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, 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, 18), 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 DNFase 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-
The expression, purification, activation, and inhibition of recombinant forms of DFF—His-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 μM 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 was incubated on ice for an additional 15 min. Nuclease activity was assayed as described previously (27).

EXPERIMENTAL PROCEDURES

Expression, Purification, Activation, and Inhibition of Recombinant Forms of DFF—His-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 μM 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 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 69121407, respectively), Jurkat T cell leukemia cells (DSMC ACC 282), HeLa S cervical carcinoma cells (ATTC CCL 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 μg/ml actinomycin (Sigma). To induce apoptosis, HL60 cells were incubated for 5 hours with 34 μM 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 μM 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 × g. Cell lysates were then centrifuged for 15 min at 10,000 × g, and the resulting supernatants are referred to as cytoplasmic and nuclear extracts. In some experiments nuclei were denatured using a 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 μg 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 μM 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 × g, and the resulting supernatants were referred to as nuclear extracts.

Western Blot Analyses—Total cell protein (80 μg/lane) and the corresponding fractions of cytoplasmic extracts, nuclei and nuclear extracts, or protein from cytoplasmic extracts alone (50 μg/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 ~1 μg/ml in 50 mM HEPES, pH 7.5. Immediately before imaging, 2-μl aliquots of diluted protein samples were introduced to the surface of freshly cleaved mica and rinsed with 200 μl 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 μg of protein/lane) were separated on linear 4–24% gradient polyacrylamide gels in 0.1 mM 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 (34 kDa), 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, ~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 membrane. Membrane-immobilized proteins were probed with antibodies as described above. In one experiment (in-gel DFF40 oligomerization) proteins from cytoplasmic extracts were electrophoresed briefly (~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
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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-
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 (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. 3D) (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 DFF45 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 “hetero-dimer” molecules possess a homogeneous, 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...
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-nm$^3$ 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 $\sim 150$ nm$^3$ 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 due to its loss during cell fractionation because the same fraction contained DFF40 after Western blot 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 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. 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 by 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.](https://www.jbc.org/)

![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.](https://www.jbc.org/)

| Protein species | Cell lines studied | DFF40 | DFF45 | DFF35 |
|-----------------|--------------------|-------|-------|-------|
|                 | HL-60 | HeLa | Jurkat |
| DFF40           | 1.00$^a$ | 1.00$^a$ | 1.00$^a$ |
| DFF45           | 6.45 ± 2.22 | 6.76 ± 1.15 | 1.59 ± 0.48 |
| DFF35           | 1.42 ± 0.88 | 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.
analysis. As shown in Fig. 6B, 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 DFF 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 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. 7A, 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 homooligomerization 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 homooligomerization and catalytic activity.

The DFF activation pathway, subunit stoichiometries, and inhibition mechanisms suggest that multiple fail-safe steps in the DFF activation stages of DFF, perhaps these supramolecular complexes form as terminal events, participating in apoptotic body formation and the protection of neighboring cells from nucleosome leakage and diffusion.

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**REFERENCES**
43427–43432
46. Kawane, K., Fukuyama, H., Yoshida, H., Nagase, H., Ohsawa, Y., Uchiyama, Y., Okada, K., Iida, T., and Nagata, S. (2003) Nat. Immunol. 4, 138–144
47. McIlroy, D., Tanaka, M., Sakahira, H., Fukuyama, H., Suzuki, M., Yamamura, K., Ohsawa, Y., Uchiyama, Y., and Nagata, S. (2000) Genes Dev. 14, 549–558
48. Zhang, J., Wang, X., Bove, K. E., and Xu, M. (1999) J. Biol. Chem. 274, 37450–37454
49. Slane, J. M., Le, H. S., Vorhees, C. V., Zhang, J., and Xu, M. (2000) Brain Res. 867, 70–79
50. Cloé, J., Westwood, J. T., Becker, P. B., Wilson, S., Lambert, K., and Wu, C. (1999) Cell 63, 1085–1097
51. Widlak, P., Palvoda, O., Kumala, S., and Garrard, W. T. (2002) J. Biol. Chem. 277, 21683–21690
52. Smith, G. C. M., Cary, R. B., Lakin, N. D., Hann, B. C., Teo, S., Chen, D. J., and Jackson, S. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11134–11139
53. Mukae, N., Enari, M., Sakahira, H., Fukuda, Y., Inazawa, J., Teb, H., and Nagata, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9125–9128
54. Paine, P. L., Ausaterberry, C. F., Desjarlais, L. J., and Horvitz, S. B. (1983) J. Cell Biol. 97, 1240–1242
55. Nagase, H., Fukuyama, H., Tanaka, M., Kawane, K., and Nagata, S. (2003) Cell Death Differ. 10, 142–143
56. Sabel, S. L., Li, R., Lee, T. Y., and Abdul-Khalek, R. (1998) Biochem. Biophys. Res. Commun. 253, 1551–1558
57. Chen, D., Stetler, R. A., Cao, G., Pei, W., O’Horo, C., Yin, X.-M., and Chen, J. (2000) J. Biol. Chem. 275, 38568–38571
58. Yang, Y., Wang, H., and Erie, D. A. (2000) Methods 29, 175–187
59. Mitamura, S., Ikawa, H., Mizuno, N., Kuzoro, Y., and Itoh, H. (1998) Biochem. Biophys. Res. Commun. 243, 480–484
60. Wohrl, W., and Hacker, G. (1999) Biochem. Biophys. Res. Commun. 254, 552–558
61. McCarty, J. S., Toh, S. Y., and Li, P. (1999) Biochem. Biophys. Res. Commun. 264, 176–180
62. McCarty, J. S., Toh, S. Y., and Li, P. (1999) Biochem. Biophys. Res. Commun. 264, 181–185
63. Boatright, K. M., Renatus, M., Scott, F. L., Sperandio, S., Shin, H., Pedersen, I. M., Ricci, J. E., Edris, W. A., Sutherlin, D. P., Green, D. R., and Salvesen, G. S. (2003) Mol. Cell 11, 529–541
64. Lugovskoy, A. A., Zhou, P., Chou, J. J., McCarty, J. S., Li, P., and Wagner, G. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 747–755
65. Meiss, G., Schulz, S. R., Korn, C., Gimaudtudinow, O., and Pingoud, A. (2001) Nucleic Acids Res. 29, 3901–3909
66. Kern, C., Scholz, S. R., Gimaudtudinow, O., Pingoud, A., and Meiss, G. (2002) Nucleic Acids Res. 30, 1325–1332

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