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Inhibition of Apoptosis-Associated DNA Fragmentation Activity in Nonapoptotic Cells: The Role of DNA Fragmentation Factor-45 (DFF45/ICAD)

Steven L. Sabol,[1] Renee Li,[2] Tamara Y. Lee,[2] and Rolla Abdul-Khalek[2]

Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, National Institutes of Health, Bldg. 36, Room 1C06, Bethesda, Maryland 20892

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We have investigated the mechanism whereby nuclear DNA fragmentation activity emerging during early apoptosis is inhibited during normal cell life. In a cell-free system, cytosol fractions from diverse nonapoptotic human cell lines (Jurkat T-cell leukemia, HeLa carcinoma, SK-N-MC neuroblastoma, and WI-38 embryonic lung fibroblast) potently neutralized the nuclear DNA fragmentation activity of cytosol from apoptotic anti-Fas treated Jurkat cells. Recombinant human DNA fragmentation factor 45 kDa subunit (DFF45/ICAD), an inhibitor of the caspase-activated DNase DFF40/CAD, substituted for healthy cytosol in inhibiting DNA fragmentation. An antiserum against human DFF45 detected 44 and 34 kDa proteins (major and minor, respectively) in the cytosols but not in the nuclear or membrane fractions of various cultured human cells. Cytosols depleted of DFF45/ICAD by immunoadsorption had little or no inhibitor of nuclear DNA fragmentation activity and no caspase-activated DNA fragmentation activity. We conclude that immunoreactive DFF45/ICAD is the principal inhibitor of apoptotic DNase activity in the cytosol of healthy cells.

During apoptosis a series of orchestrated biochemical reactions results in the rapid and tidy destruction of the cell's nucleus and genome. This process includes chromatin condensation, fragmentation of DNA into large fragments (50-300 kbp) and later into oligonucleosomal ("ladder") fragments, and nuclear condensation and disintegration (1,2). These events are not required for apoptotic cell death *per se* (3) but are evidently of biologically importance, possibly to facilitate phagocytosis and eliminate potentially damaged DNA (4). As they can occur in the absence of ongoing RNA and protein biosynthesis (5), the responsible enzymatic machinery must be present but inhibited in healthy cells and activated when apoptosis is triggered through caspase protease activation (6,7). Thus, it is critical that the mechanism of nuclear apoptosis be inhibited during normal cell life.

Until recently, the identities of endonuclease(s) responsible for apoptotic DNA fragmentation and mechanisms of their inhibition have not been clear (8). One approach to identify relevant enzymes is that of cell-free systems in which postnuclear extracts from apoptotic cells elicit the morphological and biochemical changes of apoptosis in healthy nuclei (9–15). These cell-free systems appear to emulate faithfully the nuclear changes occurring in apoptotic cells. We have used a system employing cytosol from Jurkat human leukemia cells made apoptotic by Fas ligation (13,16). While characterizing the nuclear DNA fragmentation activity in apoptotic cytosol, we found that it could be substantially inhibited by cytosols from nonapoptotic cells.

While this work was in progress, other laboratories reported the isolation from healthy human and mouse cells of a novel protein complex with caspase-3-activated nuclear DNA fragmentation activity. Originally termed DNA fragmentation factor (DFF) by Liu et al. (17), it consists of two subunits, DFF45 (actual MW 36.5 kDa), which is cleaved into three 12-kDa fragments by caspase-3, and DFF40, which is not cleaved by caspases. DFF45 was found to inhibit endonuclease activity of cytosol from apoptotic Jurkat cells (18). Enari et al. (19,20) characterized a novel caspase-

[1] To whom correspondence should be addressed. Fax: 301-402-0270. E-mail: sabol@codon.nih.gov.

[2] NIH Summer Internship Program in Biomedical Research. Present addresses: R.L., Washington University School of Medicine, St. Louis, MO; T.Y.L., Northwestern University, Evanston, IL; R.A.-K., College of William and Mary, Williamsburg, VA.

Abbreviations used: PBS, phosphate-buffered saline minus calcium and magnesium ions; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Ac-DEVD-cho, acetyl-Asp-Glu-Val-Asp-aldehyde; DFF, DNA fragmentation factor; CAD, caspase-activated DNase; ICAD, inhibitor of caspase activated DNase.

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activated DNase (CAD) with a putative nuclear localization signal and strong homology to DFF40 (21–23), as well a CAD inhibitor ICAD, which is homologous to human DFF45 and has two forms (331 and 265 amino acid residues) which probably arise from alternative mRNA splicing. DFF45/ICAD binds to and inhibits DFF40/CAD, but during apoptosis it is proteolytically inactivated by effector caspases, releasing active DNase (17,19,20).

In this report we have utilized recombinant DFF45/ICAD protein and an antiserum against it to address the questions of whether DFF45/ICAD accounts for the majority of the protection against nuclear DNA fragmentation in healthy cells, whether two forms of DFF45/ICAD are found in a variety of human cells, and where DFF45/ICAD is localized within the cell.

MATERIALS AND METHODS

Cell lines. Jurkat T-cell leukemia, HeLa cervical adenocarcinoma, SK-N-MC neuroblastoma, and WI-38 embryonic lung fibroblast cells (passage 22), originally from American Type Culture Collection (Rockville, MD), were cultured in RPMI 1640 medium containing 7% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin in a 5% CO_2 incubator at 37°C.

Induction of apoptosis and preparation of cytosols. Jurkat cells (generally 8×10^6 cells/ml) in fresh medium were cultured with CH-11 anti-human Fas monoclonal IgM (100 ng/ml, Upstate Biotechnology, Lake Placid, NY). When 20-30% of cells exhibited apoptotic morphology under microscopy at 250X (usually 3-5 h), the cells were harvested and cytosols (~200,000 × g supernatant fractions) prepared usually as described by Lazebnik et al. (9). Several cytosols were alternatively prepared from apoptotic Jurkat cells mechanically homogenized in isotonic buffer without a freeze-thaw step, and they were found to have similar levels of activity. Nonapoptotic cytosols from untreated Jurkat cultures (2-4% apoptotic cells) were prepared by the same procedures. Cytosols were diluted to 15 mg/ml in Buffer A (10 mM HEPES buffer (pH 7.0), 40 mM β-glycerophosphate, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, and 2 μg/ml each of leupeptin, pepstatin A, and aprotinin) plus 1 mM dithiothreitol and stored over liquid nitrogen. Protein was determined by use of the BCA kit (Pierce) with bovine serum albumin as standard.

Preparation of ^{3}H-labeled nuclei. Six tubes, each containing $5–8 \times 10^6$ Jurkat cells, were cultured for 4-5 hours in 3 ml/tube fresh medium containing 30 μCi [methyl-^{3}H]thymidine (6.7 Ci/mmole, ICN) and 20 mM HEPES, pH 7.4. The cells were washed twice with PBS and suspended in 16 ml of cold Buffer NB (10 mM PIPES buffer (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$, 10 μM cytochalasin B, 2 μg/ml each of leupeptin, pepstatin A, and aprotinin). After incubation on ice for 15 minutes, they were lysed in a Dounce homogenizer by 10 strokes of a loose-fitting pestle. KCl was added to a concentration of 0.11 M, and 4 ml of lysate was layered onto a 3-ml cushion of 25% sucrose in Buffer NB in each of four tubes. The tubes were centrifuged for 20 min at 1000 × g, and nuclei were washed twice by centrifugation and resuspended in Buffer A plus 0.25 M sucrose. The nuclei were stored at 2-4°C and were found to be usable in the DNA fragmentation assay for at least 1 month.

Cell-free assay of nuclear DNA fragmentation. Reaction mixtures were prepared in duplicate U-bottom microtiter wells. Approximately 25,000 nuclei (8000-25,000 cpm) from [^{3}H]thymidine-labeled healthy Jurkat cells were incubated with cytosol (5–80 μg protein) and other components in 25-μl reaction mixtures containing 10 mM HEPES (pH 7.0), 40 mM β-glycerophosphate (disodium salt), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 2 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine phosphate, 1 mM dithiothreitol, and 5 μM each of pepstatin A, leupeptin, and aprotinin. (KCl could replace NaCl, and inorganic phosphate or α-glycerophosphate could replace β-glycerophosphate.) Assays of the inhibition of nuclear DNA fragmentation contained 10 μM zVAD-fmk (Enzyme Systems Products, Livermore CA). After incubation at 32–35°C (the optimal temperature range) for 60–90 min, the reactions were rapidly filtered (24 wells at a time) with water by a PHD Cell Harvester (Cambridge Technology) onto glass fiber filters, which retain unfragmented chromatin but not fragments of chromatin or DNA (24). A reduction of filter-bound ^{3}H radioactivity is termed here "nuclear DNA fragmentation activity." The assay was validated by gel electrophoretic analyses of nuclear DNA. The appearance of apoptotic morphological changes in the nuclei incubated with cytosol from apoptotic but not healthy Jurkat cells was confirmed by fluorescence microscopy after nuclear staining with Hoechst 33342 dye.

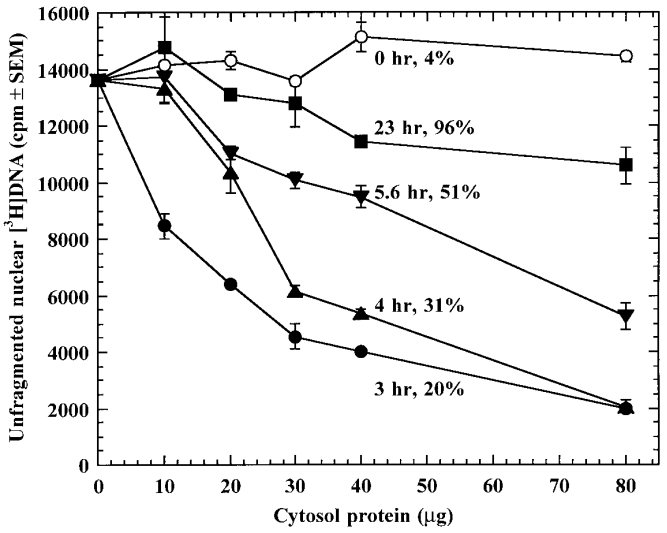

FIG. 1. Nuclear DNA fragmentation elicited in the cell-free system by cytosol fractions from Jurkat cells harvested after the indicated durations of anti-Fas treatment. The percents of cells exhibiting morphological features of apoptosis under 250× magnification at each time of harvest are indicated. In another experiment, cytosol from a culture with 10% of cells exhibiting apoptosis had no DNA fragmentation activity (not shown).

Preparation of His_6-tagged DFF45 protein. A human DFF45 cDNA clone was kindly donated by Dr. Xiaodong Wang (University of Texas, Dallas). The coding region was amplified by PCR and subcloned into the NdeI and XhoI sites of pET-15b vector (Novagen), as described (17). The expected sequence was confirmed by automated DNA sequencing. A 200 ml culture of *E. coli* strain BL21(DE3) containing this construct was induced with 1 mM isopropyl-β-D-thiogalactoside, and His_6-tagged protein (44 kDa) was purified on a nickel-agarose column (His-Bind Kit, Novagen) to approximately 97% purity (by SDS-PAGE). Eluted protein (5.8 mg) was concentrated by a Centricon-10 membrane (Amicon), dialyzed vs. PBS, and clarified by microcentrifugation.

Preparation of antiserum against DFF45. Purified undenatured His_6-DFF45 was used for immunization of two female New Zealand white rabbits by Spring Valley Laboratories (Woodbine, MD). Primary immunization and three boosts (150 μg/injection) occurred over 2 months. The antiserum with the greater titer and specificity on Western blots (from rabbit "Daffne") was chosen for use. Antiserum Daffne stained human and monkey but not mouse or rat DFF45/ICAD.

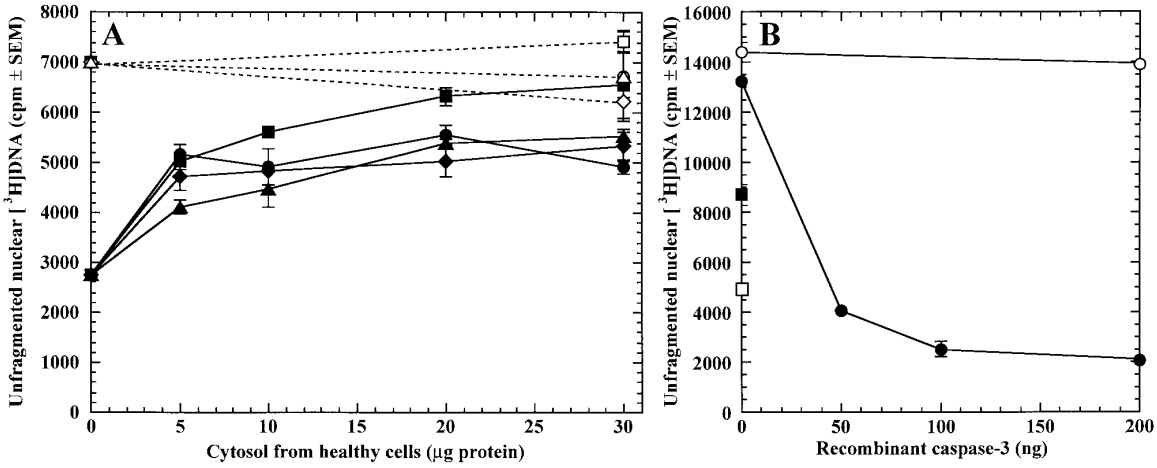

FIG. 2. Effect of cytosol fractions from nonapoptotic human cells on the nuclear DNA fragmentation activity of apoptotic Jurkat cytosol in the cell-free system. A. Reaction mixtures contained either 24 μg apoptotic Jurkat cytosol plus indicated amounts of cytosols from healthy Jurkat (●), HeLa (■), SK-N-MC (◆), or WI-38 (▲) cells; or the healthy cytosols alone (corresponding open symbols). B. Effect of indicated amounts of recombinant caspase-3 (Pharmingen), added to the cell-free system without preincubation, on the inhibition of nuclear DNA fragmentation elicited by 15 μg cytosol protein from nonapoptotic Jurkat cells (●). For comparison, DNA fragmentation values in the presence of no cytosol (○), 12 μg apoptotic Jurkat cytosol alone (□), and nonapoptotic cytosol (15 μg) plus apoptotic cytosol (12 μg) (■) are shown.

Immunoblotting. SDS-PAGE was performed on precast 8-16% gradient gels (Novex, San Diego, CA). Proteins were transferred to Immobilon-P membranes (Millipore) using a Novex transfer apparatus and procedures recommended by Novex. Blots were blocked with 10% non-fat milk, incubated with antiserum Daffne (1/10,000 final dilution), and subsequently with horseradish peroxidase-conjugated goat anti-rabbit IgG (1/5000 dilution of 1 mg/ml stock, New England Nuclear), in PBS containing 10 mg/ml bovine serum albumin and 0.2% Tween-20. Labeled bands were detected by chemiluminescence through the use of the ECL kit (Amersham) or the Renaissance kit (New England Nuclear) and exposure of Hyperfilm-ECL (Amersham).

Subcellular fractionation. Healthy Jurkat cells (2×10^8) were Dounce-homogenized as described for the preparation of nuclei. Nuclei were purified as described above. The post-nuclear supernatant was centrifuged at 10,000 × g for 30 min and the pellet (crude mitochondrial fraction) was washed in Buffer A by two further similar centrifugations, followed by a centrifugation at 500 × g to remove traces of nuclei. The post-mitochrondrial supernatant was centrifuged at 200,000 × g for 1 h, and the pellet (microsomal fraction) was washed twice in Buffer A by two similar ultracentrifugations, followed by a centrifugation at 500 × g to remove aggregates. HeLa cells were fractionated similarly, except that a single membrane/organelle fraction was obtained by centrifuging the post-nuclear supernatant at 200,000 × g and washing the pellet twice in Buffer A by similar ultracentrifugations. The nuclear fraction was sonicated to reduce the viscosity prior to SDS-PAGE.

Immunoadsorption. Protein A-Sepharose CL-4B (12.5 mg, Pharmacia) which had been previously washed and equilibrated in 300 μl Buffer A was incubated with 100 μl of either preimmune or immune serum from rabbit Daffne at 4°C for 2 h with continuous inversion on a rotator. The beads were then washed six times by centrifugation in 1 ml Buffer A. To each pellet of beads was added 100 μl healthy Jurkat cytosol (15 mg/ml). The mixtures were continuously inverted at 4°C for 3.5 h, after which they were centrifuged in a microfuge and the supernatant fractions recovered.

RESULTS AND DISCUSSION

Nuclear DNA fragmentation activity. Ligation of Fas on Jurkat cells with anti-Fas IgM antibodies triggered rapid apoptosis and generated nuclear DNA fragmentation activity in the cytosol fraction, as reported previously (12,13,15). Because previous reports did not specify the conditions yielding optimal activity, we determined that peak nuclear DNA fragmentation activity in the fractionated cytosol was reached when only approximately 20% of the cells exhibited morphological apoptosis prior to harvest (Fig. 1). More advanced apoptosis was associated with reduced activity, suggesting that the responsible endonuclease activity appears early but is degraded as apoptosis progresses. General endonuclease activity in the same cytosols, measured with plasmid DNA rather than nuclei, dramatically rose and fell in parallel with the nuclear DNA fragmentation activity (not shown).

The nuclear DNA fragmentation activity was resistant to high concentrations (100 μM) of the broad-spectrum caspase inhibitor z-VAD-fmk and the caspase-3 inhibitor Ac-DEVD-cho, both of which in our hands blocked anti-Fas-induced apoptosis in cultured Jurkat cells (not shown). Thus, DNA fragmentation activity is downstream of caspase action, although generated by caspase action prior to cell harvest. The activity was sensitive to thiol reagents (e.g., N-ethylmaleimide, IC_{50} 0.4 mM) and to several serine protease inhibitors (tosyl-phenylalanyl-chloromethylketone, IC_{50} 100 μM; 3,4,-dichloroisocoumarin, IC_{50} 10 μM), but not to other representative protease inhibitors (not shown). Similar inhibition profiles were obtained for the degradation of plasmid DNA and for nuclear morphological changes elicited by apoptotic cytosol (not shown). These results suggest that the same enzyme may be responsible for the three activities. Its reactivity with some serine protease inhibitors does not necessarily

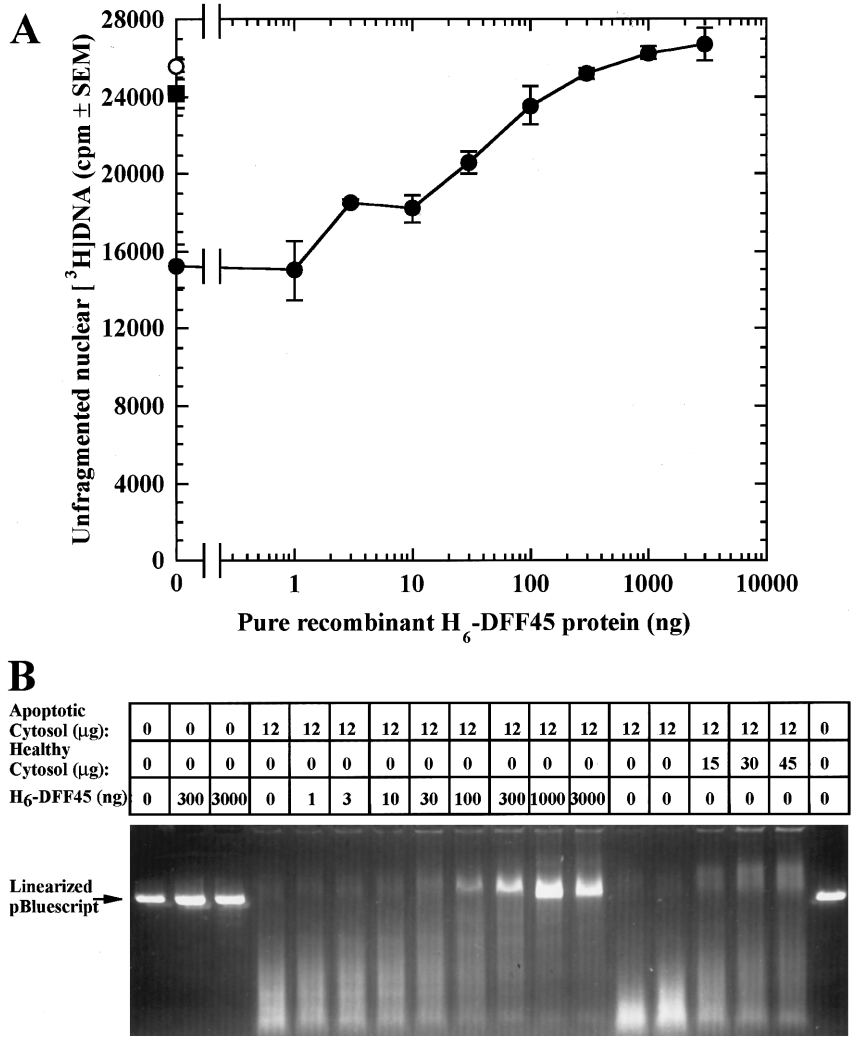

FIG. 3. Inhibition by pure recombinant His_6-DFF45 of endonuclease activities of apoptotic Jurkat cytosol. A. Nuclear DNA fragmentation in the cell-free system was assayed in the presence of 12 μg apoptotic cytosol, 10 μM zVAD-fmk, and indicated amounts of His-tagged DFF45 (●), or 15 μg healthy Jurkat cytosol (■). The level of unfragmented DNA in the absence of any cytosol (○) is also shown. B. Endonucleolytic cleavage of 0.4 μg EcoRI-linearized pBluescript II by 12 μg apoptotic cytosol was performed in incubations (20 μl, 33°C, 60 min) containing the indicated components and 15 μg/ml bovine serum albumin in TCME buffer (20 mM TrisHCl (pH 8.0), 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 mM EGTA) plus 10 μM z-VAD-fmk. DNA was analyzed by 1% agarose gel electrophoresis and staining with ethidium bromide.

implicate a protease but may reflect the known reactivity of these inhibitors at moderate-high concentrations toward a critical thiol group of an endonuclease.

Inhibitor of nuclear DNA fragmentation. Cytosol fractions from several nonapoptotic cultured human cells potently neutralized the nuclear DNA fragmentation activity of apoptotic Jurkat cytosol in the cell-free system (Fig. 2A). In a panel of four cell lines of different lineages, HeLa cytosol appeared to provide the most complete inhibition, but HeLa, Jurkat, and SK-N-MC had similar levels of inhibition at submaximally effective concentrations. Cytosol from WI-38 cells, a nonmalignant and mortal line, was less inhibitory at submaximally effective concentrations than that of the other other lines (Fig. 2A). Inhibitory activity was found also at varying levels in cytosols from several mouse tissues and rodent cell lines but not with bovine serum albumin (not shown). The inhibitor was partially heat stable; after heating to 100°C for 15 min and centrifugation of precipitated protein, approximately 50% of the original activity remained in the soluble fraction (not shown).

Treatment of nonapoptotic Jurkat cytosol with recombinant caspase-3 not only eliminated the inhibitor but generated new nuclear DNA fragmentation activity (Fig. 2B), as shown previously (18,25,26).

Membrane fractions (crude mitochondrial and microsomal fractions) from Jurkat cells, both healthy and apoptotic, also potently inhibited the nuclear DNA fragmentation activity of apoptotic cytosol (not shown).

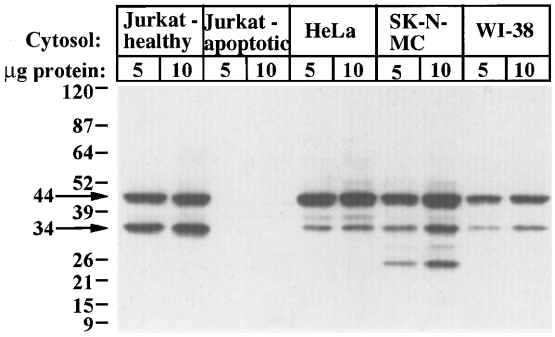

FIG. 4. DFF45 immunoreactivity in cytosol fractions from cultured human cell lines. Cells were healthy except in the case of apoptotic anti-Fas-treated Jurkat cells. Indicated amounts of protein were analyzed by Western blotting. The positions of standards (BenchMark Prestained Protein Ladder, GIBCO-BRL) are shown at left. The blot was kept in the dark for 30 min, after which the film was exposed for 10 sec. Assigned M_r values for DFF45 bands are averages from multiple experiments.

However, inhibition by these fractions was not affected by caspase-3 pretreatment and thus may not be regulated by apoptosis.

Anti-DNase activity of recombinant DFF45/ICAD. To determine whether DFF45/ICAD can entirely substitute for crude cytosol in inhibiting nuclear DNA fragmentation, we prepared purified N-terminally His_6-tagged-DFF45 protein. When added to the cell-free system this protein inhibited nuclear DNA fragmentation by apoptotic Jurkat cytosol (Fig. 3A). Half-maximal and maximal inhibitions were obtained with approximately 30 and 1000 ng protein, respectively. DFF45/ICAD also inhibited the general endonuclease activity of apoptotic cytosol, assayed by plasmid degradation, at similar concentrations (Fig. 3B). These results qualitatively confirm previous reports (18,19). Healthy Jurkat cytosol also exhibited anti-DNase activity toward the plasmid substrate, but it was less effective than pure DFF45/ICAD.

Immunoblot analysis of DFF45/ICAD in human cell lines and subcellular fractions. To study the expression of DFF45/ICAD, we generated a sensitive and specific rabbit antiserum against human DFF45. The antiserum labeled two bands of 44 ± 1 and 34 ± 2 kDa (relative to prestained standards) on blots of cytosol protein from human Jurkat, HeLa, SK-N-MC, and WI-38 cells (Fig. 4). Thus, human cells, like mouse cells (19), consistently and perhaps universally express two forms of DFF45/ICAD. The longer species was much more abundant than the shorter one in HeLa, SK-N-MC, and WI-38 cytosols, but only slightly more abundant in Jurkat cytosol, suggesting cell-specific variations in the extent of alternative RNA splicing. The relative intensities of DFF45 in the four cell lines approximately paralleled the relative abundances of the nuclear DNA fragmentation inhibitory activity (Fig. 3A, 5 μg points). For SK-N-MC cytosol, an additional band at 24 kDa may be a fragment produced by a single caspase-3 cut (17). Cytosol from apoptotic Jurkat cells (Fig. 4), as well as caspase-3-treated cytosol from healthy cells (not shown), exhibited only faint immunoreactivity in the 10-12 kDa range, reflecting complete caspase-3 cleavage to fragments which either are less immunoreactive or lost from the blot during electrophoresis.

In Western blots of subcellular fractions from Jurkat and HeLa cells, the 44 kDa and 34 kDa DFF45 immunoreactive species were detected exclusively in the cytosol and not in purified nuclei or the washed mitochondrial membrane and microsomal fractions (Fig. 5). This result is at odds with two recent reports that demonstrate histochemically a nuclear localization for transfected DFF45 (which lacks a typical nuclear localization signal) in a variety of healthy cell types (22,27). Our results show absolutely no DFF45 immunoreactivity at 44 or 34 kDa, even in overexposed films, in Jurkat and HeLa nuclei which have been purified (in

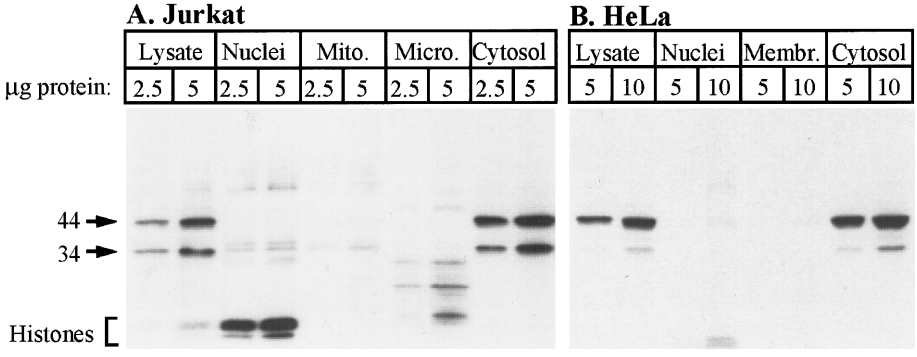

FIG. 5. Subcellular distribution of DFF45 immunoreactivity in healthy Jurkat (A) and HeLa (B) cells. Cells were lysed in hypotonic medium with a Dounce homogenizer and fractionated as described in Materials and Methods. Abbreviations: Mito., washed crude mitochondrial fraction; Micro., washed microsomal fraction; Membr., unseparated washed crude mitochondrial and microsomal fractions. Film exposure times for panels A and B were both 5 sec. Staining of nuclear bands coinciding with presumed histone bands in the 12-15 kDa range (strongly visualized with Ponceau S dye) was of variable intensity in repeated experiments and thus is probably nonspecific.

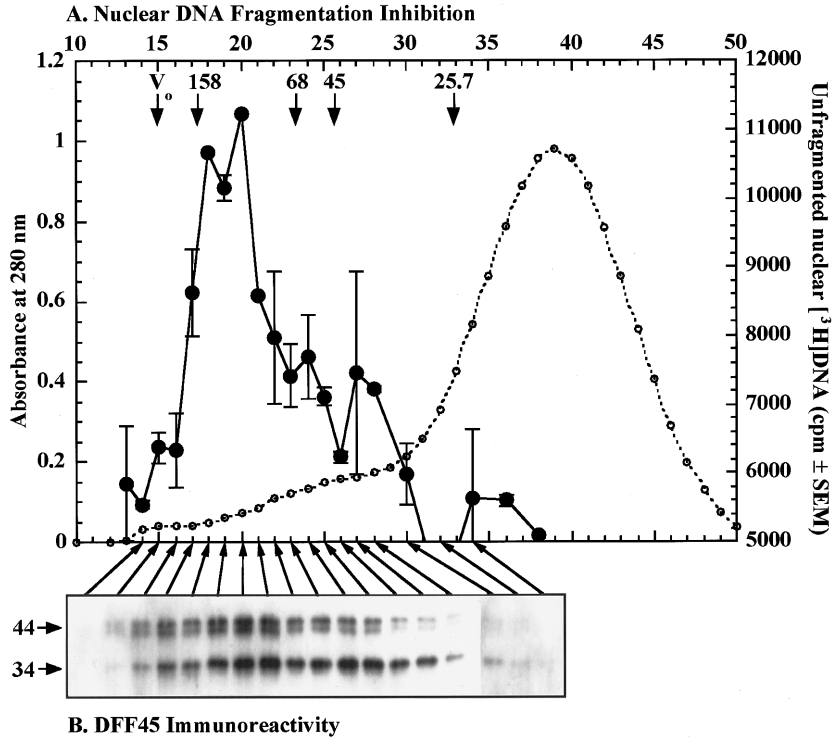

FIG. 6. Sephadex G-200 chromatography of heat-soluble protein from cytosol from healthy Jurkat cells and assays of inhibition of nuclear DNA fragmentation and DFF45 immunoreactivity. A 0.30 ml aliquot of cytosol containing 5.7 mg protein was heated to 100°C for 15 min, cooled, and centrifuged at 14,000 × g for 30 min. Part of the supernatant (0.20 ml, ~ 0.7 mg protein) was fractionated on a G-200 column (18 × 0.65 cm, Pharmacia) in Buffer A at 4°C. A. Fractions of 0.20 ml were collected, and the absorbance at 280 nm was determined (dotted line). Aliquots of 10 μl were assayed for the inhibition of nuclear DNA fragmentation (●) in the presence of 22.5 μg apoptotic cytosol. The amounts of unfragmented DNA in the absence of cytosol and in the presence of apoptotic cytosol alone were 15,600 and 4920 cpm, respectively (not plotted). B. Western blot depicting DFF45 immunoreactivity in each column fraction (8 μl). Film exposure time was 5 sec. The appearance of the 44 kDa band as a doublet reflects a change due to storage at 4°C for 5 days, because a preliminary immunoblot performed immediately after the column run exhibited the usual single band.

the presence of cytochalasin B) from homogenized cells by centrifugation through a sucrose cushion.

Gel filtration of the inhibitor of nuclear DNA fragmentation. To estimate the molecular mass of the inhibitor and relate it to that of DFF45/ ICAD, we chromatographed on Superose-12 FPLC and Sephadex G-200 either untreated healthy cytosol or the soluble fraction after heating it to 100°C for 15 min. As shown in Fig. 6 for the heat-soluble fraction run on G-200, the inhibitor chromatographed as a major peak of an apparent M_r 120-160 kDa, along with poorly resolved smaller peaks or shoulders of 40-70 kDa. For both Superose-12 and G-200, the profiles for inhibitor in unheated cytosol were essentially identical to those of the soluble fraction after boiling (not shown). The size of the major peak is in contrast to that (75 kDa) reported for partially purified ICAD (19). DFF45 immunoreactivity (Fig. 6B) was localized to the column fractions having inhibitory activity, with the peak fractions of 44 kDa immunoreactivity consistent with the peak fractions (120-160 kDa) of inhibitory activity, within the experimental error of the two assays. These data suggest that in the crude cytosol DFF45/ICAD chains associate with either themselves as trimers or tetramers or with other polypeptide chains, and that these associations exist even after heating to 100°C. Pure recombinant His_6-DFF45 also chromatographed on a G-200 column as an apparent series of multimers (not shown).

Removal of nuclear DNA fragmentation inhibitor and caspase-3-activated DNase by DFF45/ICAD immunodepletion. To determine whether the cytosolic inhibitor of nuclear DNA fragmentation is mostly or entirely DFF45/ICAD, healthy Jurkat and HeLa cytosols were depleted of immunoreactive DFF45/ICAD by adsorption with anti-DFF45 IgG. Cytosol that was completely immunodepleted of DFF45, as confirmed by Western blotting (Fig. 7A), had lost most or all (≥ 80%) of the inhibitory activity, while cytosol that was sham-immunodepleted with preimmune IgG from the same rabbit fully retained the inhibitory activity (Fig. 7B). These results supports the hypothesis that the cytosolic inhibitor is mostly or entirely DFF45/ICAD (long and short forms). It is reasonable to assume that the

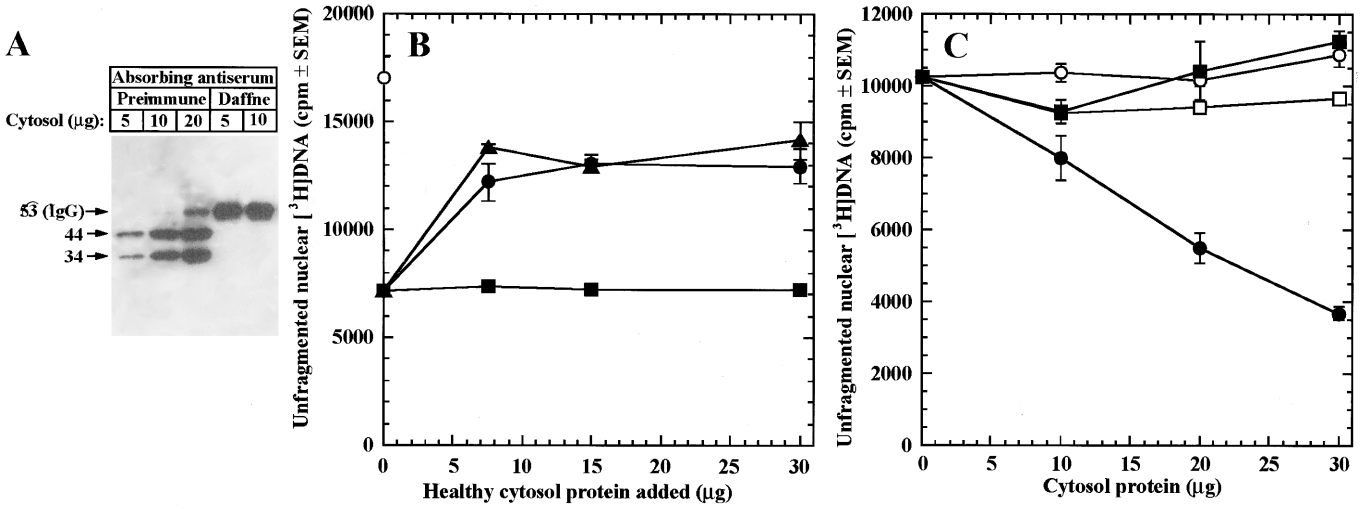

FIG. 7. Activities of nonapoptotic Jurkat cytosol immunodepleted of DFF45. A. Western blot analysis of the extent of DFF45 removal. The 53 kDa band is IgG heavy-chain molecules dissociated from the Protein A-Sepharose. B. Inhibition of nuclear DNA fragmentation elicited by apoptotic Jurkat cytosol (15 μg) assayed in the presence of indicated amounts of nonapoptotic cytosol that was untreated (▲) or preadsorbed with preimmune IgG (●) or anti-DFF45 IgG (■). The level of unfragmented DNA in the absence of cytosol is shown (○). In replications of this experiment, the removal of inhibitory activity was ≥ 80% at the lowest concentration of cytosol tested (7.5 μg). Identical results were obtained with HeLa cytosol (not shown). C. Nuclear DNA fragmentation activity of originally nonapoptotic Jurkat cytosol that was either preadsorbed with preimmune IgG and then assayed in the absence (○) or presence (●) of 100 ng caspase-3, or preadsorbed with anti-DFF45 IgG and then assayed in the absence (□) or presence (■) of 100 ng caspase-3.

inhibitor measured in the nuclear DNA fragmentation assay consists of free or excess DFF45/ICAD not already complexed with endogenous DFF40/CAD.

Healthy cytosol immunodepleted of DFF45/ICAD and then treated with recombinant caspase-3 was found to be also totally depleted of caspase-3-activated nuclear DNA fragmentation activity, while sham-immunodepleted cytosol retained this activity (Fig. 7C). This result indicates that apparently all of the latent nuclear DNA fragmentation activity in healthy cytosol is bound to DFF45/ICAD immunoreactive protein(s). This is consistent with the model in which the principal apoptotic nuclease is DFF40/CAD, which in healthy cells is bound in an inactive form to DFF45/ICAD.

CONCLUSIONS

Our findings support a model (18,19) in which DFF45/ICAD is an inhibitor of apoptotic DNA fragmentation. Furthermore we conclude that this protein (existing as long and short forms) is the principal shield whereby cells during normal life protect their genome from the constitutively expressed caspase-activated DNase. Our data also suggest that the level of DFF45/ICAD expression, possibly in conjunction with DFF40/CAD expression, is regulated according to cell type, perhaps in direct relation to "readiness" to undergo apoptosis. This is suggested by our finding that Jurkat and HeLa cells, which can readily undergo apoptosis, have higher levels of DFF45/ICAD immunoreactivity than that of WI-38 cells, which as fibroblasts would be expected to undergo apoptosis less readily. Appropriate tools are now available to test this hypothesis rigorously.

ACKNOWLEDGMENTS

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