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Study of DFF45 in Its Role of Chaperone and Inhibitor: Two Independent Inhibitory Domains of DFF40 Nuclease Activity

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CAD/DFF40, the nuclease responsible for DNA fragmentation during apoptosis, exists as a heterodimeric complex with DFF45/ICAD. This study determines the molecular mechanisms of regulation of DFF40 via the chaperone and inhibition activities of DFF45. We analyze proteins corresponding to the fragments (D1, D2, and D3) of DFF45 generated by cleavage at the caspase consensus sites in DFF45. Either D1 or D2, as an isolated domain, is capable of inhibiting DFF40 nuclease activity while double domain fragments D1-2 and D2-3, as well as full-length DFF45, bind to DFF40 with high affinity and are much more effective inhibitors. The chaperone activity of DFF45 resides in part in its ability to maintain DFF40 as a soluble protein. In addition, D1 of DFF45 was found to be critical for the expression of active DFF40 *in vivo*, suggesting a role for DFF45 in binding nascent DFF40.

Key Words: **apoptosis; CAD and ICAD; DNA fragmentation; BIAcore; DFF40 and DFF45.**

Cleavage of chromosome DNA into nucleosomal multimer fragments is one hallmark of apoptosis (1), which has recently been demonstrated to be mediated by the DNA fragmentation factor, DFF (2, 3). Identified by biochemical analysis, DFF is a heterodimeric protein complex consisting of DFF45 (or ICAD) (2–4) and DFF40 (or CAD or CPAN) (2, 5–7). Full length DFF45 contains two conserved caspase cleavage sites, DETD and DAVD at amino acid positions 117 and 224, respectively (3, 4). During apoptosis, DFF45 can be cleaved by activated caspase 3 at these sites to yield three fragments of 10–15 Kd (3, 4) resulting in the activation of DFF40 as a nuclease (2, 5, 7). DFF45 therefore acts as an inhibitor for DFF40 enzymatic activity under physiological conditions. It was observed that DFF45 was required not only to inhibit DFF40 activity but was also necessary for expression of DFF40 (2, 7, 8). Expression of DFF40 alone in mammalian cells such as CHO and Jurkats cells (2) or in insect cells (5) produced low levels of DFF40 with corresponding low levels of nuclease activity. Furthermore, when DFF40 alone was expressed in bacteria, it aggregated into inclusion bodies and became insoluble (7). In contrast, co-expression of DFF45 with DFF40 in various cell lines significantly increased the amount of soluble DFF40 with corresponding increases in nuclease activity (2). Moreover, co-expression of DFF45 and DFF40 from a single plasmid (7, 9) generated active DFF40 in bacteria. Based on these limited observations, it has been postulated that DFF45 works as a specific chaperone necessary for generating functional DFF40 (2, 7). However mechanisms underlying the role of DFF45 as both a chaperone and an inhibitor of DFF40 nuclease is unclear.

The study presented here addresses the nature of DFF45's chaperone activity for DFF40 and repression of DFF40 nuclease activity. We generated protein fragments of both DFF40 and DFF45 and characterized their interaction *in vivo* and *in vitro*. We show that DFF40/45 interaction is mediated by binding of three functional domains of DFF45 to two domains of DFF40. Synergistic interaction of individual domains of DFF45 to DFF40 results in strong inhibition of DFF40's nuclease activity.

MATERIALS AND METHODS

Plasmid construction. Truncated forms of DFF45 were generated by PCR amplification with the corresponding oligonucleotide primers that introduced unique restriction sites and were inserted into Nde I and Sma I sites of pBKS-Flag and subcloned into pCMV-5 and pET-15b (Novagen) bacterial expression vector. Full length DFF45 or individual domains were further subcloned from pET-15b after Nde I and Hind III digestion into pET-DFF40his (16) to express both DFF40 and DFF45 or its truncated forms from a single plasmid. For the double plasmid system, DFF40 was co-expressed with DFF45

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encoded by pB13-DFF45 constructed by subcloning DFF45 into pB13 (10) that contains a pACYC replication origin and Kanamycin resistance allowing stable maintenance in bacteria with the pet15b vectors.

Bacterial protein production and purification. Procedures for the purification of bacterially expressed proteins were essentially as described by Toh *et al.* (16). DFF40/45, DFF45, DFF45 fragments and caspase 3 were purified as an initial step with Ni^{++} agarose (Qiagen). DFF45 was then further purified by mono Q and fractions that contain near homogeneous DFF45 were dialyzed against Buffer D (20 mM Hepes/KOH pH 7.5, 50 mM KCl, 1 mM β-mercaptoethanol, and 2 mM EDTA) for use in enzymatic assays and BIAcore experiments. DFF45 fragment proteins were further purified by S200 chromatography in Buffer D. Active DFF40 was generated by co-expression of pB13-DFF45 and pET15b-DFF40 in BL21(DE3). After initial purification of His-tagged DFF40 and DFF45 with a Ni^{++} agarose (Qiagen) followed by a monoQ column, fractions containing DFF40 were mixed with caspase-3 Ni^{++} agarose eluate and allowed to incubate for several hours at 37°C. DFF40 was then purified to near homogeneity by additional chromatography on mono S, phenyl sepharose and S200 gel filtration columns (Pharmacia) and stored in 50% glycerol.

DFF40 enzymatic assay and inhibition assay. Plasmid DNase assay was performed in 25 μl reactions containing 2 μg supercoiled maxiprep DNA in Buffer B (Buffer D with 100 mM KCl, 5 mM $MgCl_2$ and 0.1 mg/ml BSA). DFF40 was diluted in Buffer D containing 50% glycerol and was added in 5 μl to the reaction (glycerol was not found to substantially effect the DNase reaction at concentrations up to 20%). Proteins tested for inhibitor effect were diluted in Buffer B and added in 2.5 μl. DFF40 (0.5 pmole) and inhibitor proteins were preincubated 15 minutes at 37°C prior to the addition of DNA. After 30 min incubation at 37°C, DNA loading dye containing 50 mM EDTA was added and the DNA fragments separated on a 2% agarose gel by electrophoresis for 7 min at 150 volts.

Cell culture, transfection, immunoprecipitation, and Western blot analysis. 293T cells were maintained in RPMI medium containing 10% heat inactivated fetal calf serum. Transfection was performed using Dosper (Boehringer) according to manufacturer's instruction. Cells were plated at 50% confluence in 60 mm culture dish and transfection was performed one day after plating. 1.5 μg of pCMV-DFF40 and 1.5 μg of pCMV encoding DFF45 or corresponding domains were cotransfected into each plate and lysate was harvested in lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100 and 1 mM PMSF) 24 hours after transfection. 10 μl of Flag monoclonal antibody conjugated sepharose beads (Kodak, Sigma) was added into lysate and the binding reaction allowed proceeding for 2 hours at RT or 4°C overnight. The beads were subsequently washed with lysis buffer 5 times and resuspended in 50 μl of SDS loading buffer. Samples were then loaded on to 10 or 15% SDS-PAGE and separated by electrophoresis. Proteins were transferred to high-bond nitrocellulose membrane (Amersham) and analyzed by standard Western blot analysis using polyclonal antibodies against DFF40 and DFF45 and binding visualized using ECL kit (Amersham).

RESULTS AND DISCUSSION

Capase cleavage fragments of DFF45 are domains that can inhibit DFF40 nuclease activity. To generate large quantities of highly purified and active DFF40, we developed an expression system in which DFF40 and DFF45 expression are driven from two separate plasmids in the same cell (10). Nuclease activity of the purified DFF40 was assayed by a plasmid DNA degradation system (Fig. 1A). In this assay, the level of DFF 40 activity was visualized by the sigmoidal distribution

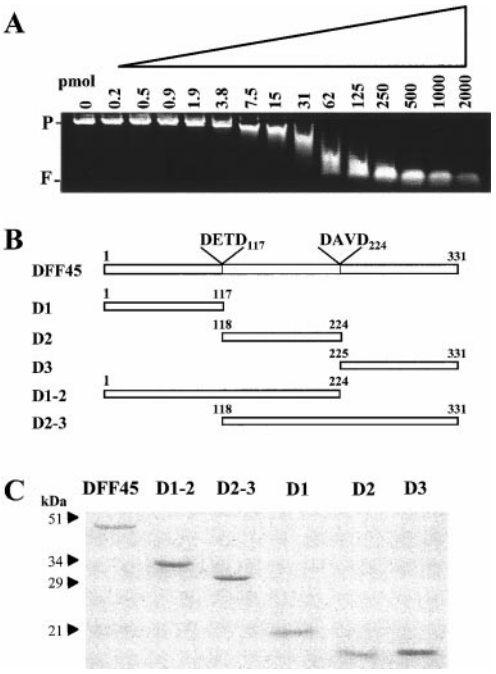

FIG. 1. Purified DFF40 nuclease activity, schematic for expression, and purification of DFF45 and its truncated forms. (A) DFF40 nuclease activity is quantified in a plasmid DNA degradation assay at various DFF40 concentrations. P and F represent the full length, supercoiled plasmid and the fully digested DNA fragments, respectively. (B and C) Truncated forms of DFF45 were generated through genetic constructs to yield five fragments guided by two internal caspase sites in DFF45 (DETD117 and DAVD224). These fragments were expressed in bacteria and purified *in vitro.*

of fragment banding as the concentration of DFF40 is diluted during seven sequential, two-fold dilutions (resulting in a total of 128 fold dilution). The terminal stage of normal fragmentation appears to be represented by the appearance of *ca.* 80 bp fragments, though at high concentrations of DFF40 or after prolonged incubation, these fragments disappeared (J. S. M. and P.L., unpublished data).

It has been shown that cleavage of DFF45 by caspase 3 at the corresponding caspase consensus sequences resulted in the activation of FF40 nuclease (2, 3). To determine how caspase cleavage alters DFF40's ability to inhibit DFF40 nuclease activity, we generated truncated DFF45 proteins guided by the caspase cleavage sites (Fig. 1B). This allowed us to isolate and work with proteins corresponding to the N-terminal region (D1), the middle region (D2), the C-terminal region (D3) of DFF45 as well as proteins corresponding to the first two (D1-2) and the last two (D2-3) fragments with wild type linkage. These proteins, as well as full length DFF45, were purified to homogeneity (Fig. 1C) and assayed for their ability to inhibit the DFF40 nculease activity.

In the enzymatic inhibition assay, DFF40 concentration was kept constant at a level that was just suffi-

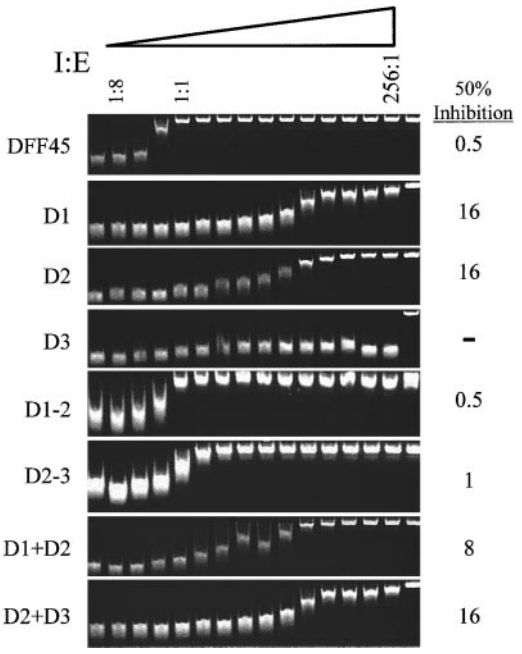

FIG. 2. The ability of DFF45 and purified protein fragments (*I*nhibitor) to repress DFF40 (*E*nzyme) nuclease activity analyzed in an *in vitro* assay. DFF40, present at constant concentration sufficient to totally convert plasmid from full-length to 80 bp, was preincubated with the indicated full-length or fragment proteins prior to the addition of plasmid substrate. Differential repression ability is quantitated by the different Inhibitor:Enzyme (I:E) ratios, increasing in two-fold increments from 1:8 to 256:1. The stoichiometric amount of inhibitor, relative to DFF40, yielding approximately 50% inhibition of DFF40 is indicated to the right. The first and last lanes represent controls containing either no inhibitor or DFF40, respectively.

cient to fully digest the plasmid DNA. Unlike the sigmoidal distribution of DNA fragments observed over a broad range of DFF40 concentrations (Fig. 1A), addition of full length DFF45 resulted in a strong inhibition of DFF40 activity with an abrupt change from fully digested DNA to uncleaved DNA over a four fold range (Fig. 2). Similar sharp transitions in DFF40 activity were observed for D1-2 and D2-3. In contrast, much broader distributions of fragment size, similar in appearance to the sigmoidal distribution associated with dilution of DFF40, were observed during titration with D1 and D2 corresponding to the N terminal and middle regions of DFF45. No inhibition of DFF40 nuclease activity was observed for titration of D3 (up to 256 molar excess) corresponding to the C-terminal region of DFF45, though it also is capable of binding DFF40 (11). Addition of D1 together with D2 or D2 together with D3 did not result in significant increase in their ability of inhibiting DFF40 nuclease activity compared with the isolated domain alone. These results demonstrate that D1-2, D2-3 and full-length DFF45 are strong repressors of DFF40 with 50% inhibition at inhibitor concentrations corresponding between 0.5 to 1 times of DFF40 (Fig. 2), suggesting these proteins bind to DFF40 with high affinity and equimolar stoichiometry. D1 and D2 required more than 10 fold excess to achieve 50% inhibition of nuclease activity suggesting that these domains may bind to DFF40 with lower affinity. These results suggest a mechanism of DFF40 activation by caspase 3 that involves disruption of overall DFF45 binding strength through cleavage at domain boundaries which results in the disruption of a synergistic binding strength. These results and conclusions are further supported by the accompanying study of the physical interaction of DFF45 with DFF40 by Biacore analysis (11).

DFF45 functions as a chaperone for folded and nascent DFF40. It was suggested previously that DFF45 might function as a chaperone for DFF40 (2). Chaperones have been shown to function either to prevent non-productive protein-protein interactions during protein translation (with nascent proteins) or with mature proteins (12). We tested the latter putative chaperone function for DFF45 by observing its effect in the solubility of purified, active DFF40. In this assay, GST tagged full length or truncated forms of DFF45 were incubated with active DFF40 (maintained as soluble stock in 50% glycerol) at 37°C for one hour in the presence of bacterial lysate proteins. The reaction was then centrifuged and the amount of soluble (in the supernatant) and aggregated (in the pellet) DFF40 was determined by western blot analysis. As shown in Fig. 3A, purified DFF40 partitioned to the pellet when incubated at low glycerol concentration with bacterial

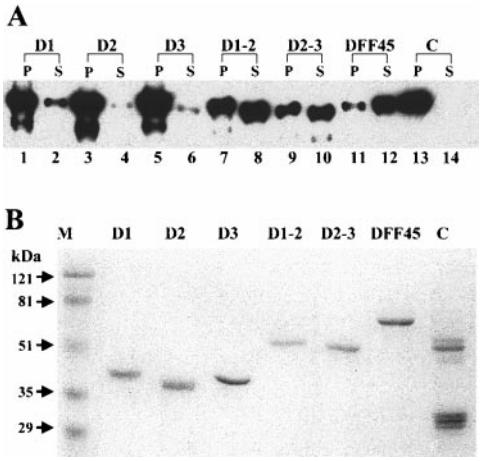

FIG. 3. DFF45 functions as a chaperone for the mature DFF40 *in vitro.* (A) Purified, active DFF40 (stored in 50% glycerol) was mixed with GST fusion proteins of either full length DFF45 or fragments in the presence of bacterial lysate. The mixture was incubated and recleared in a microcentrifuge. S and P represent DFF40 in the soluble phase or in the pellet phase, respectively visualized by using anti-DFF40 antibodies. GST-RGS16 was used as a control (C) protein in this assay. (B) Purified GST fusion proteins of DFF45 and its truncated forms used in the experiment shown in panel A visualized by coomassie staining.

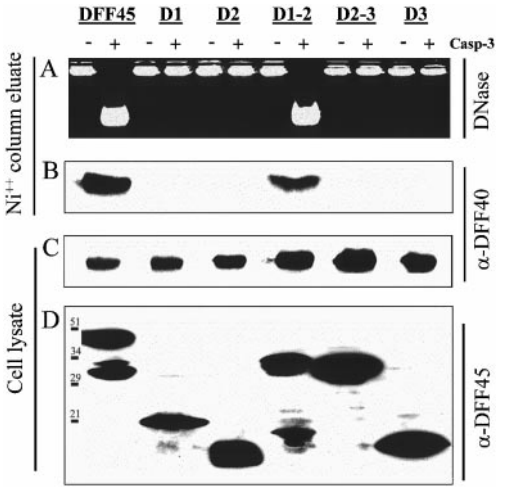

FIG. 4. DFF45 functions as a chaperone during expression of DFF40 in bacteria. His-tagged DFF40 and DFF45 or its truncated forms were co-expressed from a single plasmid in *E. coli* and proteins were purified from the lysate on a Ni^{++} column. (A) Nuclease activity of the purified DFF40 was determined after the Ni^{++} column purification. +/− represent the presence or absence of caspase-3 in the nuclease assay. (B) The Western blot analysis of Ni^{++} eluate fractions using anti-DFF40 antibody recapitulates the enzyme assay. (C and D) Expression of DFF40 and DFF45 or its truncated forms in cells was monitored by Western blot analysis with the appropriate antibody.

lysate proteins and control protein. In contrast, the majority of DFF40 remained in the supernatant phase in the presence of full length DFF45 (lanes 11/12). D1-2 and D2-3 were also able to maintain a large portion of the DFF40 in the supernatant (lanes 7–10), while isolated domains of DFF45 alone were not capable of preventing the majority of DFF40 partitioning to the pellet (lanes 1–6).

In a further test of DFF45's chaperone function, we analyzed the importance of the individual domains during bacterial expression of DFF40. Consistent with previous reports, we were unable to isolate active DFF40 when expressed in the absence of DFF45 (data not shown). In contrast, co-expression of DFF45 allowed DFF40 to be recovered in the supernatant of lysed cells and subsequently to bind to the Ni^{++} column (Fig. 4A and 4B). The eluate from the Ni^{++} column was active as a nuclease in the presence of caspase 3. The fragments of DFF45 were then tested in this ability to function as a chaperone for DFF40 *in vivo* to yield active protein. Only strains expressing the D1-2 fragment resulted in purification of active DFF40. Notably, co-expression of D2-3 with DFF40 was not able to yield active DFF40 even though D2-3 exhibited strong inhibition to DFF40 nuclease activity (Fig. 2) and acts as a chaperone to maintain DFF40 solubility *in vitro* (Fig. 3A). These results indicate that the N-terminal D1 region is essential in regard DFF45 activity as a chaperone for nascent DFF40. In all cases, expression of DFF40 protein as well as the DFF45 protein fragments were equal (Fig. 4C and 4D).

Chaperone activity of DFF45 domains for DFF40 was identical in mammalian cells (Fig. 5A) as to that observed in the bacterial expression system. This conclusion is reached from an experiment in which flag-tagged full-length DFF45 or its fragments were transiently co-expressed with DFF40 in 293 cells. Only cells expressing full length DFF45 or D1-2 fragment had accumulated substantial levels of DFF40 in the cell extracts (Fig. 5A) and show nuclease activity (data not shown). Under these conditions, however, expression of full length DFF45 allowed a higher level of DFF40 expression relative to D1-2 (*ca.* 5X). As expected from the presence of endogenous DFF45, low level expression of DFF40 was observed for cells co-expressing D1, D2, D3 or D2-3 (Fig. 5A). The data from mammalian cells are consistent with that obtained from bacterial expression system, supporting the conclusion that D1 plays a crucial role as a chaperone for *de novo* synthesized DFF40. These results on chaperone activity differ from a recently published studies of DFF45/DFF40 interaction. Sakahiri *et al.* (13) and Gu *et al.* (14) observed that a naturally occurring splice variant of DFF45, DFF35, roughly equivalent to the D1-2 fragment used here, did not display an *in vivo* chaperone activity towards DFF40. Indeed, we observed this fragment to display a weaker ability to act as a chaperone for DFF40. Alternatively, this difference may result from the additional C-terminal residues in DFF35 not found in D1-2.

The direct interaction between DFF40 and domains of DFF45 was tested by immunoprecipitation using

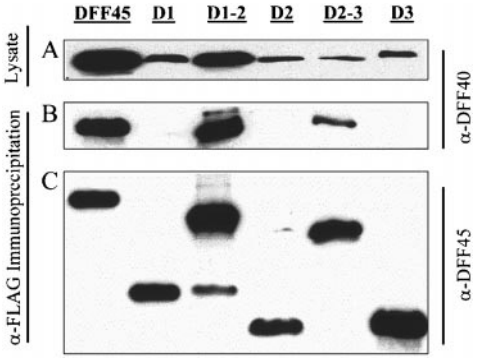

FIG. 5. The ability of DFF45 and its protein fragments to act as a chaperone during expression of DFF40 in mammalian cells and the direct interaction between DFF45 and DFF40 evaluated by coimmunoprecipitation assay. 293 cells were transiently transfected with plasmids encoding DFF40 and FLAG-tagged DFF45 or its fragments. (A) The dependence of DFF40 expression in 293 cells on DFF45 and its fragments was monitored by Western analysis of total cellular lysate. Five-fold lower amounts of total lysate were applied for full length DFF45 (first lane). (B) Co-immunoprecipitation of DFF40 was performed on these lysates utilizing the anti-FLAG antibody. (C) DFF45 levels were visualized through Western blot analysis and levels used to normalize amounts of cell material for panels A and B.

anti-Flag antibody and the level of associated DFF40 was determined on Western blot using anti-DFF40 antibodies (Fig. 5B). Only full length DFF45, D1-2, and D2-3 co-precipitated appreciable amounts of DFF40 at levels correlating to the total amount in the cell. Isolated domains D1, D2 or D3 showed little or no ability to immunoprecipitate DFF40 from the cell lysate. These results indicate that only proteins containing at least two domains of DFF45 (D1-2, D2-3 or full-length DFF45) bind to DFF40 with high affinity. This is additional evidence that covalent linkage of individual domains plays an important role in stabilizing the interaction between DFF45 and DFF40.

In conclusion, we found that DFF45 was necessary both for expression *in vivo* of DFF40 but for its subsequent maintenance *in vitro* in the presence of cellular lysate proteins. We interpret this to indicate that the chaperone activity of DFF45 centers around its ability to prevent DFF40 aggregation. It is intriguing to observe that D1-2 and D2-3 fragments of DFF45 possess DFF40 chaperone activity sufficient to maintain soluble DFF40 and inhibition activity approaching to that of full-length DFF45 protein. This *in vitro* observation correlates well to a recent study of MCF7 cells (15) showing that DFF45 can be cleaved by two distinct caspases and that cleavage at the first caspase site of DFF45 was not sufficient to induce DNA fragmentation. Thus, after the critical nascent protein stage, linked binding full length DFF45 to DFF40 is not necessary for DFF40 expression and repression. Finally, it would appear that D1 of DFF45 plays a particular role *in vivo*, presumably during the nascent phase of translation. This conclusion is in agreement with mapping analysis in the accompanying paper (11) that localizes the binding site of DFF45 D1 to the first 80 translated residues of DFF40.

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