/CAD complexes predominantly with the short form of ICAD, cleavage of the DFF35/ICAD<sub>3</sub> counterpart was associated with an induced DFF40/CAD activity that resulted in internucleosomal DNA fragmentation and morphological nuclear changes (Fig. 4). Correspondingly, neuron-differentiated PC12 cells that were stably transfected with the caspase-resistant rat DFF35/ICAD<sub>3</sub> mutants exhibited remarkable resistance to apoptotic DNA degradation induced by STS or VP-16 and delayed the cell death significantly (Fig. 4). Hence, the DFF35/ICAD<sub>3</sub> counterpart and the associated DFF40/CAD in these systems are indeed functional. Taken together, these results dispute the contention that DFF45/ICAD<sub>L</sub> is the exclusive molecule that can bind to functional DFF40/CAD, especially in tissues lacking DFF45/ICAD<sub>L</sub>. Although the existence of other chaperone(s) for DFF40/CAD in tissues, such as the brain, cannot be excluded, DFF35/ICAD<sub>3</sub> may serve as such a chaperone under certain conditions. Alternatively, DFF45/ICAD<sub>L</sub> or other possible molecules in selected tissues may still be the primary chaperones that assist DFF40/CAD for proper folding but are unable to stay in stable association with DFF40/CAD due to rapid degradation (hence the lower expression in these cells). DFF35/ICAD<sub>3</sub> may then substitute for the degraded primary chaperone(s) to bind to the now functional DFF40/CAD and maintain its functional configuration. Consistent with this notion, we found in the in vitro assay that although endogenous DFF40/CAD can be inactivated by exogenous rat DFF35/ICAD<sub>L</sub>, it resumed its DNase activity when the latter was cleaved by caspase-3 (Fig. 3).

Although it is known that the ICAD-CAD complex is activated by caspase-3, the precise mechanism by which this complex is processed in apoptotic cells has not yet been fully elucidated. It has been proposed that caspase-3 cleaves DFF45/ICAD<sub>L</sub> and thus releases the bound DFF40/CAD, which is then translocated to the nucleus to execute its activity (17, 20). However, our current data strongly suggest that DFF40/CAD activation in PC12 cells is a process that takes place in the nucleus and involves the cleavage of nuclear DFF35/ICAD<sub>3</sub>. First, we found that in normal non-apoptotic cells DFF40/CAD was mainly localized in the nucleus in complex with DFF35 and that there was no evidence of further translocation of DFF40/CAD to the nucleus during the course of apoptosis (Fig. 6). Second, the amount of nuclear DFF40/CAD is apparently sufficient to degrade nucleosomal DNA in the presence of active caspase-3, and the depletion of cytosolic DFF40/CAD did not seem to affect this process (Fig. 7). Third, DFF35/ICAD<sub>3</sub> was completely cleaved in the nuclei of apoptotic cells, presumably by activated caspase-3 or -7 translocated into the nuclei (Fig. 6). Finally, the activated form of caspase-3 was indeed found in the apoptotic PC12 cells following STS treatment (Fig. 6), and the time course of caspase translocation coincided with that of the cleavage of nuclear DFF35/ICAD<sub>3</sub>.

These results suggest that nuclear translocation of caspase-3 or caspase-7 may constitute an important step in the activation of DFF40/CAD during neuronal apoptosis. This result parallels other nuclear proteins, such as poly(ADP-ribose) polymerase, which are also cleaved by caspase-3/-7 early in the process of apoptosis before nuclear degradation. It is unknown, however, whether such nuclear translocation of caspase-3 is a passive and diffusive process as a result of nuclear membrane damage or an active protein-transporting process via the action of a caspase carrier. Nevertheless, nuclear translocation of caspase-3 appears to be dependent on the action of cytosolic components. As determined in the cell-free assay, purified recombinant active caspase-3 (p17) alone was unable to enter the isolated intact nucleus. However, in the presence of cytosolic protein extracts, caspase-3, mainly the p17 active peptide, moved into the nucleus and degraded DFF35/ICAD<sub>3</sub> and other nuclear proteins (Fig. 7). These results suggest that certain cytosolic factor(s) may enable caspase-3 to translocate into the nucleus. Such a cytosolic component could be another caspase that degrades nuclear membrane-bound proteins and, consequently, increases the membrane permeability for macromolecules. Alternatively, it could be a specific caspase-3/-7 carrier bearing nuclear translocation signals. Taken together, our data favor a model in which DFF40/CAD is activated in neuronal apoptosis via a pathway involving nuclear translocation of caspase-3.

Multiple factors may be responsible for the apoptotic nuclear events (15, 16, 24, 32). Whereas DFF40/CAD is important for the small DNA fragmentation at the nucleosomal junction, it also seems to be involved in the nuclear fragmentation (Fig. 5). Whereas nuclear condensation seems to be a process independent of DFF40/CAD, our results suggest that it is still a caspase-
dependent event (Fig. 5). These results thus are consistent with the recent report by Sahara et al. (33) that a distinct caspase-activated factor may be responsible for nuclear condensation in apoptosis. It should be pointed out that DNA fragmentation is a part of the execution of apoptosis but not a factor in determining whether cells will die after a death stimulus. Thus PC12 cells expressing the caspase-resistant DFF35/ICADS showed resistance to DNA fragmentation, but the development of cell death was only delayed, not prevented (Fig. 4). This observation is consistent with the evidence from cells deficient in DFF45 (22).

In summary, a counterpart of DFF35/ICADS has been cloned from the rat brain. This protein is the predominant form of ICAD expressed in the rodent brain and inhibits apoptotic DNase activity of DFF40/CAD by heterodimerization. During neuronal apoptosis, active caspase-3 and/or caspase-7 translocates to the nucleus, cleaves nuclear DFF35/ICADS, and consequently activates DFF40/CAD. Expression of caspase-resistant DFF35/ICADS cDNA constructs can inhibit inter-nucleosomal DNA degradation and nuclear fragmentation and significantly delay the process of cell death in caspase-mediated neuronal apoptosis. These results thus establish that DFF35/ICADS is the endogenous inhibitor of DFF40/CAD in neurons.

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