DNA degradation during apoptotic execution generally occurs at two levels: early as high molecular weight (HMW) fragments and later on as oligonucleosomal fragments. Two nucleases, CAD/CPAN/DFF40 and endonuclease G, can digest nuclear chromatin to produce the oligonucleosomal fragments, and it has been suggested that CAD might be responsible for HMW DNA cleavage. To more clearly define the role of CAD in nuclear disassembly, we have generated CAD−/− sublines of chicken DT40 cells in which the entire CAD open reading frame has been deleted. These cells grow normally and undergo apoptosis with kinetics essentially identical to wild type cells. However, they fail to undergo detectable HMW DNA cleavage and early stage apoptotic chromatin condensation against the nuclear periphery proceed normally in the absence of CAD. However, the final stages of chromatin condensation and nuclear fragmentation do not occur. Our results demonstrate that CAD is essential for this stage of DNA cleavage, at least in DT40 cells. Other aspects of nuclear disassembly, including HMW DNA cleavage and early stage apoptotic chromatin condensation against the nuclear periphery proceed normally in the absence of CAD. However, the final stages of chromatin condensation and nuclear fragmentation do not occur. Our results demonstrate that CAD is essential for this stage of DNA cleavage, at least in DT40 cells. Other aspects of nuclear disassembly, including HMW DNA cleavage and early stage apoptotic chromatin condensation against the nuclear periphery proceed normally in the absence of CAD. However, the final stages of chromatin condensation and nuclear fragmentation do not occur. Our results demonstrate that CAD is required for complete disassembly of the nucleus during apoptosis and reveal the existence of one or more as yet unidentified second factors responsible for HMW DNA cleavage and the early stages of apoptotic chromatin condensation.

DNA degradation was the first biochemical hallmark of apoptotic execution to be identified (1). In most cells two stages of DNA degradation are observed (1, 2). Early in the process, the DNA is cleaved to high molecular weight (HMW)1 fragments that may be indicative of a large scale domain organization of the chromatin (3). Later on, in most cell types the chromatin is cut into shorter fragments, referred to here as oligonucleosomal DNA fragmentation (1). Importantly, some cell lines exhibit only HMW DNA cleavage (4, 5).

To date, the best characterized nuclease with a clearly demonstrated role in cell death in vertebrates is CAD/CPAN/DFF40 (6–8). This 40-kDa polypeptide was first isolated from cytoplasm of healthy cells together with a second polypeptide of 45-kDa as part of a complex called DFF (DNA fragmentation factor) (9). The 40-kDa subunit was subsequently purified in an independent study (6), shown to have nuclease activity, and renamed CAD (caspase-activated DNase). DFF45/ICAD is both a folding chaperone and enzymatic inhibitor of CAD. If CAD is translated in the absence of ICAD, it adopts an inactive conformation and is catalytically inert (6). If CAD is translated in the presence of ICAD, it adopts an active conformation but is catalytically inactive due to the binding of the ICAD inhibitor. Upon the induction of apoptosis, ICAD/DFF45 is cleaved in two places by caspase-3 or similar caspases, thereby releasing active CAD (9, 10). Studies with purified cloned CAD have shown that CAD alone is sufficient to induce both DNA fragmentation and complete apoptotic chromatin condensation in isolated nuclei (8, 11).

The cellular location of CAD is controversial. CAD was initially isolated from cytosolic fractions (6, 9); however, it was subsequently shown that both CAD and ICAD are nuclear in healthy cells (8, 12–14). It now appears likely that the CAD-ICAD complex leaks out of nuclei during subcellular fractionation.

A second nuclease recently shown to be active in apoptosis is endonuclease G. This enzyme normally resides in mitochondria, and its translocation to the nucleus during apoptosis can lead to oligonucleosomal DNA fragmentation (15, 16). The relative contributions of CAD and endonuclease G to apoptotic DNA processing are only now being elucidated. Studies of the DFF45/ICAD knockout mouse showed that apoptotic cells had a significant reduction both in the level of oligonucleosomal DNA fragmentation and in the level of apoptotic chromatin condensation (17). This could be explained if CAD were inactive as a result of the loss of its folding chaperone. Overexpression of caspase-resistant ICAD in cells also resulted in a suppression of DNA cleavage during apoptosis, presumably because the ICAD remained bound to CAD despite the presence of active caspases (18). Interestingly, in both of these studies, the HMW cleavage was also suppressed, suggesting that CAD itself might be responsible for both stages of cleavage (18, 19).

It remains possible that the phenotypes seen with the ICAD/DFF45 knockout and overexpression of uncleavable ICAD arose not due to the lack of CAD activity but as a reflection of an as yet unknown function of ICAD (11). To clarify this issue, we have generated sublines of chicken DT40 cells in which the entire CAD open reading frame has been deleted. Our results reveal that CAD is essential for oligonucleosomal DNA fragmentation in apoptosis but that another nuclease (or nucleases) is responsible for HMW cleavage. Furthermore, differences between the phenotypes observed following elimination

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**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF406761.**

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1 The abbreviations used are: HMW, high molecular weight; DFF, DNA fragmentation factor; CAD, caspase-activated DNase; ICAD, inhibitor of CAD; bp, base pair(s); kb, kilobase(s); kbp, kilobase pair(s); EST, expressed sequence tag; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PARP, poly(ADP-ribose) polymerase; DAPI, 4′,6-diamidino-2-phenyl-indole.
of CAD and those observed following the elimination of ICAD or expression of uncleavable ICAD mutants raise the possibility that ICAD may do more than simply regulate CAD activity.

**EXPERIMENTAL PROCEDURES**

**Library Screening and Construction of Targeting Vector**—Plaques of lacdam Fix II carrying a genomic DNA fragment from DT 40 cells were screened with a 532-bp DNA fragment from CAD EST (dkf426_18p13r1). The phage DNA insert (G3B, 18 kb), which contains chicken CAD genomic DNA, was totally sequenced by GATC Biotech AG (Germany). S- and 3′-flanking regions of 1.8 and 3 kb were amplified from the phage DNA using oligo primers aagccgaggctctgctgc and ataggatccgctctctttggaagcagcttc. The GenBank TM accession number for the cCAD gene is AF406761.

**Transfection and Selection of KO Cell Line—**1.5 × 10^7 DT40 cells (grown in RPMI media plus 10% fetal bovine serum, 1% chicken serum, penicillin, and streptomycin) were suspended into 0.5 ml of OptiMEM per 1 ml of OptiMEM (4 mm). 50 μg of linearized DNA was added to each cuvette and kept on ice for 5 min before and after electroporation (300 V; 950 microfarads in a GenePulsor, Bio-Rad). After 24 h, cells were transferred to 96-well plates containing selective media (puromycin or blasticidin). Nuclei of HeLa S3 or DT40 cells were transferred to 96-well plates containing selective media (puromycin or blasticidin) and kept on ice for 5 min before and after electroporation.

**In Vivo Analysis of Apoptotic Cells**—Cells were labeled with either a TUNEL fluoros staining kit or an annexin V-fluorescence staining kit (Boehringer Molecular Biochemicals) following the manufacturer’s instructions. Microscopy was done as described previously (11).

**In Vitro Analysis**—Cells were treated with either 50 μM etoposide (Calbiochem) for 3.5 h or 25 mM staurosporine for 16 h, and extracts were prepared as described previously (20). The protein concentration of each extract measured by the Bradford assay ranged from 9.3 to 15.6 mg/ml. Active caspases in extracts were labeled with Z-EK(bio)D-amk and affinity labeled of caspases in those extracts were prepared from cells treated with either etoposide or staurosporine. Affinity labeling of caspases in those extracts demonstrated that caspase activation was similar in CAD−/−, CAD+/−, and CAD+/+ cells in the induction of annexin V staining during 2-h treatment with etoposide (Fig. 1C). For in vitro studies, cytosolic extracts were prepared from cells treated with either etoposide or staurosporine. Affinity labeling of caspases in those extracts demonstrated that caspase activation was similar in CAD−/−, CAD+/−, and CAD+/+ cells (Fig. 1D). When isolated HeLa nuclei were incubated in extracts made from these apoptotic cells, the chromatin-condensed and PARP cleavage occurred normally (Fig. 1, E and F). These results suggested that induction of apoptosis, including activation of caspases, is normal in CAD−/− cells.

**Lack of Internucleosomal DNA Cleavage in CAD−/− Cells**—Two enzymes, CAD/DF40/CPAN and endonuclease G have been reported to induce oligonucleosomal DNA cleavage in apoptosis (6–8, 15, 16). To assess the relative contributions of the two nuclease in chromatin fragmentation in DT40 cells following exposure to etoposide or staurosporine, we examined the process of internucleosomal DNA cleavage in CAD−/− cells (Fig. 2A). Upon treatment with 50 μM etoposide, CAD+/− and CAD+/+ cells showed robust DNA ladder formation after 2 h. Heterozygous cells showed a slight delay compared with CAD+/− cells. In contrast, CAD−/− cells showed no ladder formation even after 3 h (box), indicating that CAD is largely responsible for DNA ladder formation in DT40 cells. This result was also confirmed by in vitro studies. When exogenous nuclei (either HeLa or DT40) were incubated with cytosolic extracts from apoptotic CAD+/− or CAD−/− cells, CAD−/− extracts induced formation of an oligonucleosomal ladder, while CAD+/− extracts did not (Fig. 2B and data not shown). Although CAD is a nuclear protein, no obvious difference in DNA cleavage was observed between DT40 CAD+/+ or CAD−/− nuclei. This could be explained if CAD leaks out during preparation of the substrate nuclei.

**High Molecular Weight DNA Cleavage Occurs in CAD−/− Cells**—During apoptosis, cleavage of the chromosomes into fragments of 200–300 kb and 30–50 kb is observed prior to oligonucleosomal DNA fragmentation. Studies with ICAD knockout mice or cells expressing caspase-uncleavable ICAD, in which the HMW cleavage was not observed (17–19, 24), suggested that CAD might contribute to the HMW DNA cleavage. However, CAD is unlikely to be the sole factor for HMW DNA cleavage, because ICAD and CAD are not expressed in all tissues. Furthermore, some cell types only show HMW DNA cleavage (4, 25). Were CAD activated, it is expected that these cells would also show oligonucleosomal DNA fragmentation.

To determine whether CAD is required for HMW DNA cleav-
We induced apoptosis in CAD−/− cells by treatment with etoposide for 30 min to 4 h and then subjected the DNA to pulsed-field gel electrophoresis. HMW cleavage occurred in CAD−/−, CAD+/−, and CAD+/+ cells with essentially identical kinetics (Fig. 2, C and D). After 30 min of etoposide treatment, the chromosomes of CAD−/− cells showed 50- to 300-kbp fragments and by 1 h 30- to 50-kbp fragments became dominant. In CAD+/− and CAD+/+ cells the HMW DNA fragments began to decline after 2 h as the chromosomes were degraded further to produce oligonucleosomal DNA fragments. In CAD−/− cells, the HMW DNA cleavage pattern remained stable for at least 4 h (Fig. 2D).

This result demonstrates clearly that CAD is dispensable for HMW DNA cleavage during drug-induced apoptosis. We do not know the nature of the factor responsible for this cleavage in CAD−/− DT40 cells; however, it is unlikely to be an enzyme that produces DNA ends with a free 3′-OH. Such ends are detected by the TUNEL reaction, and apoptotic CAD−/− cells were negative for TUNEL labeling (Fig. 3, H–J).

Stage I Chromatin Condensation Still Occurs in CAD−/− Cells—CAD induces not only DNA cleavage but also chromatin condensation (8, 11). Furthermore, apoptotic DNA condensation was reported to be significantly repressed in cells of the ICAD knockout mouse (17). Thus we examined whether apo-
DNA Cleavage and Chromatin Condensation without CAD/DFF40

Fig. 2. Internucleosomal DNA cleavage is absent in CAD−/− cells, however, high molecular weight (HMW) cleavage of the chromosomes occurs normally. A, DNA from cells treated with 50 μM etoposide was subjected to conventional agarose gel electrophoresis. The white box in the upper gel shows the region of the gel where oligonucleosomal DNA fragmentation would be expected in CAD−/− cells after 3 h. B, DNA from HeLa nuclei incubated with apoptotic extracts from +/+ , +/−, and −/− cells for 1 h at 37 °C was subjected to conventional agarose gel electrophoresis. Extracts from −/− cells failed to induce oligonucleosomal DNA fragmentation. C, DNA from cells treated with 50 μM etoposide was subjected to pulsed-field gel electrophoresis. A range of DNA fragments ≈ 50 kb in size was observed at early times (0.5 h) in +/+ and +/− cells and at all times in the −/− cells. These fragments gradually disappeared in the +/+ and +/− cells starting at 1 h, giving rise to the oligonucleosomal DNA ladder seen in A. D, results of a similar experiment in which loss of the ≈ 50-kb DNA had begun at 1 h in +/+ cells, while the −/− cells still showed only HMW DNA fragmentation even at 4 h. In both C and D, the region containing HMW DNA fragments is designated by white brackets.

DISCUSSION

We have isolated sublines of chicken DT40 lymphoma cells in which the entire coding region of the CAD gene has been deleted. CAD is not essential for growth, and CAD−/− cells proliferate normally. Upon etoposide or staurosporine treatment, CAD−/− cells become positive for annexin V staining, activate caspasmes, and cleave PARP in a manner indistinguishable from CAD+/+ or CAD+/− cells. Therefore, induction of apoptosis and the general events of apoptotic execution are normal in CAD−/− cells.

All phenotypes observed in CAD−/− cells (see below) were reversed upon transfection of either a cDNA encoding chicken CAD, or a cDNA encoding murine CAD fused to GFP. This confirmed that deletion of the CAD gene was responsible for the various phenotypes. Furthermore, the demonstration that murine CAD can replace the chicken protein must also mean that chicken ICAD can act both as folding chaperone and inhibitor of murine CAD. Because we previously showed that murine ICAD can inhibit chicken CAD in our in vitro system, these data confirm that CAD and ICAD are interchangeable between birds and mammals.

Because CAD has been proposed to be the nuclease responsible for DNA fragmentation during apoptotic execution (6, 28),

ptotic chromatin condensation still occurs in CAD−/− cells. Upon etoposide or staurosporine treatment, these cells reproducibly showed stage I chromatin condensation (chromatin condensed against the nuclear periphery, Fig. 1G) but never reached stage II (formation of discrete apoptotic bodies, Fig. 3A). The same treatment readily induced stage II chromatin condensation in CAD+/+ or CAD+/− cells (Fig. 3B and data not shown). Similar data were obtained when isolated HeLa nuclei were incubated in apoptotic extracts prepared from either CAD+/+ or CAD−/− cells (Fig. 1E). Lamins are cleaved relatively late during apoptotic execution and are eventually dispersed throughout the cytoplasm following nuclear lysis (26, 27). Lamina disassembly was observed during apoptotic execution both in CAD+/− cells, which underwent stage II chromatin condensation (Fig. 3C), and in CAD−/− cells, in which chromatin condensation became arrested at stage I (Fig. 3D). This control shows that the CAD−/− cells examined were in the late stages of apoptotic execution despite the block in chromatin condensation at stage I. These data reveal that significant levels of apoptotic chromatin condensation can occur in the absence of CAD.

A CAD cDNA Can Rescue the Phenotype of CAD−/− Cells—To confirm that the phenotypes observed in CAD−/− cells were due to loss of CAD, we transiently transfected CAD−/− cells with a plasmid encoding both the chicken CAD cDNA and GFP under separate promoters. Transfected cells were recognized by the expression of GFP. When we induced apoptosis in cCAD/GFP-transfected CAD−/− cells with etoposide, cells showed stage II chromatin condensation indistinguishable from CAD+/+ cells (Fig. 3F). Control CAD−/− cells

2 K. Samejima, S. Tone, and W. C. Earnshaw, unpublished results.
we first examined the state of DNA cleavage in CAD−/− cells in vivo and in vitro. CAD−/− cells did not show oligonucleosomal DNA fragmentation even after exposure to 50 μM etoposide for 3 h, which renders the culture fully apoptotic. Therefore, CAD is essential for oligonucleosomal DNA fragmentation in DT40 cells following treatment with etoposide. This is noteworthy, because etoposide is known to induce the mitochondrial cell death pathway (29). Our results indicate that the mitochondrial endonuclease G does not appear to play a significant role in apoptotic execution in these cells.

Next we looked at cleavage of the chromosomes into high molecular weight fragments (HMW DNA cleavage). Previous studies had shown that either disruption of the DFF45/ICAD gene (28) or expression of mutant ICAD that cannot be cleaved by caspases (18) severely suppressed HMW DNA cleavage, suggesting that CAD is required for this event. However, CAD−/− DT40 cells clearly showed HMW DNA cleavage with wild type kinetics upon etoposide treatment. Thus, CAD cannot be essential for HMW DNA cleavage.

It should be noted that, even though CAD−/− cells underwent HMW DNA cleavage and stage I chromatin condensation (see below), the cells remained TUNEL-negative. TUNEL labeling detects free 3′-OH ends and is a widely used marker for apoptotic cells. The absence of labeling of apoptotic CAD−/− cells indicates one of the following: 1) the nuclease responsible for HMW cleavage does not produce a free 3′-OH; or 2) if free 3′-OH ends are produced, they are inaccessible to the enzymes used for the TUNEL reaction; or 3) if free 3′-OH ends are produced, cleavage sites are so rare that they are not detected by the TUNEL method.

The factor responsible for HMW DNA cleavage in CAD−/− cells is unknown. One candidate is DNA topoisomerase IIα (30, 31), although the role of this enzyme in irreversible apoptotic DNA cleavage has been questioned (32). Topoisomerase IIα produces a free 3′-OH when it cleaves DNA (33). Furthermore, in preliminary results, we have found that addition of ICRF-187, a catalytic inhibitor of topoisomerase IIα (34), did not suppress the HMW DNA cleavage in the cells treated with staurosporine (data not shown). Thus we believe it unlikely that the HMW nuclease is DNA topoisomerase IIα.

At this point, the most likely candidate for the HMW nuclease is AIF, or factors activated downstream from it. AIF, a flavoprotein that is released from mitochondria during the induction of apoptosis (35) has been shown to be essential for programmed cell death during cavitation of mouse embryoid bodies and when ES cells are exposed to low serum (36). AIF acts in a pathway parallel to CAD in apoptotic execution, is required for HMW DNA cleavage in vivo in the presence of uncleavable ICAD and can induce this cleavage in isolated nuclei (35). It is not known whether AIF-induced DNA breaks can be detected by TUNEL.³

³G. Kroemer, personal communication.
yet unknown functions during apoptotic execution. For example, ICAD might modulate the accessibility to chromatin of not only CAD but also one or more other factors. Our CAD−/−cell lines should provide useful tools to further characterize the processes of HMW DNA cleavage and chromatin condensation during apoptotic execution.

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