

Subunit Structures and Stoichiometries of Human DNA Fragmentation Factor Proteins before and after Induction of Apoptosis*

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DNA fragmentation factor (DFF) is one of the major endonucleases responsible for internucleosomal DNA cleavage during apoptosis. Understanding the regulatory checkpoints involved in safeguarding non-apoptotic cells against accidental activation of this nuclease is as important as elucidating its activation mechanisms during apoptosis. Here we address these issues by determining DFF native subunit structures and stoichiometries in human cells before and after induction of apoptosis using the technique of native pore-exclusion limit electrophoresis in combination with Western analyses. For comparison, we employed similar techniques with recombinant proteins in conjunction with atomic force microscopy. Before induction of apoptosis, the expression of DFF subunits varied widely among the cell types studied, and the chaperone/inhibitor subunits DFF45 and DFF35 unexpectedly existed primarily as monomers in vast excess of the latent nuclease subunit, DFF40, which was stoichiometrically associated with DFF45 to form heterodimers. DFF35 was exclusively cytoplasmic as a monomer. Nuclease activation upon caspase-3 cleavage of DFF45/DFF35 was accompanied by DFF40 homo-oligomer formation, with a tetramer being the smallest unit. Interestingly, intact DFF45 can inhibit nuclease activity by associating with these homo-oligomers without mediating their disassembly. We conclude that DFF nuclease is regulated by multiple pre- and post-activation fail-safe steps.

Apoptosis, or programmed cell death, is a fundamental process essential for both development and maintenance of tissue homeostasis (for review, see Refs. 1 and 2). Two major apoptotic pathways exist, the death receptor pathway and the mitochondrial pathway (3, 4). Multiple apoptotic stimuli, which include activation of Fas receptors, serum starvation, ionizing radiation, or various drugs that target DNA, trigger the activation of proteases called caspases, which in turn initiate and execute the apoptotic program (5).

One of the hallmarks of the terminal stages of apoptosis is

DNA breakdown, which has functional significance (6–9). Although cell death can occur without significant DNA degradation, cell-autonomous DNA breakdown of tumor or virally infected cells functionally prepares the resulting apoptotic corpses for engulfment by phagocytes and as such eliminates the transforming potential of any hazardous oncogenes. Interestingly, patients or an animal model with defective apoptotic DNA processing have a predisposition to auto-immune disease, which is known to be associated with the appearance of anti-DNA and anti-nucleosomal antibodies (10, 11). Finally, the execution of apoptotic DNA fragmentation and the cell death program appears to be reversible at a low frequency, and under such conditions chromosomal translocations mediated by non-homologous end joining of proto-oncogenes are thought to be one mechanism of cellular transformation (12). In particular, it is well documented that treatment of leukemias with pro-apoptotic drugs, which target DNA, can lead to secondary tumors that exhibit new chromosomal translocations (13). Taken together, it seems clear that understanding the regulation of proteins that are involved in DNA processing events during apoptosis is of fundamental importance, as well as how these processes are safeguarded from accidental activation in non-apoptotic cells. In the current study we address these issues for a major apoptotic nuclease complex, termed DFF.¹

Apoptotic cell genomic DNA cleavage is known to occur in at least two stages, initial cleavage at ≥ 50 -kilobase intervals, a size consistent with chromatin loop domains, followed by a second stage of internucleosomal DNA cleavage (also called DNA laddering) (14). Apoptosis-inducing factor (15), topoisomerase II (16), endonuclease G (17), and DFF (18–20) have each been implicated in the higher order DNA cleavage reaction. Nucleosomal DNA laddering, on the other hand, has been associated with several endonucleases, including DFF (21–28), endonuclease G (17, 29–31), and DNase I (32).

In non-apoptotic cells, DFF is thought to exist as a heterodimer, composed of a 45-kDa chaperone and inhibitor subunit (DFF45/ICAD-L), and a 40-kDa latent nuclease subunit (DFF40/CAD/CPAN) (21–25). During translation, Hsp70 and Hsp40 also participate in chaperoning the functional assembly of the putative heterodimer (33). Both DFF45 and DFF40 reside in the cell nucleus (22, 34–36), and each subunit possesses its own nuclear localization sequence (34–36). A 35-kDa splic-

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¹ The abbreviations used are: DFF, DNA fragmentation factor; AFM, atomic force microscopy; CAD, caspase-activated deoxyribonuclease (also termed DFF40); DFF45, 45-kDa subunit of DFF; DFF40, 40-kDa subunit of DFF; DFF35, 35-kD subunit of DFF; ICAD-L and ICAD-S, inhibitors of CAD, long and short forms (also termed DFF45 and DFF35, respectively); CPAN, caspase-activated nuclease (also termed DFF40 and CAD).

ing variant of DFF45 (DFF35/ICAD-S) also exists that resides in the cytoplasm because of a spliced-out nuclear localization sequence in its C terminus (23, 35, 37–39). Apoptotic activation of caspase-3 or -7 results in the cleavage of DFF45/DFF35 and release of active DFF40 nuclease (21–26, 40). DFF40 nuclease activity on naked DNA substrates can be further activated by specific chromosomal proteins, such as histone H1 or high mobility group 1/2 (22, 26, 27, 41) and topoisomerase II (27, 42). Other gene products also appear to participate in the DFF pathway. Two proteins encoded by genes called CIDEs (cell death-inducing DFF45-like effectors) that exhibit homology to the N-terminal domain of DFF45 can activate apoptosis in a DFF45-inhibitable fashion, but their precise functions and mechanisms of action remain to be elucidated (43).

It should be noted that the physiological significance of DFF in triggering DNA laddering during apoptosis has been unequivocally proven. Homozygous deletions of the single copy genes encoding DFF45 or DFF40 have been created in the mouse germ line and in cultured chicken cells (44–46). In addition, dominant negative forms of DFF45 have been expressed in a cell line and transgenic mice (18, 47). Significantly, cells engineered in each of these ways exhibit greatly reduced DNA laddering when exposed to several apoptotic stimuli, both *in vivo* and *in vitro*, proving that DFF is a principal mediator of internucleosomal DNA fragmentation (18, 44–48). Perhaps unexpected, initial characterization of such knockout mice has not revealed any obvious developmental or immune system defects (44, 46), except for an increase in the granule cell number in the brain hippocampus (49). However, further investigation has uncovered that during apoptosis DFF-deficient cells are efficiently killed and engulfed by macrophages, which through the action of lysosomal DNase II degrade the DNA of the ingested corpses (46, 47). Interestingly, DFF40/DNase II double knockout mice exhibit a severe defect in thymic development (46). In conclusion, it is well established experimentally that DFF plays a major and regulated role in cell-autonomous apoptotic DNA fragmentation.

Developing an understanding of the steps involved in safeguarding normal cells against accidental activation of this nuclease is as important as elucidating its activation mechanisms during apoptosis. To gain insight on the regulation of these processes, in the present study we have determined the native subunit structures and stoichiometries of these proteins before and after induction of apoptosis using the technique of native pore-exclusion limit electrophoresis in combination with Western analyses (50) and have utilized similar techniques with recombinant proteins along with atomic force microscopy. We find that DFF activation is regulated by multiple fail-safe steps and that its inhibitory subunits unexpectedly are expressed at vast stoichiometric excess relative to its latent nuclease subunit in several tumor cells studied.

EXPERIMENTAL PROCEDURES

Expression, Purification, Activation, and Inhibition of Recombinant Forms of DFF—His₆-tagged human recombinant DFF40/DFF45 heterodimer, human recombinant DFF45 monomer, and hamster recombinant caspase-3 were expressed in *Escherichia coli* and purified using nickel affinity columns as described previously (22). DFF40/DFF45 heterodimer was incubated with caspase-3 at room temperature in reaction buffer consisting of 10 mM KCl, 100 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.5. After 30 min of incubation caspase-3 was inhibited with 10 μ M acetyl-Asp-Glu-Val-Asp-aldehyde. In some experiments fresh DFF45 (10-fold molar excess as compared with the starting DFF40/DFF45 heterodimer concentration) was then added, and the reaction mixture was incubated on ice for an additional 15 min. Nuclease activity was assayed as described previously (27).

Cell Culture and Treatments—Several established human cell lines were used for experiments: HL60 and K562 myeloid leukemia cells

(DSMZ ACC 107 and EC ACC 89121407, respectively), Jurkat T cell leukemia cells (DSMZ ACC 282), HeLa S3 cervical carcinoma cells (ATTC CC1 2.2), MCF7 breast adenocarcinoma cells (IZSBS BS TCL 37), A549 lung carcinoma cells (DSMZ ACC 107), and Me45 melanoma cells. As non-cancer cells, we used primary fibroblasts (passage 15), resting total lymphocytes (80% T cells), and proliferating T lymphocytes. Lymphocytes and leukemia cells were cultured in RPMI1640 medium, whereas other cells were cultured in Dulbecco's modified Eagle's medium, all supplemented with 10% fetal calf serum, at 37 °C under 5% CO₂. Lymphocytes were freshly isolated from circulating blood by centrifugation on Ficoll Lymphoprep™ (ICN). To obtain proliferating T cells, isolated lymphocytes were incubated for 3 days in RPMI1640 medium supplemented with 10% fetal calf serum and 10 mg/liter lectin (Sigma). To induce apoptosis, HL60 cells were incubated for 5 h with 34 μ M etoposide (Sigma). DNA fragmentation in such cells was assayed as described previously (51).

Isolation of Cytoplasmic and Nuclear Extracts—Cell growing in log phase were collected and washed twice with ice-cold phosphate-buffered saline. Washed cells were incubated for 15 min on ice in lysis buffer consisting of 0.5% Nonidet P-40, 0.25 M sucrose, 10 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.5, supplemented with a mixture of protease inhibitors Complete™ (Roche Applied Science). After incubation, nuclei were collected by centrifugation for 5 min at 600 \times g. Cell lysates were then centrifuged for 15 min at 10,000 \times g, and the resulting supernatants are referred to as cytoplasmic extracts. In some experiments nuclei were obtained by a non-detergent lysis procedure in which HeLa cells washed with phosphate-buffered saline were suspended in the lysis buffer depleted of Nonidet P-40 and sucrose, incubated with 10 μ M cytochalasin B for 15 min on ice, then lysed by passing 5 times through 25-gauge needles. Nuclei were washed with the lysis buffer depleted of Nonidet P-40 by low speed centrifugation and incubated for 15 min on ice in the nuclear extract buffer consisting of 0.5% Triton X-100, 0.25 M sucrose, 10 mM KCl, 200 mM NaCl, 4 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.5, supplemented with a mixture of protease inhibitors (Complete™ (Roche Applied Science)). Nuclei were then centrifuged for 15 min at 10,000 \times g, and the resulting supernatants were referred to as nuclear extracts.

Western Blot Analyses—Total cell protein (80 μ g/lane) and the corresponding fractions of cytoplasmic extracts, nuclei and nuclear extracts, or protein from cytoplasmic extracts alone (50 μ g/lane) were separated on SDS, 14% polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Amersham Biosciences or Schleicher & Schuell). Membrane-immobilized proteins were probed with the following commercial antibodies: rabbit anti-human DFF40 polyclonal antibodies (Pharmingen or Axxora), rabbit anti-human DFF45 N terminus polyclonal antibodies (Pharmingen), rabbit anti-human DFF45 C terminus polyclonal antibodies (Axxora), and mouse anti-human β -actin monoclonal antibody (Oncogene). The antigen-antibody complexes were visualized using enhanced chemiluminescence (ECL) Western-blotting detection reagents (Amersham Biosciences).

Atomic Force Microscopy—Protein samples were diluted to a final concentration of \sim 1 μ g/ml in 50 mM HEPES, pH 7.5. Immediately before imaging, 2- μ l aliquots of diluted protein samples were introduced to the surface of freshly cleaved mica and rinsed with 200 μ l of H₂O followed by dehydration using a graded series of ethanol solutions consisting of 20, 40, 80, and 100% ethanol. Images were collected under ambient conditions in tapping mode using a Nanoscope IIIa (Digital Instruments Inc., Santa Barbara, CA) equipped with a vertical engage J-scanner operated at a scan rate of 1.97 Hz. Volume data were calculated as previously described (52).

Native Pore-exclusion Limit Electrophoresis—Native pore-exclusion limit electrophoresis was performed as described elsewhere (50), with minor modifications. Briefly, soluble proteins from cytoplasmic extracts (300 μ g of protein/lane) were separated on linear 4–24% gradient polyacrylamide gels in 0.1 M Tris borate, pH 9.5, 1 mM β -mercaptoethanol. Gels were calibrated by co-electrophoresis of ovalbumin (45 kDa), bovine serum albumin (66 kDa), lactate dehydrogenase (140 kDa), with α -actin (monomer of actin G and a series of its oligomers) and also with Kaleidoscope pre-stained standards (Bio-Rad 161-0324). Electrophoresis was continued for 16 h at 300 V, \sim 10 mA, at 4 °C. Gels were soaked with 0.1% SDS, 25 mM Tris-Cl, 250 mM glycine, pH 8.5, and then proteins were electrophoretically transferred onto nitrocellulose membranes. Membrane-immobilized proteins were probed with antibodies as described above. In one experiment (in-gel DFF40 oligomerization) proteins from cytoplasmic extracts were electrophoresed briefly (\sim 1 cm) into a native 5% polyacrylamide gel, and the appropriate gel fragment was excised. The gel fragment was then incubated for 30 min at room

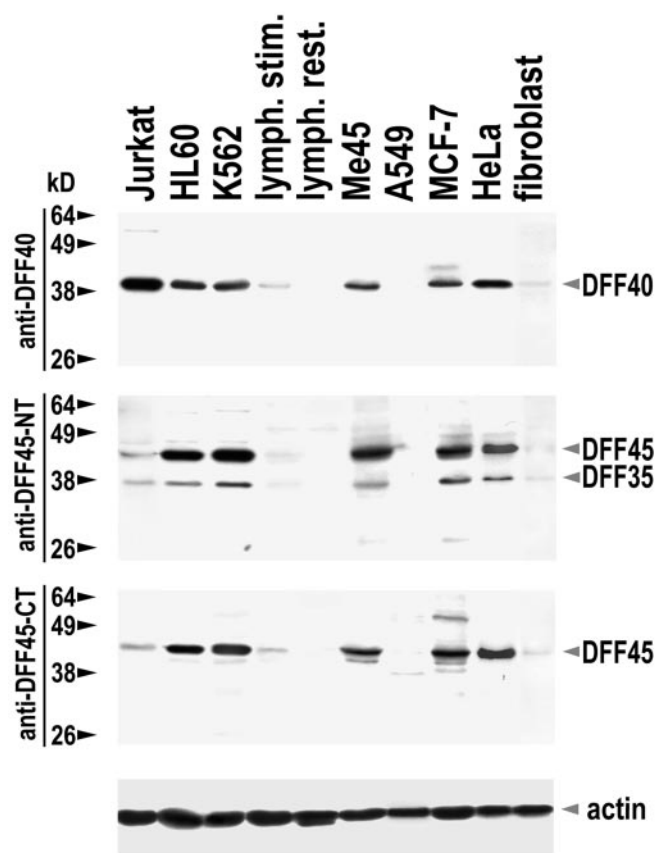


FIG. 1. Comparison of the levels of DFF40, DFF45, and DFF35 in different human cells. A Western blot of cellular extracts separated on SDS-PAGE gels was probed with antibodies specific for DFF40 (*anti-DFF40*), both DFF45 and DFF35 (*anti-DFF45-NT*), and DFF45 (*anti-DFF45-CT*). Black arrowheads represent the positions of molecular mass standards, and gray arrowheads depict the positions of DFF subunits.

temperature with 10 μ g of caspase-3, washed, and polymerized at the top of a 5–24% gradient gel. When cytoplasmic extracts were analyzed by two-dimensional electrophoresis, protein were first separated on native gradient gel, the gel fragment was soaked in 2% SDS, 0.1 M β -mercaptoethanol, and 50 mM Tris-HCl, pH 6.8, and placed on the top of standard SDS-14% polyacrylamide gel.

RESULTS

DFF Levels Vary Widely among Both Normal and Transformed Human Cells—Previous studies have noted varied expression of DFF45/35 and DFF40 among different human and mouse cell lines and tissues by Northern analyses of mRNA levels but not by Western analyses of protein levels (36, 48, 53). Because we were interested in determining the native subunit structures and relative stoichiometries of these proteins, it was first necessary to survey several cell sources to identify those that possessed adequate levels for subsequent analyses. For this purpose we performed Western analyses on suitably prepared cell extracts using antibodies specific for DFF40, DFF45 (with antibodies against the C terminus of DFF45), and both DFF45 and DFF35 (with antibodies against the N terminus of DFF45). As shown in Fig. 1, DFF species levels varied more than 10-fold between the cell sources studied, with levels undetectable in resting lymphocytes and A549 lung carcinoma cells, barely detectable in fibroblasts and stimulated lymphocytes, and quite robust in MCF-7 breast adenocarcinoma, HeLa cervical carcinoma, and Jurkat, HL-60 and K562 leukemia cells. Thus, DFF levels in some tumor cells, but not others, far exceed the levels exhibited by normal cells. Whether there is any relationship between these differences in DFF levels and

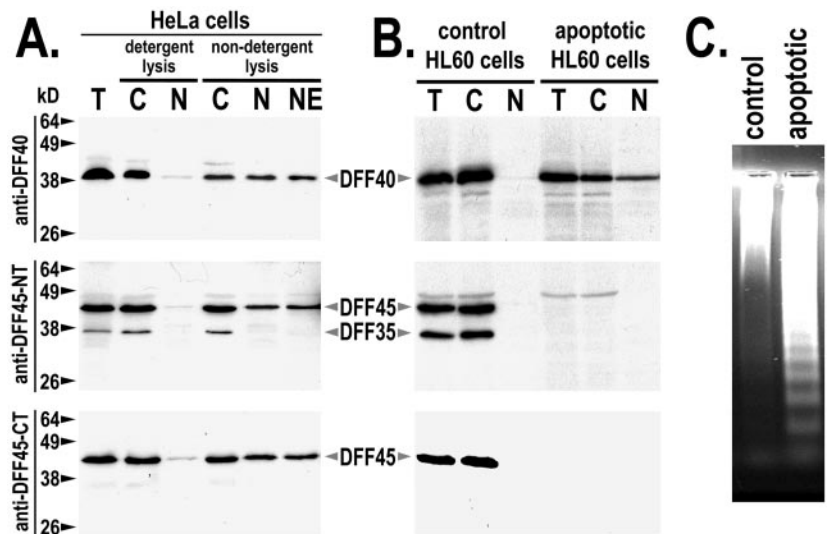
sensitivities to killing by various apoptotic stimuli remain to be determined. Nevertheless, based on these results, we selected Jurkat, HeLa, and HL-60 cells for further studies. By relating results on native subunit structures and relative stoichiometries of DFF proteins from these cell lines of different origin to those obtained with corresponding recombinant proteins, we hoped to establish the generality of our observations to apoptosis.

Subcellular Fractionation Reveals That DFF Rapidly Leaks out of Nuclei upon Mild Detergent Lysis of Cells—Based on early biochemical fractionation experiments, it was initially thought that DFF subunits resided exclusively in the cytoplasm of non-apoptotic cells (21, 23). However, immunocytochemical and GFP fusion protein expression experiments have unequivocally established that both DFF45 and DFF40 reside predominantly in the nucleus (22, 34–36). In addition, very careful nuclear preparations employing cytochalasin B for subcellular fractionation have also shown that DFF45 is exclusively nuclear (34).

Because our experiments would be facilitated if all DFF species could be the quantitatively extracted from cells as native complexes in a single fractionation step, we investigated the subcellular distribution of DFF species after lysis of cells under several conditions, including exposure to 0.5% Nonidet P-40 or cytochalasin B. Significantly, we found that the detergent lysis technique essentially quantitatively released all DFF species into the cytoplasmic fraction from HeLa or HL-60 non-apoptotic cells (Fig. 2, A and B) and also Jurkat non-apoptotic cells (data not shown). This observation is striking because our previous extensive studies show that nuclei prepared by this mild detergent lysis technique have fully intact morphology as viewed by phase contrast microscopy and 4,6-diamidino-2-phenylindole staining, and besides the histones, contain abundant amounts of non-histone chromosomal proteins (51). Evidently, DFF species belong to a class of nuclear proteins that are easily lost from nuclei upon subcellular fractionation (54). After induction of apoptosis and DNA laddering in HL-60 cells (Fig. 2C), DFF45 and DFF35 subunits disappeared as expected because of caspase cleavage events, whereas a fraction of DFF40 became localized in the nuclear fraction (Fig. 2B). We interpret this change in subcellular partitioning to reflect a partial resistance of activated DFF40 to leak out of nuclei due to its formation of high molecular weight complexes upon activation (see below). Nevertheless, for subsequent studies we have taken advantage of the observed quantitative nuclear leakage of DFF species upon mild detergent lysis of non-apoptotic cells for analyses of the subunit structures and stoichiometries of its components.

DFF Subunit Structures and Stoichiometries in Non-apoptotic Cells Reveal That Free DFF45/35 Proteins Are Monomers and That DFF45/35 Subunits Are in Vast Molar Excess of DFF40 Subunits, Which Are Stoichiometrically Associated with DFF45 to Form Heterodimers—The stoichiometries of expression of DFF45/35 subunits have not been previously determined with respect to DFF40 subunits. Furthermore, the analyses of DFF45 and DFF40 gene promoter regions suggest that they may be differentially regulated (37), although from individual gene knockouts there is evidence for the mutual regulation of the two proteins at the post-transcriptional level (55). Finally, previous studies utilize only gel filtration to assess the subunit structures of DFF species and conclude that the elution patterns of DFF45 species *per se* are either di- or trimeric (23, 56), whereas DFF45-DFF40 complexes are probably heterodimeric (21, 23, 26). These uncertainties have led us to investigate these issues by the technique of native pore exclu-

FIG. 2. Comparison of the levels of DFF40, DFF45, and DFF35 in different cellular fractions of HeLa and HL-60 cells. *A*, HeLa cells were fractionated after lysis either with mild detergent (Nonidet P-40) or without detergent by passing through a syringe needle in the presence of cytochalasin B. *B*, control and apoptotic (etoposide-treated) HL60 cells were fractionated after mild detergent lysis. *T*, total cellular proteins; *C*, cytoplasm; *N*, nuclei; *NE*, nuclear extract. *C*, DNA fragmentation before (*control*) and after 5 h of etoposide treatment of HL-60 cells to induce apoptosis.



sion limit electrophoresis in combination with Western analyses (50).

Fig. 3 shows the results of the analyses of the native subunit structures and stoichiometries of various DFF species after separation by gel electrophoresis under non-denaturing conditions. As shown in Fig. 3A, when probed with anti-DFF40, purified recombinant DFF45-DFF40 and the corresponding native complexes in extracts of HeLa, HL-60, and Jurkat non-apoptotic cells each co-migrated with an estimated mass of about 100 kDa relative to protein standards. We demonstrate by protein cross-linking and AFM that this complex is a heterodimer (see Fig. 3C and Fig. 5B, panels 1 and 3). An identical migration position was seen for DFF species extracted from nuclei under conditions of cell lysis without detergent (data not shown). It is significant that no free DFF40 monomer was detectable by this analysis (Fig. 3A). When the same blot was probed with antibodies against the N-terminal region of DFF45, besides the heterodimer signals we observed very significant signals from free DFF45 species in the cell extract samples that co-migrated with free recombinant DFF45 monomer with an estimated mass relative to protein standards of about 48 kDa (Fig. 3B). In addition, free DFF35 monomer was also present in these cell extracts, which migrated with an estimated mass of about 36 kDa relative to protein standards (Fig. 3B) (experiments employing cell lysis without detergent revealed that DFF35 was exclusively cytoplasmic; data not shown). We have quantitated these results by densitometry of several anti-N-terminal DFF45-probed blots from such experiments. As summarized in Table I, we found that DFF45 subunits were in vast stoichiometric excess of DFF40 in both HL-60 and HeLa cells, and free DFF35 subunits are close in levels to DFF40 itself. We conclude that DFF45 can exhibit an unexpected high level of overexpression relative to its DFF40 partner, which exists exclusively as a heterodimer.

To further investigate the subunit structure of DFF45-DFF40 complexes, we performed protein cross-linking with glutaraldehyde and separated the products on SDS denaturing gels relative to protein standards. The resulting Western blot probed with antibodies against either DFF40 or DFF45 revealed that cross-linked products migrated with an average mass of 86 kDa as a heterodimer complex composed of DFF45 and DFF40 (Fig. 3C, asterisk).

DFF35 Does Not Participate in Heterodimer Formation—Biochemical fractionation of cell extracts from non-apoptotic cells and co-expression experiments with insect or mouse cells suggest that DFF45, as opposed to DFF35, is the predominant

form associated with DFF40 to form a potentially activable nuclease (21, 38, 55). These observations do not support the claim that DFF35 has a higher binding affinity for DFF40 than does DFF45 (39) and is the major species participating in functional DFF40 complexes (57). To determine whether human DFF35 indeed forms complexes with DFF40 *in vivo*, we again utilized native pore-exclusion limit electrophoresis but in conjunction with a second-dimension of electrophoresis in SDS gels. For this analysis we chose extracts from Jurkat cells because of their relatively high level of DFF35 in proportion to DFF45 (Fig. 3B; Table I). Because the heterodimer band was rather broad it was possible that besides DFF45, DFF35 could also participate in complex formation with DFF40 (e.g. Fig. 3B). However, this possibility was discounted after performing a second dimension of electrophoresis in SDS gels because the heterodimeric species only contained DFF45 (Fig. 4). We conclude that DFF35 does not participate significantly in heterodimer formation with DFF40 in these cells. These results are in agreement with previous suggestions (21, 38, 55) but not others (39, 57).

DFF40 Activation in Apoptotic Cells Is Accompanied by High Molecular Weight Complex Formation like That Exhibited by Recombinant Protein Activation in Vitro—The active form of DFF has been reported to be a monomer (23), but we have evidence based on gel filtration of caspase-3-treated recombinant DFF that nuclease activation is accompanied by DFF40 homo-oligomer formation (26). To investigate further the molecular nature of activated DFF nuclease we have again utilized native pore-exclusion limit electrophoresis on recombinant protein but before and after its activation by treatment with recombinant caspase-3 and compared these results to volume measurements obtained on similar complexes by AFM. As shown in Fig. 5A, caspase-3 treatment of recombinant DFF results in proteolysis and loss of DFF45 with the concomitant formation of DFF40 homo-oligomers, which exhibit a very broad size distribution. Enzymatic assays reveal that these species represent fully activated DFF nuclease (e.g. see Fig. 7). The smallest of these DFF40 oligomeric species migrates approximately as tetramers. None of these homo-oligomers could be dissociated by the mild detergent treatment that we have used for cell lysis (data not shown). AFM volume measurements calculated from micrographs reveal that before caspase-3 treatment, recombinant DFF45-DFF40 “heterodimer” molecules possess a homogenous, tight distribution at 72 nm³ (Fig. 5B, panels 1 and 3), which is very close to that observed for Ku80, an 82-kDa protein, when used as a protein

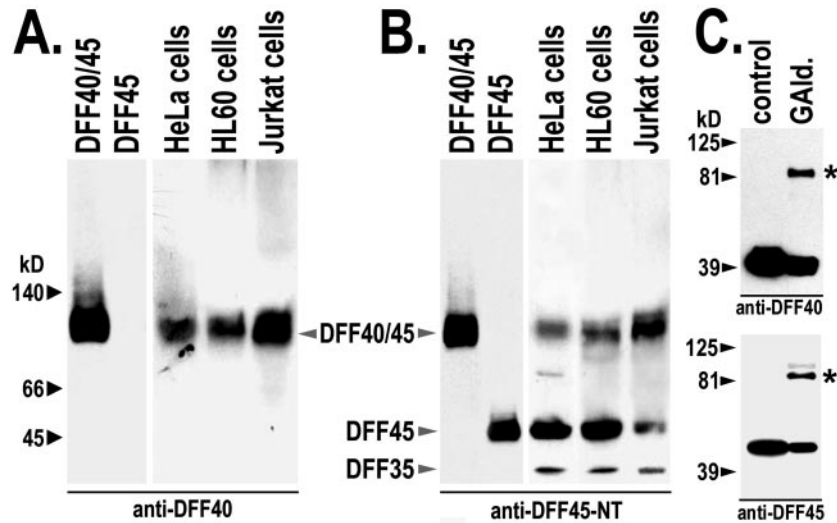


FIG. 3. Subunit structure and stoichiometry of human recombinant and endogenous DFF. Recombinant DFF (*left lanes*) and cytoplasmic extracts from the indicated non-apoptotic cells prepared by lysis with mild detergent were separated using native pore-exclusion limit electrophoresis, and Western blots were probed with antibodies specific for DFF40 (*panel A*) and DFF45/DFF35 (*panel B*). *Black arrowheads* represent the positions of molecular mass standards, and *gray arrowheads* show the positions of DFF subunits. *C*, recombinant DFF was incubated with 0.05% glutaraldehyde (*GALd.*) for 10 min at 20 °C, the reaction was terminated by adding 0.1 M glycine and SDS-gel loading buffer, samples were denatured and run on 10% polyacrylamide-SDS gel, and the resulting Western blot was probed with antibodies specific for DFF40 and DFF45.

TABLE I

Relative proportions of DFF40, DFF45, and DFF35 subunits in non-apoptotic cells

Average values from 2–4 independent experiments.

Protein species	Cell lines studied		
	HL-60	HeLa	Jurkat
DFF40	1.00 ^a	1.00 ^a	1.00 ^b
DFF45	6.45 ± 2.22	6.76 ± 1.15	1.59 ± 0.48
DFF35	1.42 ± 0.68	1.37 ± 0.28	0.48 ± 0.21

^a The absolute levels of DFF40 are similar in HL-60 and HeLa cells.

^b The absolute level of DFF40 is about 2-fold higher in Jurkat cells compared to former ones.

standard (data not shown). Furthermore, according to the equation $V = (1.2 \times \text{molecular mass}) - 14.7$ (58), which relates AFM volume measurements to molecular mass based on a standard curve created with 13 different protein complexes; the 72-nm³ volume corresponds to a protein complex of 72 kDa, a value in reasonable agreement with 79.5 kDa, the actual mass of the DFF heterodimer complex calculated from the corresponding amino acid sequences. Taken together, these results strongly support the conclusion that native DFF is indeed a heterodimer. In marked contrast, after caspase-3 treatment of recombinant DFF45-DFF40 heterodimers, which results in the cleavage and dissociation of DFF45 (26), the resulting DFF40 particles are larger and more heterogeneous and exhibit a multi-modal distribution, suggestive of the presence of several oligomeric species (Fig. 5B, panels 2 and 4). Based on the observed volume of DFF heterodimers, the first peak in this histogram, representing the predominant species, at ~150 nm³ is consistent with the predicted volume of a DFF40 homotetramer. We conclude that these subunit structure results with recombinant DFF obtained by two independent techniques are in general agreement.

Does endogenous DFF also form large complexes *in vivo* during apoptosis like those exhibited by its caspase-3-treated recombinant counterpart *in vitro*? To address this question we performed Western analyses after electrophoretic separation under non-denaturing conditions of extracts from control and apoptotic HL-60 cells. As shown in Fig. 6A, neither DFF45/35 nor DFF40 species was detectable in the apoptotic extracts after such analyses. The absence of detectable DFF40 was not

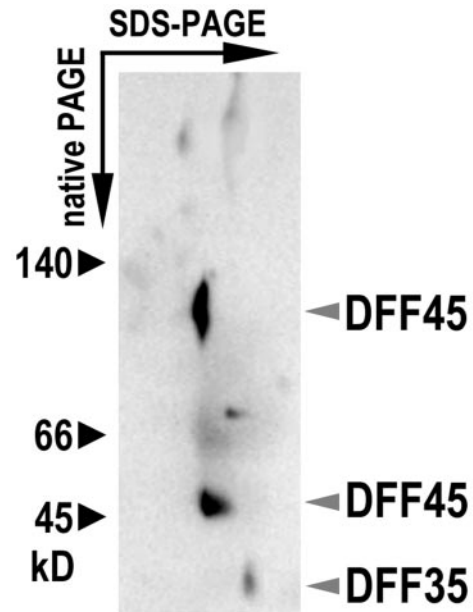


FIG. 4. DFF35 does not associate with DFF40 in Jurkat cells. A cytoplasmic extract prepared from non-apoptotic Jurkat cells lysed in mild detergent was separated in two dimensions as indicated, and the resulting Western blot was probed with antibody specific for DFF45/DFF35. *Gray arrowheads* show the positions of DFF subunits. The *upper* DFF45 spot migrated slower than free-DFF45 in the first dimension because it was associated originally with DFF40 as a heterodimer.

due to its loss during cell fractionation because the same fraction contained DFF40 after Western analysis of SDS gels (Fig. 2B). Moreover, DFF40 species were not detectable even when cytoplasmic extracts from non-apoptotic cells were incubated with caspase-3 before electrophoresis under non-denaturing conditions (data not shown). These results suggested that the complexes formed by DFF40 during apoptosis or in cell extracts could not readily enter non-denaturing gels and were larger than those of the activated recombinant protein. To test this proposal, we briefly electrophoresed an extract from non-apoptotic HL-60 cells into a native gel, treated a segment of it with recombinant caspase-3, and repeated the above electrophoretic

FIG. 5. Recombinant DFF heterodimers form homo-oligomers of DFF40 after caspase-3 activation. *A*, Western blot probed with the indicated antibodies after native pore-exclusion limit electrophoresis of purified recombinant DFF before and after treatment with recombinant caspase-3 as indicated. *B*, 1, DFF heterodimers imaged by AFM reveal a uniform particle size. *B*, 2, after treatment with recombinant caspase-3, AFM images reveal that recombinant DFF forms heterogeneous larger structures, consistent with the formation of DFF40 homo-oligomers. *B*, 3 and 4, histograms of particle volume distributions calculated from the AFM micrographs of *B*, 1 and 2.

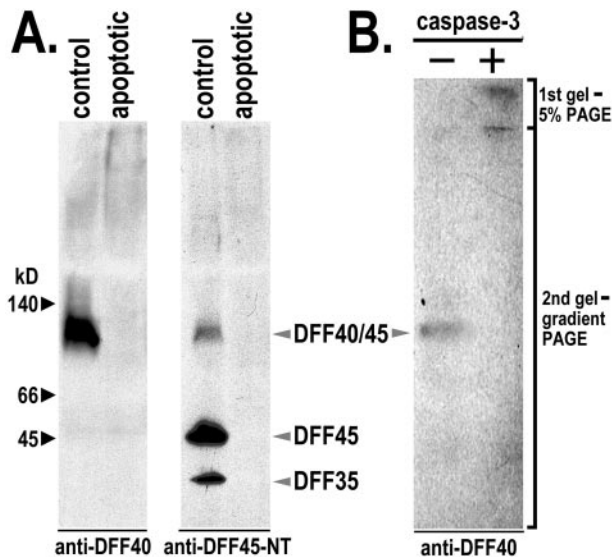
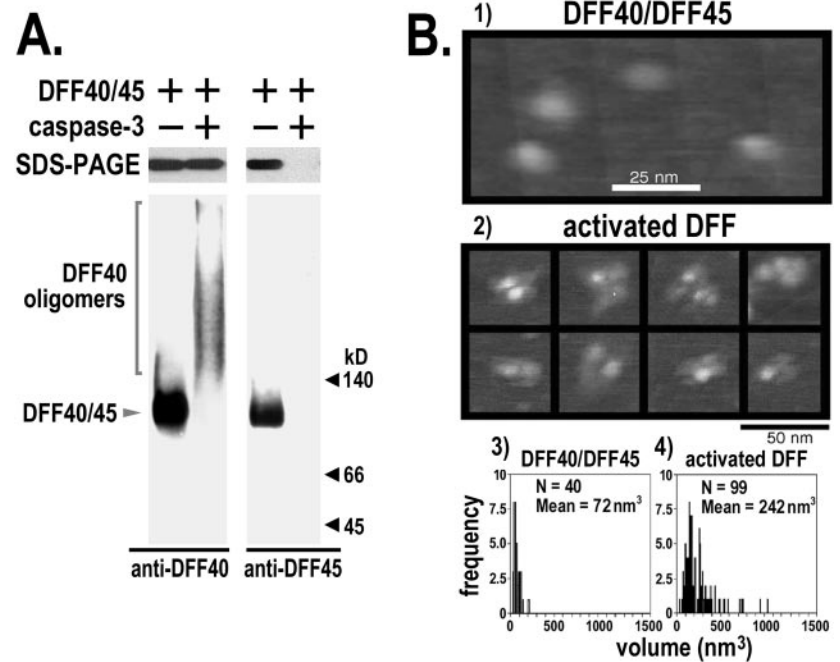


FIG. 6. Human endogenous DFF40 forms large complexes when activated with caspase-3. *A*, cytoplasmic extracts from non-apoptotic and apoptotic (etoposide-treated) HL-60 cells were separated using native pore-exclusion limit electrophoresis, and Western blots were probed with antibodies specific for DFF40 and DFF45/DFF35. *B*, cytoplasmic extracts from non-apoptotic HL60 cells were in-gel treated with caspase-3 to induce DFF40 oligomerization (first gel) and then separated using native pore-exclusion limit electrophoresis (second gel), and the resulting Western blot was probed with antibodies specific for DFF40.

analysis. As shown in Fig. 6*B*, the DFF40 complexes generated by such treatment were very large and primarily remained in the starting gel after caspase-3 treatment as compared with the control. We conclude that in apoptotic cells DFF40 forms supramolecular complexes. Whether cell proteins in addition to DFF40 *per se* participate in the formation of such complexes remains to be determined.

DFF45 Inhibits Activated DFF40 Nuclease by Binding to Homo-oligomers without Mediating Their Dissociation—Previous studies demonstrate that recombinant DFF45 is capable of inhibiting previously activated DFF40 nuclease (23, 34, 56, 59–62) and that in so doing DFF45 binds to DFF40 (61, 62). In

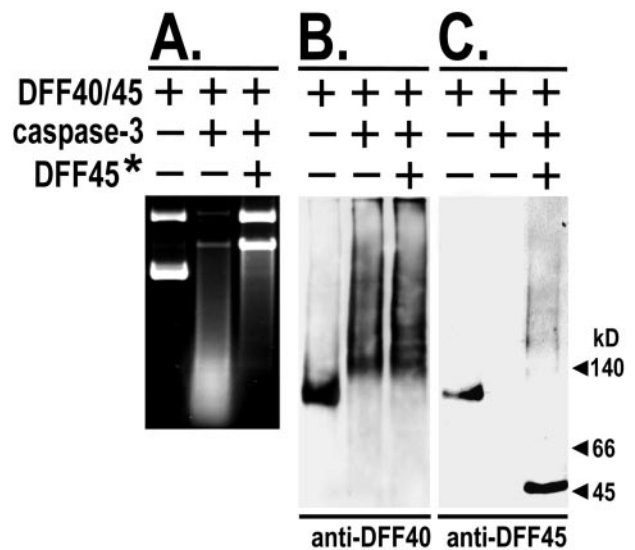


FIG. 7. Inhibition of activated DFF by DFF45 does not involve depolymerization of DFF40 and reconstitution of DFF40-DFF45 heterodimers. Intact DFF40-DFF45 heterodimer, the heterodimer treated with caspase-3, and caspase-activated DFF incubated with excess of DFF45 (DFF45*) in the presence of acetyl-Asp-Glu-Val-Asp-aldehyde were tested for nucleolytic activity (panel *A*) and subunit structures of complexes containing DFF40 (panel *B*) and DFF45 (panel *C*).

view of our observations that DFF40 forms large complexes upon nuclease activation and that intact DFF45 prevents homo-oligomer formation when associated with DFF40 as a heterodimer, one might imagine that inhibition of nuclease activity would be accompanied by the depolymerization of DFF40 multimers, with the attendant formation of DFF heterodimers. To test this hypothesis, we activated purified recombinant DFF *in vitro* by treatment with recombinant caspase-3, added acetyl-Asp-Glu-Val-Asp-aldehyde to block further caspase-3 activity, and then added purified recombinant DFF45. As shown in Fig. 7*A*, the added recombinant DFF45 successfully inhibited the activated DFF nuclease, in agreement with previous reports (23, 34, 56, 59–62). Western analysis after separation by native pore-exclusion limit electrophoresis of the inhibited

sample revealed that it still existed as large complexes like the control and had not become depolymerized by DFF45 to form heterodimers (Fig. 7B). Furthermore, such inhibited complexes possessed associated DFF45 (Fig. 7C). We conclude that DFF45 can bind to and inhibit activated DFF40 nuclease without mediating disassembly of its multimers.

DISCUSSION

For the first time we have established the native subunit structures and stoichiometries of various DFF species in several cell lines. We demonstrate that free DFF45 and free DFF35 exist as monomers, in contrast to previous reports (23, 56). We have established by AFM, native pore-exclusion limit electrophoresis, and protein cross-linking studies on recombinant or endogenous proteins that DFF45-DFF40 complexes are indeed heterodimers, in agreement with earlier predictions based on gel filtration data (21, 23, 26). We further demonstrate that both recombinant and native forms of DFF form large complexes of DFF40 upon caspase-3 activation, and in the case of the recombinant species the smallest unit of homo-oligomerization observed is a tetramer. We have found that in the cell lines studied free DFF45 and DFF35 subunits unexpectedly are in vast excess of DFF40. This indicates that the level of these inhibitors is not strictly regulated stoichiometrically by the level of DFF40 as might be inferred from previous results (55). We further demonstrate that free DFF45 can associate with homo-oligomers of caspase-3-activated DFF40 nuclease and block its activity without triggering its depolymerization. This latter result indicates that DFF45 has separate domains that can interact with DFF40 to block its homo-oligomerization and catalytic activity.

The DFF activation pathway, subunit stoichiometries, and inhibition mechanisms suggest that multiple fail-safe steps exist to protect non-apoptotic cells from the corresponding catastrophic accidents. DFF45 must be co-expressed with DFF40 and serve as a molecular chaperone to allow for the appropriate folding of DFF40 to become a potentially activable nuclease (22–24, 44). The chaperone associates with DFF40 to form a heterodimer and in turn serves as an inhibitor to block the homo-oligomerization of DFF40, which is known to accompany its nuclease activation. Several rare events could lead to the inappropriate activation of DFF40 nuclease in non-apoptotic cells, which might include accidental trace activation of caspases-3 or -7, infrequent spontaneous misfolding of DFF40 to form auto-catalytic species, or the low frequency dissociation of DFF45 inhibitor from DFF40. The vast excess of free DFF45 in the nucleus and DFF35 in the cytoplasm coupled with the abilities of these proteins to inhibit activated DFF40 homo-oligomers suggest that these species serve as fail-safe nuclear and cytoplasmic guardians for reversing the hypothetical accidental DFF40 nuclease activation pathways discussed above and protecting cells from its consequences.

It is interesting that induction of oligomerization is a reoccurring theme for the activation pathways of several apoptotic proteins, including caspases (63), Apaf1 (4), and DFF. The domains of DFF40 that mediate homo-oligomer formation remain to be physically mapped. The DFF40 N-terminal region is known to have a self-interacting CIDE (cell death-inducing DFF45-like effector) N domain (64), but based on AFM images it is clear that multiple segments of DFF40 must participate in interactions that lead to the formation of large globular-like structures. Interestingly, previous site-directed amino acid changes have been made in residues at the DFF40 catalytic site that do not block homo-oligomer formation (65, 66). Clearly, other mutations that may hypothetically block homo-oligomerization of DFF40 might be misinterpreted to also reside at its nuclease catalytic site. The complexes formed by DFF40 are

extraordinarily large, particularly those generated in apoptotic cells or caspase-3-treated normal cell extracts. Because inter-nucleosomal DNA processing occurs rapidly upon the early activation stages of DFF, perhaps these supramolecular complexes form as terminal events, participating in apoptotic body formation and the protection of neighboring cells from nuclease leakage and diffusion.

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