Communication

Functional Interaction of DFF35 and DFF45 with Caspase-activated DNA Fragmentation Nuclease DFF40*

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DNA fragmentation factor (DFF) functions downstream of caspase-3 and directly triggers DNA fragmentation during apoptosis. Here we described the identification and characterization of DFF35, an isoform of DFF45 comprised of 268 amino acids. Functional assays have shown that only DFF45, not DFF35, can assist in the synthesis of highly active DFF40. Using the deletion mutants, we mapped the function domains of DFF35/45 and demonstrated that the intact structure/conformation of DFF45 is essential for it to function as a chaperone and assist in the synthesis of active DFF40. Whereas the amino acid residues 101–180 of DFF35/45 mediate its binding to DFF40, the amino acid residues 23–100, which is homologous between DFF3/5 and DFF4, may function to inhibit the activity of DFF40. In contrast to DFF45, DFF35 cannot work as a chaperone, but it can bind to DFF40 more strongly than DFF45 and can inhibit its nuclease activity. These findings suggest that DFF35 may function in vivo as an important alternative mechanism to inhibit the activity of DFF40 and further, that the inhibitory effects of both DFF35 and DFF45 on DFF40 can put the death machinery under strict control.

Apoptosis is fundamentally important in a variety of physiological and pathological processes. Apoptotic cells undergo an orchestrated cascade of events characterized by distinct morphological changes including membrane blebbing, cytoplasmic and nuclear condensation, chromatin aggregation, and formation of apoptotic bodies (1, 2). Activation of the caspase cascade is a key molecular event in the process of apoptosis (3, 4). Apoptotic signals, including growth factor and interleukin deprivation, activation of Fas, ionizing radiation, and a series of chemicals acting as upstream signals, can convert the precursors of caspases into the active enzymes (2). Several important downstream substrates of caspase, such as gelsolin (5), p21-activated kinase-2 (PAK-2) (6), and DNA fragmentation factor 45 (DFF45) (7), whose cleavages clearly induce specific well characterized steps in apoptosis, have been recently identified. The cleavage of chromatin into the nucleosomal fragments, which distinguishes apoptosis from oncosis and necrosis, is a key element in the cell death process and is believed to be mediated by Mg$^{2+}$/Ca$^{2+}$-required and Zn$^{2+}$-sensitive nuclease (8–12).

We have previously identified a triplet of nuclease proteins named NP42–50 that causes DNA degradation in vitro when cells undergo apoptosis (13). The similarity in molecular weight and biochemical characteristics between NP42–50 and the recently identified DFF40 led us to further investigate these molecules. DFF is a heterodimeric protein composed of DFF45 and DFF40 subunits. DFF45 has been found to be the substrate of caspase-3, and DFF40 has also been cloned and found to be a DNA fragmentation nuclease (7, 14, 15). Cleavage of the DFF45 by caspase-3 during apoptosis releases DFF40 that degrades chromosomal DNA into nucleosomal fragments. Similar findings have also been described recently in the mouse. The mouse DFF is composed of three molecules: one caspase-activated DNase (CAD) and two forms of CAD inhibitors (ICAD-L and ICAD-S) (16, 17). Mouse CAD and ICAD-L are apparently the counterpart of human DFF40 (CPAN) and DFF45, respectively, whereas the human counterpart of mouse ICAD-S has not been identified.

In the present study we described the cloning of human DFF35, the short isoform of DFF45 and mapped the function domains of DFF35/45. Functional analysis has shown that the intact structure/conformation of DFF45 is essential for it to function as a chaperone and assist in the synthesis of active DFF40. In contrast, DFF35, although it can bind to DFF40 and inhibit its nuclease activity, cannot assist in the synthesis of active DFF40. While DFF35 binds to DFF40 more strongly than does of DFF45, suggesting that DFF35 may function in vivo as an additional inhibitor of DFF40, augmenting the regulatory control of DFF45. Taken together, our studies suggest that the inhibitory effects of both DFF35 and DFF45 on DFF40 place this important component of the cell death machinery under strict control.

EXPERIMENTAL PROCEDURES
cDNA Cloning, Expression, Mutation of DFF35/DFF45—Outer encoding region primers 5'-GCCGAGTCATTCCCCTGGT GCCGTC-3' (for both DFF35 and DFF45), 5'-GCCGAGTCATTCCCCTGGT GCCGTC-3' (for DFF35), 5'-GCCGAGTCATTCCCCTGGT GCCGTC-3' (for DFF45) were designed for amplifying both DFF35 and DFF45 from Jurkat cell total RNA and human fetal liver cDNA library by PCR. The PCR-amplified fragments were cloned into pCR2.1 (Invitrogen) for sequencing (ABI 200 sequencer). DFF35 and DFF45 were further amplified by 5'-GCCGAGTCATTCCCCTGGT GCCGTC-3' (for both DFF35 and DFF45), 5'-GCCGAGTCATTCCCCTGGT GCCGTC-3' (for DFF35) and 5'-GCCGAGTCATTCCCCTGGT GCCGTC-3' (for DFF45) and cloned into pGEX-KG (Amersham Pharmacia Biotech) for GST fusion protein.

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DFF35 were generated by two-step PCR using 20-nucleotide primers carrying the mutated nucleotides. Deletion mutants of DFF45 were also generated by two-step PCR. All the mutations were confirmed by DNA sequencing. GST fusion proteins were expressed in Escherichia coli and adsorbed by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and then eluted by either reduced glutathione (GSH) containing buffer (50 mM Tris, 20 mM GSH, pH 9.6) or thrombin (Sigma) according to the instructions of the manufacturer. The Flag-tagged DFF45 and DFF35 were generated by cloning into pCDNA3.0-Flag vector (Eastman Kodak Co.).

In Vitro Cell-free DNA Fragmentation Assay—Jurkat cells were incubated with anti-Flag monoclonal Ab 7C11 (1 ng/μl) at 37 °C for 2 h and then eluted with either reduced glutathione (GSH) containing buffer (50 mM Tris, 20 mM GSH, pH 9.6) or thrombin (Sigma) according to the instructions of the manufacturer. The Flag-tagged DFF45 and DFF35 were generated by cloning into pCDNA3.0-Flag vector (Eastman Kodak Co.).

Western Blot Analysis—A polyclonal Ab against the N-terminal peptide of both DFF35 and DFF45 (EVTDAGVPESEGTLPK) was from Upstate Biotechnology. A polyclonal Ab against intermediate peptide of DFF45 and DFF45 (SQEEGEEVDAYD) and a polyclonal Ab against C-terminal peptide of DFF45 only (KASPDPDLQQNPKRAQDP) were from Santa Cruz Biotechnology. A monoclonal Ab against epitope Flag (M5) was purchased from Sigma. 293T cells were transfected with Flag-tagged plasmids by calcium phosphate method. Jurkat cells were incubated with staurosporine (Sigma) at the final concentration of 1 μM for the indicated time. Immunoblot was performed with the horseradish peroxidase-conjugated goat anti-rabbit (N-terminal) or donkey anti-goat (internal and C-terminal) Ab using ECL system (Amersham Pharmacia Biotech).

cDNA Cloning and Sequencing of DFF40 in Vitro—The primers 5’-GCCAGGACGCTGCAGGCTGGGCGAC-3’ and 5’-AAATGATGCCCAAGTCAGGCCTCAAACA-3’ were used to amplify DFF45 and DFF45 (SQEEGEEVDAYD) and a polyclonal Ab against C-terminal peptide of DFF45 only (KASPDPDLQQNPKRAQDP) were from Santa Cruz Biotechnology. A monoclonal Ab against epitope Flag (M5) was purchased from Sigma. 293T cells were transfected with Flag-tagged plasmids by calcium phosphate method. Jurkat cells were incubated with staurosporine (Sigma) at the final concentration of 1 μM for the indicated time. Immunoblot was performed with the horseradish peroxidase-conjugated goat anti-rabbit (N-terminal) or donkey anti-goat (internal and C-terminal) Ab using ECL system (Amersham Pharmacia Biotech).

ASSAY FOR DFF40 NUCLEASE ACTIVITY AND BINDING ABILITY—To test DFF40 nuclease activity, a 5-μl aliquot of synthesized DFF40, after being treated with caspase-3 and inactivated with CHO-DEVD, was incubated with Jurkat nuclei (5 μl, 5.5 × 10^5 total) at 37 °C for 2 h and then eluted with either reduced glutathione (GSH) containing buffer (50 mM Tris, 20 mM GSH, pH 9.6) or thrombin (Sigma) according to the instructions of the manufacturer. The Flag-tagged DFF45 and DFF35 were generated by cloning into pCDNA3.0-Flag vector (Eastman Kodak Co.).

RESULTS AND DISCUSSION

In an effort to search for the short form of DFF45 and to better understand the molecular details of DNA fragmentation, we found a novel protein of 268 amino acid residues. A homology search in GenBank™ data bank indicated that this protein was identical to DFF45 (consisting of 331 amino acids) up to amino acid position 261, after which the sequences diverged (Fig. 1A), suggesting that the mRNAs encoding these two proteins were generated through alternative splicing. The two potential caspase-3 cleavage sites within the protein are exactly the same as those of DFF45. More importantly, the C-terminal region of this protein is highly homologous with the mouse short isoform of ICAD, ICAD-S (16), and has three additional amino acids, QGH, at the C-terminal when compared with mouse ICAD-S (Fig. 1B). These results clearly suggest that this protein is the human counterpart of mouse ICAD-S. We therefore designated this protein as DFF35 because it appears to be the short form of DFF45 with a molecular weight of 35 kDa.

To further confirm whether the RNA transcript and the
encoded protein exist in vivo, Jurkat cell total RNA and fetal liver cDNA were amplified by PCR using DFF45 or DFF35 outer encoding region primer sets (5′ primer is the same for both DFF35 and DFF45, but 3′ primers are different) and cloned (Fig. 2A). Sequences of the PCR products showed an exact match with the sequences of DFF45 and EST clone, respectively (data not shown). Moreover, polyclonal Ab specific for an N-terminal or intermediate peptide of DFF35/45 recognized two bands corresponding to DFF45 and DFF35 by Western blot analysis of the Jurkat cell lysate, whereas a polyclonal Ab specific for a C-terminal peptide of DFF45 recognized only one band corresponding to DFF45, but not DFF35 in the same blot (Fig. 2B). To further exclude the possibility that DFF35 is because of the degradation of DFF45, flag-tagged DFF45, DFF45, or an irrelevant control plasmid p53 was transiently transfected into 293T cells. Although the blot with anti-DFF35/45 indicated the existence of two endogenous bands, the translation of only one of these bands was observed on the blot with either anti-Flag or anti-DFF35/45 corresponding to the indicated N-terminal between the nuclease and its inhibitors (the amino acids 23–100 of DFF35/45 and the amino acids 10–87 of DFF45). These results clearly demonstrated that the novel 35-kDa isoform does exist in vivo at both mRNA and protein levels.

A modified in vitro apoptosis system that mimics the later stages of the apoptotic cascade under cell-free conditions was used to analyze the function of DFF35. Addition of cytosolic extracts prepared from apoptotic cells to Jurkat nuclei induced the formation of internucleosomal DNA fragments. GST-DFF35 fusion protein could completely block the DNA cleavage induced by apoptotic cytosolic extract but not GST-DFF35 cleaved by caspase-3. However, GST-DFF35 double mutant (at caspase-3 cleavage sites D117E and D224E) could block DNA degradation either in the absence or presence of caspase-3 (Fig. 3A). Cleavage of both DFF45 and DFF35 and subsequently generated DNA fragmentation in vivo were also observed in Jurkat cells killed by staurosporine (Fig. 3, B and C). These results indicate that the caspase-3 cleavage sites are required for dismantling the inhibitory ability of DFF35, as well as DFF45, and releasing the nuclease responsible for DNA cleavage. Such a nuclease seems to be DFF40.

DFF40 was then cloned by reverse transcriptase-PCR from Jurkat cells and ligated into pCDNA3.1 downstream of the T7 promoter and subjected to coupled in vitro transcription and translation in the absence or presence of recombinant DFF35 or DFF45. DFF40 was synthesized in vitro only in the presence of pCDNA3.1-DFF40 and bound to DFF45 or DFF35 in the subsequent GST pull-down experiment (Fig. 4A). Functional assay showed that the DFF40 synthesized in the presence of DFF45, but not DFF35 or vector only, could induce DNA fragmentation in the cell-free system assay, suggesting that DFF45 may act as a chaperone to assist DFF40 folding into a functional conformation with high DNase activity (Fig. 4B). However, both DFF35 and DFF45 could inhibit fully functional synthesized DFF40 in vitro and such an inhibition can be dismantled by caspase-3 cleavage (Fig. 4C). These results show that the excess carboxyl terminus of DFF45 is indispensable to achieve the correct folding of DFF40. To our surprise, an equal amount of GST-DFF35 can pull down much more DFF40 than DFF45 (Fig. 4A), indicating DFF35 has a much stronger ability to bind to DFF40 than DFF45 does.

By comparing the protein sequences of DFF35/45 with DFF40, we could delineate a homologous region at the N-terminal between the nuclease and its inhibitors (the amino acids 23–100 of DFF35/45 and the amino acids 10–87 of DFF40). Further comparison found that this region was also
Functional Domains of DFF35/45

Conserved in the mouse CAD and ICAD-S/L (44%) (Fig. 5B). We hypothesize that this region may be involved in mediating the binding or inhibitory effect of DFF35/45 on DFF40 or ICAD-S/L on CAD. We therefore constructed four deletion mutants of DFF45, which deleted the region of DFF45 ranging from 23–100 (DFF45D1), 101–261 (DFF45D2), 101–180 (DFF45D3), and 181–260 amino acids (DFF45D4), respectively. As shown in Fig. 5C, DFF40 was synthesized in the presence of all four deletion mutants at levels comparable with that of wild type DFF45, but only wild type DFF45, DFF45D1, and DFF45D4, but not DFF45D2 and DFF45D3, bound to DFF40 (Fig. 5C). It suggests that DFF40 binding domain is within 101–180 amino acids of DFF35/45. Functional analysis shows that only DFF40 synthesized in the presence of DFF45 but not in the presence of any mutant has nuclease activity (Fig. 5D). Although both DFF45D1 and DFF45D4, as well as DFF45, can bind with DFF40, only DFF45 and DFF45D4, but not DFF45D1, can inhibit the nuclease activity of DFF40 (Fig. 5E). It suggests that the homologous region (the amino acid 23–100 of DFF35/45) may function as the inhibitory domain of DFF35/45 to inhibit the nuclease activity of DFF40. It is reasonable to hypothesize that the physical interaction of DFF35 or DFF45 with DFF40 is a prerequisite for their inhibition and/or folding function. Thus, DFF45D2 and DFF45D3 cannot inhibit the activity of DFF40 because of their failure to bind to DFF40 (Fig. 5E). DFF35 and the deletion mutants of DFF45 cannot assist in the synthesis of active DFF40, suggesting that an intact structure/conformation of DFF45 is essential for its function as a chaperone involved in the folding DFF40 into an active enzyme.

Several enzymes including cyclophins, DNase I, and DNase II have been shown to be partially involved in DNA degradation during apoptosis (18–20). Both DFF45 and DFF35 can completely block nuclease activity of DFF40 but have no inhibitory effect on Dnase I or II (data not shown), suggesting that the inhibitory effect of both DFF45 and DFF35 is DFF40-specific. Further, our findings suggest that DFF45 can both bind DFF40 and assist in the folding of nascent DFF40 into a functional nuclease with high activity and inhibit its activity as well. DFF40 is a highly active nuclease that is harmful or even lethal to normal cells. Thus, it is not surprising that DFF35 or DFF45 with its strong binding ability can also serve as an important safety net to ensure a complete control of DFF40 activity in cells.

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