Apoptotic Release of Histones from Nucleosomes*

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Chromatin structure is influenced by histone modification, and this may help direct chromatin behavior to facilitate transcription, DNA replication, and DNA repair. Chromatin condensation and DNA fragmentation are the classic nuclear features but remain poorly characterized. It is highly probable that nucleosomal structure must be altered to allow these features to become apparent, but data to support this construct are lacking. We report here that in response to apoptotic signals from a death receptor (CD95 and tumor necrosis factor-α) or mitochondrial (stauorosporine) apoptotic stimulus, the core nucleosomal histones H2A, H2B, H3, and H4 become separated from DNA during apoptosis in Jurkat and HeLa cells and are consequently detectable in the cell lysate prepared using a non-ionic detergent. The timing of this histone release from DNA correlates well with the progression of apoptosis. We also show expression of a caspase cleavage-resistant form of ICAD (ICAD-DM) in Jurkat and HeLa cells abolished DNA fragmentation and also dramatically reduced histone release in apoptotic cells. However, we demonstrate that apoptotic histone release is not an inevitable consequence of CAD/DFF-40-mediated DNA destruction as DNA fragmentation but not histone release occurs efficiently in tumor necrosis factor-α- and etoposide-treated NIH3T3 cells. Furthermore, in an in vitro apoptotic assay, incubation of apoptotic Jurkat cellular extract with non-apoptotic Jurkat nuclei led to nuclear DNA fragmentation without obvious histone release. Taken together, these data demonstrate that CAD/DFF-40 functions indirectly in mediating nucleosomal destruction during apoptosis.

With few exceptions, apoptotic cell death is executed by a group of cysteine proteases called caspases. By cleavage of key cellular protein substrates, caspase activity ultimately induces the typical apoptotic features, such as membrane blebbing and DNA fragmentation. Classic apoptotic DNA fragmentation is mediated largely by CAD1 (caspase-activated DNase/DFF-40 (DNA fragmentation factor) and may also be induced by other apoptotic nucleases, including endonuclease G (3–5). CAD or DFF is composed of two subunits, a catalytic subunit CAD/DFF-40 and an inhibitory subunit ICAD (inhibitor of CAD)/DFF-45 (6–8). Normally the CAD/ICAD dimer exists without DNase activity. Proteolytic processing of two sites on ICAD/DFF-45 by caspase-7 and caspase-3 in vivo results in the release of ICAD/DFF-45 from the CAD/DFF-40 subunit (9, 10). CAD/DFF-40 then matures into an active multiple complex (11), which cleaves chromatin DNA into typical apoptotic fragments. In addition to acting as an inhibitory subunit, ICAD/DFF-45 also functions in stabilization of the CAD/DFF-40 subunit, and CAD/DFF-40 has been shown to be unstable in its absence (11, 12). A large body of evidence indicates that CAD/DFF-40 is the major apoptotic nuclease (6, 13). In support of this construct, in vitro depletion of CAD activity abolished DNA fragmentation (3), whereas expression of ICAD/DFF-45 or a non-cleavable form of ICAD (ICAD-DM) prevented DNA fragmentation in apoptotic Jurkat and HeLa cells (4, 6). Conversely, reintroduction of caspase-3 into caspase-3 mutant MCF-7 cells activated CAD/DFF-40 and led to DNA fragmentation in apoptotic MCF-7 cells (14). Finally, inactivation of ICAD/DFF-45 by gene targeting in transgenic mice significantly, if not completely, inhibited DNA fragmentation in mouse thymocytes (15).

In mammalian cells, DNA is organized into nucleosomes by wrapping around a histone octamer, containing two copies of each of the core histones H2A, H2B, H3, and H4, whereas histone H1 is located outside the nucleosome on the internucleosomal DNA (3). Histone modification can influence chromatin structures to facilitate transcription, DNA replication, and DNA repair (16–19). It has been reported that CAD/DFF-40 activity prefers chromosomal DNA as the substrate because the presence of histones increases CAD/DFF-40 activity severalfold (12, 20). In this regard, it has been postulated that apoptotic chromatin condensation and DNA fragmentation might also be a consequence of histone modification. Phosphorylations of histone H2A, H2B, and H3 and also dephosphorylation of histone H1 have been observed under some circumstances in apoptosis.

Apoptosis is a fundamental process of eukaryotic multicellular organisms (1, 2). The change from life to death is the single most momentous event for any living thing, from single cells upwards. Consequently, it is not surprising that dramatic biochemical and morphological changes occur in apoptotic cells, both outside and inside of the nucleus. In the nucleus, the classic apoptotic features are chromatin condensation and DNA fragmentation.

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1 The abbreviations used are: CAD, caspase-activated DNase; ICAD, inhibitor of caspase-activated DNase; ICAD-DM, caspase cleavage-resistant form of ICAD; DFF, DNA fragmentation factor; ZVAD, N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; TNFα, tumor necrosis factor-α; PARP, poly(ADP-ribose) polymerase; CHX, cycloheximide; STS, stauorosporine; ETP, etoposide; FAS, fatty-acid synthase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; CPP, caspase-3.
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(21–24). However, how histone phosphorylation, essential for mitotic chromatin condensation, might function in promoting apoptotic chromatin conformations and DNA fragmentation is unclear. On the other hand, it has been suggested recently that apoptotic chromatin changes might be completely different from mitotic changes (25), and mitotic-style histone modifications may have no role in apoptotic chromatin changes.

In this study, we have found that core nucleosomal histones separate from chromatin in apoptotic cells, but that this is not simply a by-product of DNA fragmentation. This event is indirectly related to CAD/DFF-40 in the sense that CAD/DFF-40 is required but is insufficient for the apoptotic histone release.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Materials—HeLa, MCF-7, WEHI-3, and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. WEHI-3 cell cultures were supplemented with 2 μM glutamine and 0.05 μM β-mercaptoethanol. Jurkat cells were cultured in RPMI 1640 containing 10% fetal calf serum. The retroviral packaging line was AmphiPack-293 (CLONTECH). ICAD and ICAD-DM were kindly provided by Dr. Shiogakuzi Nagata (Osaka University Medical School, Osaka, Japan). Jurkat cells were infected in the retroviral packaging line with a retrovirus that was amplified by reverse transcriptase-polymerase chain reaction using Jurkat RNA and inserted into pBabe, and the DNA sequence was confirmed as described previously (14).

N-Benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone (ZVAD), a pan-caspase inhibitor, was from Enzyme System Products. Staurosporine (STS), etoposide (ETOP), and cycloheximide (CHX) were from Sigma, whereas tumor necrosis factor-α (TNF-α) was from Invitrogen.

Induction of Apoptosis—Jurkat cells were treated with an anti-FAS antibody, CH-11 (either 50 ng/ml or 100 ng/ml, Kamiya Biomedical), or with STS at 1 μM for times specified in the relevant figure legends. Apoptotic cell death of HeLa, MCF-7, WEHI-3, and NIH3T3 lines was initiated by 1 μM STS or 100 ng/ml TNF-α plus CHX at 10 μg/ml for the times specified in the relevant figure legends. Cells were also treated with ETOP at 10 μM for 24 h to initiate apoptotic cell death. Apoptosis was determined by terminal deoxynucleotidyltransferase-mediated dUTP end nick labeling (TUNEL) assay or WST-1 cell viiability assay.

TUNEL and WST-1—TUNEL assays were performed using a commercial kit (Roche Molecular Biochemicals) as previously published (26). Jurkat cell extract was purchased from Roche Molecular Biochemicals. 10 μl of the WST-1 reagent was added to 100 μl of cultured medium in 96-well plates and incubated in a tissue culture incubator for 2 h. Viable cells were then determined by a microplate reader at 490 nm as a reference wavelength.

Western Blotting—After induction of apoptosis, cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, and 10 μg/ml aprotinin on ice for 20 min. Cell lysate was harvested after centrifugation at 20,000 g for 10 min at 4°C. The clear cytosolic fraction was carefully harvested without disturbing the nuclear pellet and adjusted to 50 mM NaCl. Aliquots of this were frozen at −80°C for later use. Nuclei were prepared from normal Jurkat cells. Cells were harvested and suspended in buffer A, followed by swelling on ice for 20 min. Cells were disrupted by gentle homogenization with a Dounce homogenizer. Liberated nuclei were then layered over a cushion of 30% sucrose in buffer B (10 mM HEPES, pH 7.4, 80 mM KC1, 20 mM NaCl, 5 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM PMSF) and centrifuged at 800 × g for 10 min and resuspended in nuclear storage buffer (10 mM HEPES, pH 7.4, 80 mM KC1, 20 mM NaCl, 5 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM PMSF, and 50% glycerol) at 2 × 106 nuclei/mL. An in vitro apoptotic assay was carried out by incubating different amounts of cell extract with 5 × 105 nuclei for 2 h at 37°C. Nuclei were then recovered by a low-speed centrifugation. Nuclear DNA was subsequently isolated and analyzed on a 1.5% agarose gel for DNA fragmentation as we have published previously (28). In vitro apoptotic histone release was carried out by analyzing both the cytosolic and nuclear fractions after the in vitro apoptotic assay. Harvested nuclei were lysed in a 1% Triton X-100 lysis buffer. The cytosolic and Triton X-100-soluble nuclear fractions were separated by 15% SDS-PAGE and Coomassie Blue-stained for histone release.

RESULTS

Small Molecular Weight Proteins Appear in Massive Amounts in Some Cell Lines after Exposure to Apoptotic Stimulation—Initially, we were interested in the fact that apoptosis is accompanied by dramatic changes in both lipid membranes (such as the loss of membrane asymmetry) and in nucleic acids (such as DNA fragmentation) but not in proteins, although this is consistent with the fact that caspases only selectively proteolyze limited numbers of key proteins. In the course of pursuing this, we treated Jurkat cells with STS at 1 μM for an 8-h time course, and cell lysate prepared in a lysis buffer containing 1% Triton X-100 was analyzed on Coomassie Blue-stained 15% SDS-PAGE. Surprisingly, four small polypeptides ranging from 10 to 15 kDa molecular mass were specifically stained in apoptotic cells (Fig. 1B). Kinetically, production of these peptides matched well with the progression of STS-induced apoptosis, beginning at 2 h and progressing through to 8 h of treatment, as indicated by caspase-mediated PARP cleavage and by WST-1 assay (Fig. 1, A and C). A broad range caspase inhibitor, ZVAD, at 50 μM inhibited caspase activity (Fig. 1A) and apoptosis (Fig. 1C) as well as preventing the production of Coomassie Blue-stained apoptotic proteins (Fig. 1B), confirming that generation of these polypeptides is apoptosis-specific and requires caspase activity. Similar results were obtained in a death receptor-initiated apoptosis (Fig. 2). An anti-FAS antibody, CH-11, at 50 μg/ml was found to induce apoptosis with ZVAD inhibition, apoptosis, and appearance of the four apoptotic proteins (Fig. 2B), which were again prevented in the presence of ZVAD (Fig. 2B). Besides STS, other mitochondrial apoptotic stimuli, like CHX (data not shown) and ETOP (Fig. 3B) were also able to induce the appearance of these apoptotic proteins in Jurkat cells. Taken together, these results demonstrate that appear-

Generation of Retrovirus and Infection of Cells—High titer retrovirus was generated by transfection of a retrovirus packaging line, AmphiPack-293, with pBabe-based constructs as we have described previously (28). Briefly, 5 × 107 AmphiPack-293 cells were seeded 1 day before transfection with 20 μg of plasmid DNA by the calcium phosphate method. The virus-containing medium was harvested over 12 h until 72 h post-transfection. The medium was filtered through a 0.45-μm filter. After addition of 10 μg/ml Polybrene (Sigma), the medium was used to infect NIH3T3, Jurkat, HeLa, and MCF-7 cells for 24 h.

In Vitro Apoptotic Assay—Cell extracts were prepared as described by Briefly, Jurkat cells were treated with 1 μM STS for 1 h and then pelleted at 200 × g. Cells were washed twice with 50 ml of phosphate-buffered saline, followed by a single wash with 5 ml of buffer B (10 mM HEPES, pH 7.4, 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 1 mM PMSF), suspended in 1 volume of buffer A, and incubated on ice for 20 min. Cells were then lysed with 40 strokes using a B-type pestle in a 2-ml glass Dounce homogenizer. After centrifugation at 20,000 × g for 15 min at 4°C, the clear cytosolic fraction was carefully harvested without disturbing the nuclear pellet and adjusted to 50 mM NaCl. Aliquots of this were frozen at −80°C for later use. Nuclei were prepared from normal Jurkat cells. Cells were harvested and suspended in buffer A, followed by swelling on ice for 20 min. Cells were disrupted by gentle homogenization with a Dounce homogenizer. Liberated nuclei were then layered over a cushion of 30% sucrose in buffer B (10 mM HEPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 5 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM PMSF) and centrifuged at 800 × g for 10 min and resuspended in nuclear storage buffer (10 mM HEPES, pH 7.4, 80 mM KCl, 20 mM NaCl, 5 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM PMSF, and 50% glycerol) at 2 × 106 nuclei/mL. An in vitro apoptotic assay was carried out by incubating different amounts of cell extract with 5 × 105 nuclei for 2 h at 37°C. Nuclei were then recovered by a low-speed centrifugation. Nuclear DNA was subsequently isolated and analyzed on a 1.5% agarose gel for DNA fragmentation as we have published previously (28). In vitro apoptotic histone release was carried out by analyzing both the cytosolic and nuclear fractions after the in vitro apoptotic assay. Harvested nuclei were lysed in a 1% Triton X-100 lysis buffer. The cytosolic and Triton X-100-soluble nuclear fractions were separated by 15% SDS-PAGE and Coomassie Blue-stained for histone release.
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The appearance of these proteins might be a general feature of apoptosis, at least in Jurkat cells.

To determine whether the appearance of these apoptotic proteins is limited to Jurkat cells, apoptosis was induced in HeLa, MCF-7, WEHI-3, and NIH3T3 cells using STS, TNFα, and ETOP, and the appearance of these proteins was examined by Coomassie Blue staining of a 15% SDS-PAGE gel. Results are summarized in Table I. The proteins appeared in apoptotic HeLa and murine WEHI-3 but not apoptotic MCF-7 and NIH3T3 cells, indicating that although this event takes place in lines other than Jurkat, it does not occur in all cells. This is consistent with the notion that not all apoptotic features occur in every apoptotic cell line.

These Proteins Are Histones, Separated from Chromatin—To identify these apoptotic proteins, the largest protein (15 kDa) was separated on SDS-PAGE and transferred onto a PVDF membrane, and protein sequences were determined by Edman degradation. GenBank™ searching found that an amino acid sequence of 15 residues of this protein matched to a region in histone H3 (Fig. 3A), indicating that this protein is histone H3. Comparison of the Coomassie Blue staining profile of the four apoptotic proteins (Fig. 1B) with the published histone profile on SDS-PAGE immediately suggested that the other three apoptotic proteins might be histones H2A, H2B, and H4.

To confirm this, we took two approaches. First, histone proteins were purified using sulfuric acid (27) and separated side by side with the total lysate from control and treated Jurkat cells on 15% SDS-PAGE. The four apoptotic proteins in CH-11-, ETOP-, and STS-Jurkat cell lysate matched exactly with the purified histones H3, H2A, H2B, and H4 (Fig. 3B), suggesting that they are indeed histones. Secondly, to further confirm the identities of these proteins, apoptotic Jurkat cell lysate prepared from STS-treated cells was immunoblotted with antibodies against histones H3, H2A, H2B, and H4 (Fig. 3B), indicating that these small apoptotic proteins are histones.

Because these histones form the core structure of the nucleosome, they are normally insoluble in non-ionic detergents like Triton X-100. Consequently, their detection in apoptotic Jurkat cell lysate prepared in 1% Triton X-100 indicates that they have been released or separated from chromatin. Histone H1 sits outside of the nucleosome and is also released into apoptotic cell lysate in this setting (Fig. 3C). The lower band recognized by the anti-histone H1 antibody may be due to cross-reaction with histone H3.

CAD Plays an Important Role in Mediating Histone Separation from Chromatin in Apoptosis—Because DNA is wrapped around a histone structure, these data suggest a relationship...
between DNA fragmentation and histone separation from chromatin in the apoptotic process. Kinetically, histone release matched well with STS-induced CAD activation and DNA fragmentation in Jurkat cells (Fig. 4). STS-induced CAD activation (as judged from ICAD cleavage), DNA fragmentation, and histone release started at 1.25 h and progressed through to 6 h (Fig. 4). To further explore the link between DNA fragmentation and histone release, we expressed ICAD-DM using the pBabe retrovirus system into Jurkat cells (Fig. 5A). ICAD-DM was generated by substitution of both Asp (117, 224) residues in the caspase cleavage sites with Glu residues (6). This mutant protein is therefore resistant to caspase cleavage. Because activation of CAD requires cleavage of ICAD by caspases and CAD is the major apoptotic nuclease, expression of ICAD-DM in Jurkat cells should inhibit DNA fragmentation (6). As expected, expression of ICAD-DM completely blocked STS-induced DNA fragmentation (Fig. 5D). Interestingly, ICAD-DM also substantially reduced histone release in apoptotic Jurkat cells (Fig. 5C). Expression of ICAD-DM did not affect STS-induced apoptosis (Fig. 5B), consistent with previous observations (6). Similar results were obtained from CH-11-treated Jurkat (parental, infected with pBabe or ICAD-DM) cells and TNFα- or ETOP-treated HeLa cells infected with pBabe or ICAD-DM (data not shown), indicating an essential role of CAD in dismantling chromatin in these apoptotic cells. This result is consistent with the observation that histones do not separate from chromatin in apoptotic MCF-7 cells (Table I) because of the inability to activate CAD in this line due to a lack of functional caspase-3 (30). To further consolidate a role for CAD in apoptotic histone release, caspase-3 (CPP32) was reintroduced into MCF-7 cells using the pBabe retrovirus system (Fig. 6A). STS, as expected, induced caspase-3 activation and ICAD cleavage (Fig. 6, A and B). Histone release could be detected, although at low levels, in MCF-7/CPP32 but not MCF-7/pBabe cells (Fig. 6C). Because histone release was at low levels, we also performed immunoblotting for histone H4 under these conditions to confirm that histone release was indeed occurring. Indeed, histone H4 was detected only in apoptotic lysate from capase-3 infected MCF-7 cells (CPP32) (Fig. 6D). No DNA fragmentation could be detected in either pBabe or caspase-3-infected cells (Fig. 6E) for up to 24 h of STS treatment, despite

### Table I

| Cell line | Apoptotic inducers | Apoptotic proteins | ETOP |
|-----------|--------------------|--------------------|------|
| Jurkat    | STS    | Anti-FAS | TNFα | ETOP |
| HeLa      | +      | ND       | ND   | +    |
| MCF-7     | -      | ND       | ND   | -    |
| NIH3T3    | -      | ND       | ND   | -    |
| WEHI-3    | +      | ND       | ND   | ND   |

*+, appearance of small apoptotic proteins.
ND, not studied.
-<, no appearance of small apoptotic proteins.
the fact that under such conditions more than 70% of the cells were apoptotic (data not shown). Lack of DNA fragmentation in STS-treated MCF-7/caspase-3 cells might be due to the low level of caspase-3 expression and is consistent with our observation that MCF-7 cells do not fragment DNA effectively even in the presence of caspase-3 (9). Taken together, these results demonstrate a role for CAD in releasing histones from chromatin during apoptosis.

Histone Separation from Chromatin Is Not Simply a Byproduct of DNA Fragmentation—Because CAD plays a major role in fragmenting genomic DNA in apoptotic cells, we wished to determine whether CAD leads to histone release from chromatin simply by destruction of genomic DNA. In this case, histone separation will be an inevitable consequence of apoptotic DNA fragmentation. We have generated two lines of evidence that indicate that this is not the case. First, DNA fragmentation was readily induced in NIH3T3 cells by either TNFα (Fig. 7B) or ETOP (Fig. 8B), compatible in magnitude with the DNA fragmentation observed in STS-treated Jurkat cells (Fig. 7B). However, histones were detectable only in apoptotic Jurkat cell lysate and could not be detected in apoptotic NIH3T3 cell lysate (Fig. 7A), even at conditions under which more than 65% of the DNA was fragmented in NIH3T3 cells compared with 45% fragmentation in Jurkat cells (Fig. 7C).

Because endonuclease G and other nucleases also contribute to DNA fragmentation, it is possible that failure to release histones is due to a relatively minor contribution of CAD to DNA fragmentation in NIH3T3 cells. To exclude this possibility, ICAD-DM was expressed in NIH3T3 cells using the retroviral system. Expression of ICAD-DM substantially reduced apoptotic DNA fragmentation (Fig. 8), confirming a major role of CAD in DNA fragmentation in these cells.

Second, we have observed that, kinetically, STS at 1 μM led to DNA fragmentation and histone release in Jurkat cells after 1.25 h of exposure (Fig. 4). In an in vitro assay, incubation of Jurkat cytosolic extract derived from 1-h STS-treated cells with nuclei from normal Jurkat cells resulted in DNA fragmentation but not dissociation of histones from chromatin (Fig. 9A), whereas histones were readily released from chromatin in vivo.
In pursuing this, we observed that a DNA smear is converted to clear fragmentation as the CAD activity provided is increased. Increasing the amount of the STS-treated Jurkat cellular extract in the assay converted a DNA smear into fragmentation (Fig. 9A, upper panel) in vitro. Similarly, whereas 100 ng/ml CH-11 for 2 h led to a DNA smear, a 4-h incubation led to clear DNA fragmentation (Fig. 9B, upper panel). Consequently, failure to observe histone separation from chromatin in the in vitro assay is not due to inadequate CAD-mediated DNA cleavage. Taken together, it appears that, unlike DNA fragmentation, CAD is indirectly involved in the dissociation of histone from chromatin in apoptotic cells. This might also provide an explanation of why Jurkat/ICAD-DM and MCF-7/caspase-3 cells displayed some histone release but not DNA fragmentation under apoptotic conditions (Figs. 5 and 6).

DISCUSSION

Historically, apoptosis was defined by the appearance of DNA fragmentation, which accompanied chromatin condensation in dying cells (31). Because nuclear DNA is built into a structure termed chromatin, chromatin changes logically should be associated with apoptotic DNA fragmentation. To allow for transcription, DNA replication, chromosome segregation, and damage repair, chromatin conformational changes must occur. Similarly, apoptotic chromatin condensation may influence DNA fragmentation in apoptotic cells (3). Chromatin condensation occurs in both mitotic and apoptotic cells, and the assumption has therefore been made that the same or similar histone modifications might take place in both events (32). There is some evidence supporting this construct. Deubiquitination of histone H2A has been observed in apoptotic lymphoma cells induced by proteosome inhibitors (33). Phosphorylation of histones H1, H2A, H2B, and H3 as well as dephosphorylation of histone H1 have also been found to occur in some apoptotic settings (21–24, 34). Inhibitors of histone deacetylases, at high concentration, can induce apoptosis (17). However, the role that these histone modifications may play in apoptotic chromatin condensation and DNA fragmentation is unclear. On the other hand, recent data have indicated that apoptotic chromatin condensation might be totally unrelated to mitotic chromatin condensation (25), suggesting that events specific to apoptosis may occur and alter chromatin structure.

In this study, we provide evidence that apoptotic chromatin condensation and DNA fragmentation are accompanied by dissociation of histone from chromatin in apoptotic cells. This might also provide an explanation of why Jurkat/ICAD-DM and MCF-7/caspase-3 cells displayed some histone release but not DNA fragmentation under apoptotic conditions (Figs. 5 and 6).
ent with a major role of CAD/DFF-40 in fragmenting DNA in apoptotic cells (6). Interestingly, apoptotic histone release was also significantly reduced by ICAD-DM (Fig. 5), indicating a role for CAD in this apoptotic event. This is further supported by the observation that reintroduction of caspase-3 into MCF-7 cells (which lack caspase-3) resulted in detectable apoptotic histone release (Fig. 6). Because ICAD is not only an inhibitory subunit, but also a chaperone for the CAD catalytic subunit, CAD/DFF-40, ICAD-DM may therefore inhibit CAD activity by preventing the maturation of CAD/DFF-40 (11, 12). In this regard, we cannot definitively distinguish whether CAD nuclease activity or another activity of CAD functions in promoting apoptotic histone release. ICAD/DFF-45 also plays a role in the induction of chromatin conformational changes in some cells derived from knockout mice (15). Consequently, further work will be required to determine precisely how CAD mediates histone release.

Although it clearly plays an important role in apoptotic histone release, CAD/DFF-40 is not sufficient in and of itself to cause this to occur. The two events, DNA fragmentation and histone release, could be separated both in vivo and in vitro. In vitro, DNA fragmentation, significantly reduced by ICAD-DM, occurred efficiently in apoptotic NIH3T3 cells. However, no histone release could be found (Fig. 6). Because other nucleases, including endonuclease G, are able to cleave DNA in apoptotic cells (3–5) but might not function in histone release, lack of histone release in apoptotic NIH3T3 cells might be due to a major contribution of this or other nucleases. Although we cannot exclude this possibility completely, this is very unlikely because ICAD-DM dramatically inhibited apoptotic DNA fragmentation (Fig. 8), indicating a major role of CAD in DNA fragmentation in NIH3T3 cells. This would be consistent with previous data demonstrating that knockout of ICAD prevents DNA fragmentation in apoptotic MEF cells (15). In vitro, it has been shown that reconstitution of apoptotic cellular extract with nuclei prepared from Jurkat cells results in CAD-dependent DNA fragmentation because addition of ICAD-DM blocks fragmentation (6) (our data not shown). In such an assay, we could detect DNA fragmentation but not histone release (Fig. 9). Taken together, these data indicate that whereas CAD functions directly in DNA fragmentation, it may play an indirect role in mediating histone release in apoptotic cells.

The fact that histones are not released in apoptotic NIH3T3 cells is consistent with the notion that not all apoptotic features occur in every apoptotic setting. Possibly, induction of selective apoptotic features, such as membrane blebbing or DNA fragmentation, would be sufficient to execute cells. By the same logic, inducing histone release might also be able to cause cell death. The observation that CAD plays an important but insufficient role in causing histone release and that histone release is not simply the result of DNA fragmentation offers another layer of apoptotic regulation. It may also open other pathways for exploration in the induction of apoptosis in cancer therapy. Finally, because histone release can be detected in apoptotic cells that were negative for DNA fragmentation (apoptotic Jurkat/ICAD-DM, HeLa/ICAD-DM, and MCF-7/caspase-3), detection of histones in apoptotic cellular lysate prepared in 1% Triton X-100 by simple staining of a 15% SDS-PAGE by Coomassie Blue may provide an excellent way to assay apoptosis under some conditions.

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Fig. 9. In an in vitro apoptotic assay, histone release does not coincide with DNA fragmentation. In vitro incubation of differing amounts of Jurkat cytosolic extract derived from 1-h STS (1 μM)-treated cells with nuclei from normal Jurkat cells for 2 h at 37 °C was carried out. The control (Con) was 400 μg of Jurkat cytosolic extract from normal cells. After incubation, DNA fragmentation and histone release were investigated (A). In vitro, Jurkat cells were treated with 100 ng/ml CH-11 for different times, and then DNA fragmentation and histone dissociation were determined (B).
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