Identification and Developmental Expression of Inhibitor of Caspase-activated DNase (ICAD) in *Drosophila melanogaster*

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DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase (CAD) and its inhibitor (ICAD). When cell lysates from *Drosophila* S2 cells were chemically denatured and the denatured proteins were removed after dialysis, the supernatant inhibited *Drosophila* CAD (dCAD). To identify the inhibitor, we tested recombinant DREP-1, which was previously identified using the *Drosophila* EST data base and found it also inhibited dCAD DNase. An antibody against DREP-1 inhibited the ICAD activity in the S2 cell extracts, confirming the identification of DREP-1 as a *Drosophila* homolog of ICAD (dICAD). The recombinant DREP-1/dICAD was cleaved at a specific site by human caspase 3 as well as by extracts prepared from S2 cells undergoing apoptosis. Biochemical fractionation and immunoprecipitation of dICAD from S2 cell extracts indicated that dICAD is complexed with dCAD in proliferating cells. The expression of the caspase-resistant form of dICAD/DREP-1 in a *Drosophila* neuronal cell line prevented the apoptotic DNA fragmentation. Northern hybridization and the immunohistochemical analyses revealed that the expression of the *dICAD* gene is developmentally regulated.

During animal development, many harmful or useless cells are generated, which are removed by apoptosis, or programmed cell death, to maintain homeostasis (1). Apoptosis is accompanied by morphological changes in the cells such as condensation and fragmentation of nuclei and cells and loss of microvilli from plasma membranes (2). Another hallmark of apoptosis is the extensive degradation of chromosomal DNA into nucleosomal units, giving rise to a ladder with multimers of about 180 base pairs (3, 4). Recent genetic and biochemical characterization of the apoptotic signal transduction pathway indicates that apoptosis is mediated by a family of proteases called caspases (cysteinyl aspartate-specific protease) (5). Caspases exist as inactive precursor forms (zymogens) in proliferating cells and are activated by proteolytic processing during apoptosis. The caspases involved in the apoptotic pathway can be grouped into two subfamilies, initiators and executors. The initiator caspases such as caspases 8 and 9, are activated at the plasma membrane by death factors, or at mitochondria by various apoptosis-inducing agents, whereas the executor caspases, such as caspases 3, 6, and 7, are activated by the initiator caspases.

We and others have recently identified in a mammalian system a DNase (CAD, 1 also called DFF-40 or CPAN) that is activated by a caspase (6–10) and is responsible for the characteristic degradation of chromosomal DNA into nucleosomal units. CAD is complexed with its inhibitor (ICAD, also called DFF-45) in proliferating cells. When apoptotic stimuli activate a caspase cascade, caspase 3, which acts downstream in the cascade, cleaves ICAD, which releases CAD to cleave the chromosomal DNA (11, 12).

The apoptotic signal transduction pathway appears to be well conserved among metazoans. Genetic analysis of programmed cell death in *Caenorhabditis elegans* demonstrated that it is regulated by a small set of gene products (13): Ced-3 (cell death abnormal) and Ced-4 function as executors of the process, whereas Ced-9 blocks it. Mammalian counterparts of these gene products have been identified as the caspase, Apaf-1 (apoptosis-activating factor), and Bcl-2 families, respectively (14). Each family in the mammalian system is composed of several members, thus apoptotic signal transduction seems to be more elaborate than that in *C. elegans*.

Development of *Drosophila melanogaster* is also regulated by programmed cell death (15–17). Dying cells appear at stage 11 of embryogenesis, which continue throughout embryogenesis. The cell death is also observed during metamorphosis. As in mammals and *C. elegans*, the apoptosis in *Drosophila* is accompanied by morphological changes in the cells, including DNA fragmentation. This process is also regulated by Ced-3/caspase- and Ced-4/Apaf-1-like molecules (16). At least five caspase members have been identified in *Drosophila*. Of these, DREDD and DRONC resemble the mammalian initiator caspases 8 and 9. The other three members, Dcp-1, drICE, and DECAY resemble effector or executioner caspases such as caspases 3 and 7 (18–22).

Despite the apparently well conserved apoptotic system in mammals, *Drosophila*, and *C. elegans*, the molecular mechanism of the apoptotic DNA fragmentation in *Drosophila* has not been well studied. We recently identified a DNase in *Drosophila* Schneider line 2 (S2) cells that can be activated by caspase,
and showed it to be the Drosophila counterpart of CAD (dCAD) (23). In this report, we found that Drosophila cells also carry an inhibitor of CAD (dICAD). dICAD was complexed with dCAD and could be cleaved by caspase. Analysis of the expression of the dICAD gene indicated that it is maternally regulated and highly expressed during early embryogenesis and metamorphosis. This expression pattern suggests that dICAD plays an important role in programmed cell death in Drosophila.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Materials—S2 cells from D. melanogaster (ATCC CRL1693) and its transformed lines bearing pMT-reaper (24) were maintained at 25 °C in Schneider’s Drosophila medium (Sigma) supplemented with 10% fetal calf serum (Equitech-Biological Inc.). To induce the expression of reaper, the transformants were incubated at 25 °C for 12 h in the presence of 0.5 mM CuSO4, and the cell lysates were prepared as described (24). The Drosophila neuronal cell line, ML-DmBG2-c2 (BG2-c2) (25) was maintained at 25 °C in Shields and Sang M3 medium (Sigma) containing 10% fetal calf serum and 10 μg/ml insulin. A cDNA for the caspase-resistant form of dICAD/DREP-1 was isolated by PCR from BG2-c2 cells together with the hygroycin-resistant gene. The hygromycin-resistant clones (over 200 clones) were pooled and used as stable transformed cells.

Human caspase 3 was produced in Escherichia coli and purified as described (26). Biotinylated Asp-Glu-Val-chloromethylketone was purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). A monoclonal antibody (clone M2) against the FLAG peptide was obtained from Sigma. Isolation of dICAD cDNA and Recombinant dICAD—Poly(A) RNA isolated from S2 cells was reverse-transcribed with random hexamer primers. The coding sequence of the DREP-1 cDNA was amplified by polymerase chain reaction (PCR) using the following primers: sense primer, S-1: 5′-GGCTGGAAATAAAGTGCATAGTG-3′; antisense primer, AS-1: 5′-AAAGATTCGTCGTTAGTTGAATGGCCG-3′. The PCR product of 0.9 kilobase (kb) was inserted into the pGEM-T vector (Promega), and the nucleotide sequence was determined by a DNA sequencer (PRIZM310, Applied Systems). DNA encoding a caspase 3-noncleavable DREP-1 mutant was prepared by recombinant PCR. In brief, an N-terminal portion of DREP-1 cDNA was amplified with a sense primer (S-2: 5′-CCGGATCTGGATGTTGAATGGCCG-3′) and an antisense primer carrying the mutation (AS-2: 5′-GTGGTTGGATGTTGAATGGCCG-3′) as described (26). The PCR product was cloned into the pGEX-2T vector, and the coding sequence of DREP-1 was amplified with a sense primer complementary to AS-2 and an antisense primer of AS-1. The products from the first PCR were isolated by agarose gel electrophoresis, mixed 1:1, and the secondary PCR was carried out using primers S-2 and AS-1. The coding sequence of DREP-1 cDNA was tagged with a FLAG epitope and ligated to the E. coli glutathione S-transferase (GST) gene using pGEX-2T (128/129). The GST-DREP-1 fusion proteins were expressed in E. coli AD202, and purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). To produce N-terminal six-histidine-tagged DREP-1, the DREP-1 coding sequence was inserted into the pQE-10 vector (Qiagen). The protein was expressed in E. coli M15 (pREP4) by treating the cells with 2 mM isopropyl-β-D-thiogalactopyranoside, and purified using a Ni-chelated Hi-Trap column. The N-terminal sequence of the caspase-cleaved GST-DREP-1 was determined at the Takara Shuzo Co. (Kyoto, Japan).

Assay for dCAD and dICAD—To determine the dCAD activity, plasmid DNA (1 μg) was incubated at 4 °C for 12 h and at 30 °C for 2 h with samples in 20 μl of buffer A (10 mM Hepes-KOH (pH 7.2), 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 1 mM DTT, and 1 mM (p-amino)phenylmethanesulfonyl fluoride (pAPMSF) containing 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were disrupted by three cycles of freezing and thawing in a Dounce homogenizer accompanied by grinding with a pestle during each thawing cycle. The homogenate was spun at 30,000 × g for 30 min and at 100,000 × g for 2 h. Urea (10 mM) was added to the supernatant fraction (about 100 mg of protein) to a final concentration of 8 M, and incubated at room temperature for 1 h. After adjusting the urea concentration to 5 M, the sample was loaded onto a DEAE-Sepharose FF column (10 ml, Amersham Pharmacia Biotech) that was equilibrated with buffer B containing 10% glycerol, 5 mM urea, and 50 mM NaCl. Proteins were eluted with 5–350 mM linear NaCl gradient in buffer B containing 5 mM urea. The active fractions were pooled, adjusted to 1 M (NH4)2SO4, and loaded onto a butyl-Sepharose column (18 ml) that was equilibrated with buffer B containing 5 M urea and 1 M (NH4)2SO4. Proteins were then eluted with a 1 to 0 M (NH4)2SO4 descending gradient. The active fractions were pooled and dialyzed against buffer B containing 50 mM NaCl, and the insoluble materials were removed by centrifugation and used as partially purified dCAD.

Immunohistochemistry—Immunohistochemical analysis of Drosophila embryo was carried out essentially as described (28). In brief, embryos at the stages of 0–3 h and 3–16 h were collected, dechorionated by treating for 2.5 min in 2.5% NaClO, and fixed for 20 min with 4% paraformaldehyde in phosphate-buffered saline (PBS). After blocking nonspecific sites for 1 h with 5% skim milk in PBS containing 0.02% Tween 20 (PBST), the embryos were incubated overnight at 4 °C with 2000-fold-diluted rabbit anti-dCAD antibody in PBS containing 5% skim milk. After several washes in PBST, the embryos were incubated at room temperature with 1 μg/ml biotin-conjugated goat anti-rabbit antibody (Jackson Immunoresearch). The signals were amplified using an ABC Elite kit (Vector Laboratories) according to the manufacturer’s instructions. The embryos were mounted in 70% glycerol in PBS and observed under a microscope.

RESULTS

ICAD Activity in Drosophila S2 Cells—To examine whether Drosophila cells express ICAD, the cytosolic fraction (S-100) was prepared from S2 cells. The extracts had no effect on the DNase activity of partially purified Drosophila CAD (dCAD) or of recombinant rat CAD (data not shown). We observed previously that mouse ICAD does not show ICAD activity when it is complexed with CAD (29). Because mouse ICAD but not CAD is resistant to denaturants such as 6 M guanidine hydrochloride, 8 M urea, or 0.1% SDS (6), the cytosolic fraction (S-100) from S2 cells was treated with 5 M guanidine hydrochloride or 8 M urea. When the denaturants were removed by incubated at 30 °C for 2 h. The remaining dCAD activity was determined with 1 μg of plasmid DNA as described above. The H-7-induced DNA fragmentation in BG2-c2 cells was assayed as described (23, 27). Partial Purification of ICAD from S2 Cells—S2 cells (9 × 106 cells) were suspended in buffer B (10 mM Hepes-KOH (pH 7.2), 5 mM MgCl2, 5 mM EGTA, 1 mM DTT, and 1 mM (p-amino)phenylmethanesulfonyl fluoride (pAPMSF) containing 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were disrupted by three cycles of freezing and thawing in a Dounce homogenizer accompanied by grinding with a pestle during each thawing cycle. The homogenate was spun at 30,000 × g for 30 min and at 100,000 × g for 2 h. Urea (10 mM) was added to the supernatant fraction (about 100 mg of protein) to a final concentration of 8 M, and incubated at room temperature for 1 h. After adjusting the urea concentration to 5 M, the sample was loaded onto a DEAE-Sepharose FF column (10 ml, Amersham Pharmacia Biotech) that was equilibrated with buffer B containing 10% glycerol, 5 M urea, and 50 mM NaCl. Proteins were eluted with a 5–350 mM linear NaCl gradient in buffer B containing 5 M urea. The active fractions were pooled, adjusted to 1 M (NH4)2SO4, and loaded onto a butyl-Sepharose column (18 ml) that was equilibrated with buffer B containing 5 M urea and 1 M (NH4)2SO4. Proteins were then eluted with a 1 to 0 M (NH4)2SO4 descending gradient. The active fractions were pooled and dialyzed against buffer B containing 50 mM NaCl, and the insoluble materials were removed by centrifugation and used as partially purified dCAD.

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Identification and Expression of Drosophila ICAD

Identification of DREP-1 as dICAD—Following the inhibitory activity against dCAD, we partially purified dICAD by chromatography on DEAE-Sepharose and butyl-Sepharose. Meanwhile, Inohara et al. (30) found a cDNA clone (DREP-1) in the Drosophila EST data base that has weak but significant homology with human ICAD/DFF-45. To examine whether DREP-1 possessed ICAD activity, we prepared recombinant DREP-1 in E. coli as a fusion protein with glutathione S-transferase. As shown in Fig. 2A, GST-DREP-1 efficiently inhibited the dCAD DNase activity, and this ICAD activity was destroyed by treatment with caspase 3. Furthermore, the ICAD activity of partially purified dICAD from S2 cells was inhibited by a rabbit antibody raised against recombinant DREP-1 protein (Fig. 2B). These results indicated that the DREP-1 cDNA in fact codes for dICAD.

Fig. 3A shows the amino acid sequence of DREP-1/dICAD, aligned with those of mouse and human ICAD. The sequence differed from that published by Inohara et al. (30) at two positions, but agreed with the sequence in the Drosophila Genome data base. Drosophila ICAD consists of 296 amino acids and has a molecular mass of 31,970 Da. It is rich in acidic residues, containing 23 aspartate and 27 glutamate residues, and has an isoelectric point of 4.37. Its overall identities with mouse ICAD (mICAD) and human ICAD (hICAD) are 17.6% and 17.3%, respectively. As noted previously (30), the homology between DREP-1 and ICAD/DFF-45 (∼20%) was clearly weaker than that observed among the ICAD/DFF-45 family (∼40%). (30) at two positions. Six nucleotides coding for Lys-Leu are inserted at amino acid position 195, and a nucleotide change at position 108 causes an amino acid replacement from Asp to Glu in Inohara et al. (30). B, the chromosomal gene structure of dICAD. The structure of the dICAD chromosomal gene is schematically shown with that of the gene for β-subunit of RNA polymerase III. Closed boxes and open boxes represent coding and noncoding regions, respectively.
between Drosophila ICAD and the mammalian ICADs is more pronounced in the N-terminal CAD/CIDE domain (7, 30). In situ hybridization of polytene chromosome with diCAD cDNA indicated that the diCAD gene is located at band 48EF to 49AB. In fact, we found the diCAD/DREP-1 gene in P1 clones derived from this region, and the alignment of the genomic sequence in the data base with that of the cDNA indicated that the 5.5-kb diCAD/DREP-1 gene is split by three introns and is located 1.5 kb downstream of the gene for the β-subunit of RNA polymerase III (Fig. 3B).

Cleavage of diCAD by Caspase—The abolishment of the ICAD activity by caspase 3 suggested that diCAD is cleaved by a caspase. In fact, treatment of GST-DREP-1 with caspase 3 produced two fragments of 45 and 25 kDa (Fig. 4A). Western blotting analysis of the native diCAD in the S-100 fraction or partially purified preparation from S2 cells showed a 46-kDa protein, which was cleaved by human caspase 3 to generate a band of 25 kDa. Expression of reaper in S2 cells activates caspase 3-like proteases and induces apoptosis (18, 24, 31). To confirm that Drosophila caspases can cleave diCAD, GST-DREP-1 was incubated with the cytosolic fraction from S2 cells (lanes 1 and 2) and the partially purified diCAD (lanes 3 and 4) were incubated at 30 °C for 12 h without (lanes 1 and 3) or with 90 ng of caspase 3 (lanes 2 and 4). Proteins were then resolved by electrophoresis on a 4–20% gradient polyacrylamide gel. The Western blotting was carried out with 4000-fold-diluted rabbit anti-dCAD antibody, followed by incubation with 3000-fold-diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO). Positions of molecular mass standard proteins are indicated in kilodaltons. The intact diCAD and its cleaved products are indicated by arrows on the right. B, cleavage of diCAD by S2 cells by caspase 3. The cytosolic fraction from S2 cells (lanes 1 and 3) and the partially purified diCAD (lanes 1 and 3) were incubated at 30 °C for 12 h without (lanes 1 and 3) or with 90 ng of caspase 3 (lanes 2 and 4). Proteins were then resolved by electrophoresis on a 4–20% gradient polyacrylamide gel. The Western blotting was carried out with 4000-fold-diluted rabbit anti-diCAD antibody, followed by incubation with 3000-fold-diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO). Positions of molecular mass standard proteins are indicated in kilodaltons. The intact diCAD and its cleaved products are indicated by arrows on the right. C, cleavage of diCAD by the apoptotic extracts from S2 cells. The cytosolic extracts (5–30 fraction) were prepared from transformed S2 cells induced to express reaper (lanes 1–5) or from the nontransformed parental S2 cells (lane 6). The GST-DREP-1 (30 ng of protein) was incubated at 30 °C for 3 h with 8 μg (lane 1), 16.0 (lane 2), 35.0 (lane 3), 65.0 (lane 4), and 120.0 (lane 5) of the lysates in 100 μl of buffer A containing 1 mg/ml bovine serum albumin. The GST-DREP-1 proteins were bound to glutathione-Sepharose beads (20-μl bed volume) by incubating at 4 °C for 30 min. The beads were heated at 95 °C for 5 min in Laemmli sample buffer. Proteins released from the Sepharose beads were then separated by electrophoresis on a 4–20% gradient polyacrylamide gel. Western blotting was carried out with 3000-fold-diluted anti-FLAG monoclonal antibody, followed by incubation with the anti-mouse IgG (Jackson Laboratories). The positions of molecular mass standard proteins are indicated in kilodaltons on the left.

Fig. 4. Cleavage of DREP-1/diCAD by caspase. A, cleavage of the recombinant DREP-1 by caspase 3. The GST-DREP/diCAD (2.4 μg) was incubated at 30 °C with 90 ng of human caspase 3 for the indicated periods of time. Proteins were analyzed by electrophoresis on a 4–20% gradient polyacrylamide gel and stained with Coomassie Brilliant Blue. Positions of molecular mass standard proteins are indicated on the left in kilodaltons. The intact GST-DREP/diCAD and its cleaved products are indicated by arrows on the right. B, cleavage of diCAD by S2 cells by caspase 3. The cytosolic fraction from S2 cells (lanes 1 and 2) and the partially purified diCAD (lanes 3 and 4) were incubated at 30 °C for 12 h without (lanes 1 and 3) or with 90 ng of caspase 3 (lanes 2 and 4). Proteins were then resolved by electrophoresis on a 4–20% gradient polyacrylamide gel. The Western blotting was carried out with 4000-fold-diluted rabbit anti-diCAD antibody, followed by incubation with 3000-fold-diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO). Positions of molecular mass standard proteins are indicated in kilodaltons. The intact diCAD and its cleaved products are indicated by arrows on the right. C, cleavage of diCAD by the apoptotic extracts from S2 cells. The cytosolic extracts (5–30 fraction) were prepared from transformed S2 cells induced to express reaper (lanes 1–5) or from the nontransformed parental S2 cells (lane 6). The GST-DREP-1 (30 ng of protein) was incubated at 30 °C for 3 h with 8 μg (lane 1), 16.0 (lane 2), 35.0 (lane 3), 65.0 (lane 4), and 120.0 (lane 5) of the lysates in 100 μl of buffer A containing 1 mg/ml bovine serum albumin. The GST-DREP-1 proteins were bound to glutathione-Sepharose beads (20-μl bed volume) by incubating at 4 °C for 30 min. The beads were heated at 95 °C for 5 min in Laemmli sample buffer. Proteins released from the Sepharose beads were then separated by electrophoresis on a 4–20% gradient polyacrylamide gel. Western blotting was carried out with 3000-fold-diluted anti-FLAG monoclonal antibody, followed by incubation with the anti-mouse IgG (Jackson Laboratories). The positions of molecular mass standard proteins are indicated in kilodaltons on the left.

Fig. 5. Resistance of the diCAD mutant to cleavage by caspase. Wild-type GST-DREP-1 (lanes 1 and 2) or mutant GST-DREP-1 (D119E) (lanes 3 and 4) (2.3 μg) was incubated at 30 °C for 12 h in buffer A in the absence (lanes 1 and 3) or presence of 90 ng of human caspase 3 (lanes 2 and 4). Aliquots (2 μg) were then analyzed by electrophoresis on a 4–20% gradient polyacrylamide gel and stained with Coomassie Brilliant Blue (A). The positions of the size marker proteins are shown on the left in kilodaltons. Using 12-ng aliquots, the ICAD activity was determined with the partially purified diCAD (B). The DNase activity of the partially purified diCAD is shown in lane 5.

Complex of diCAD with dCAD and Requirement of diCAD Cleavage in the Apoptotic DNA Fragmentation in Drosophila Cells—diCAD activity in the S2 cell extracts was detected only after treatment with denaturants, suggesting that diCAD is complexed with dCAD or another molecule that inhibits the
beads were thoroughly washed with buffer B containing 150 mM NaCl. The cytosolic fraction from S2 cells (S-100, 11 mg protein) was loaded onto a DEAE-Sepharose column (1 ml) equilibrated with 10 mM Hepes-KOH buffer (pH 7.2) containing 50 mM NaCl, 0.1 mM DTT, 0.15% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 0.1 mM pAPMSF. Proteins were eluted from the column with a 50–350 mM linear NaCl gradient, and 0.5-ml fractions were collected. Using 10-μl aliquots, the dCAD activity in the indicated fractions was determined in the presence or absence of 0.5 mg of caspase 3. 10-μl aliquots were also separated by electrophoresis on a 4–20% polyacrylamide gel and analyzed by Western blotting with anti-DREP/dICAD antibody. When the immunoprecipitate was treated with caspase 3, it showed strong DNase activity. These results confirmed that dCAD was complexed with dICAD in Drosophila cells (Fig. 6B).

A Drosophila neuronal cell line BG2-c2 undergoes apoptosis by treatment with a kinase inhibitor, H-7, and this process is accompanied by DNA fragmentation (27). We previously reported that dICAD is cleaved during the apoptotic process of BG2-c2 cells (23). To confirm the involvement of the dICAD-dCAD system in the DNA fragmentation during apoptosis of Drosophila cells, the expression plasmid for the caspase-noncleavable form of dICAD/DREP-1 was introduced into BG2-c2 cells, and the stable transformants expressing the mutant dICAD/DREP-1 were established (Fig. 7A). As shown in Fig. 7B, the DNA fragmentation was observed in the parental BG2-c2 cells after treatment with H-7, whereas no DNA fragmentation was induced in the BG2-c2 cell transformants expressing the caspase-resistant form of dICAD/DREP-1.

Expression of dICAD during Early Embryogenesis—To determine the expression of dICAD mRNA during Drosophila development, mRNA was prepared from embryos at various stages, larvae, and pupae. Poly(A) mRNA was also prepared from adult male and female flies as well as from S2 cells. As shown in Fig. 8A, Northern hybridization indicated that the dICAD mRNA of about 2.2 kb was expressed abundantly in the early stage of embryogenesis (0–3 h), which is before the onset of zygotic transcription, indicating that the expression of the dICAD gene was maternally regulated. The expression level of the dICAD mRNA rapidly decreased in the later stages of embryogenesis, and no dICAD mRNA was detected in the 16- to 24-h embryonic and larval stages. When the flies entered the pupal stage, they started to express dICAD mRNA again, and both male and female adult flies expressed abundant levels of dICAD mRNA. S2 cells that were established from Drosophila...
embryos also expressed dICAD mRNA.

We then examined the distribution of dICAD protein in embryos by whole-mount immunohistochemical analysis using the DREPV1-specific antibody. As shown in Fig. 8b, the dICAD protein was detected uniformly in embryos at early and late stages (Fig. 8b, A and B). No specific signal was detected when the antibody was preadsorbed by the recombinant dICAD/DREPV1 (Fig. 8b, C and D).

**FIG. 8. Expression of dICAD in Drosophila.** a, Northern hybridization with dICAD cDNA was carried out with 2 μg of poly(A) RNA from the following developmental stages: 0- to 3-h embryo (lane 1), 3- to 16-h embryo (lane 2), 16- to 24-h embryo (lane 3), first and second instar larva (lane 4), third instar larva (lane 5), early pupa (lane 6), late pupa (lane 7), adult male (lane 8), and adult female (lane 9). Lane 10 contains 2 μg of poly(A) RNA from S2 cells. The membrane filter used for hybridization was stained with methylene blue and is shown at the bottom. b, immunohistochemical analysis. The whole-mount embryos (A and C, 0- to 3-h embryo; B and D, 3- to 16-h embryo) were immunohistochemically analyzed with anti-dICAD antibody (A and B) or the anti-dICAD antibody (C and D), which had been preincubated for 1 h at room temperature with the equal amount of His-tagged DREPV1. A scale bar (100 μm) is shown below panel D.

**DISCUSSION**

In this report, we identified ICAD in *D. melanogaster*. Although the homology between DREP-1/dICAD and mouse or human ICAD is not very high (about 17% identity), DREP-1/dICAD fulfilled the criteria to be identified as ICAD. That is, it was complexed with dCAD, inhibited dCAD DNase activity, and could be cleaved by caspase 3, resulting in the loss of its inhibitory activity. There are two alternatively spliced forms (ICAD-L and ICAD-S) for human and mouse ICADs, and these are expressed to a similar extent in a variety of different cell types (6, 29, 33). In contrast, *Drosophila* S2 cells expressed only a single form of ICAD corresponding to ICAD-L. The gene structure of dICAD also ruled out the possibility of generating an alternatively spliced form corresponding to ICAD-S. We recently showed that ICAD-L but not ICAD-S has a chaperone-like activity in the production of functional CAD, although both forms of ICAD can equally inhibit the CAD DNase activity (29). The lack of ICAD-S in *Drosophila* may indicate that there is no specific role for ICAD-S beyond the regulation of CAD activity.

Human and murine ICADs carry two recognition sites for caspase 3 (6, 8), whereas only a single caspase 3 cleavage site was found in dICAD. The cleavage site in dICAD is located close to the site corresponding to the first N-terminal cleavage site in mouse and human ICADs (Fig. 3A). Human ICAD-L cleaved at both sites dramatically loses affinity for CAD, whereas ICAD-L cleaved singly at either site still has significant affinity for CAD (34). On the other hand, cleavage of dICAD by caspase 3 at the single site abolished its ICAD activity, suggesting that the cleaved dICAD loses its ability to bind dCAD. In *Drosophila*, but not in mammalian systems, CAD (dCAD) is also cleaved by caspase 3 to be activated (23). Thus, when caspase 3 is activated in *Drosophila*, the single cleavage of dICAD/DREPV1 may be sufficient to release activated, cleaved dCAD, whereas two cleavages are required in mammalian systems.

The overall similarity between dICAD and mammalian ICADs (mICAD/hICAD), is low (32.2% similarity). Accordingly, there is species specificity in the CAD-ICAD system between mammals and *Drosophila*. That is, although mouse ICAD inhibited dCAD as efficiently as mCAD, dICAD could not inhibit mCAD DNase. In addition to the inhibitory activity against CAD DNase, mouse and human ICAD function as specific chaperones to facilitate the correct folding of CAD (6, 29, 35). *Drosophila* DREP-1/dICAD also had this chaperone-like function for dCAD in COS cells or in a cell-free system. However, it did not assist in the correct folding of mouse CAD, and mICAD could not support production of functional CAD (23), indicating that the species specificity was more strict for the chaperone-like activity than for the inhibitory activity against CAD. ICAD and ICAD can interact with each other, and this interaction seems to be important for the chaperone-like activity of ICAD (35). In this regard, it is interesting to note that the CAD domains between dICAD and dCAD, and between mICAD and mCAD, are more conserved (32% and 37% identity, respectively) than between dCAD and mCAD (Fig. 9).

Expression of the caspase-resistant form of dICAD prevented the apoptotic DNA fragmentation in a *Drosophila* cell line of BG2-c2, indicating that ICAD should be cleaved by a caspase to cause the DNA fragmentation. The finding of dICAD complexed with dCAD indicates that the dICAD-dCAD system is solely responsible for the cell-autonomous DNA fragmentation in *Drosophila* cells. Massive programmed cell death occurs during the embryogenesis and metamorphosis of *Drosophila* (15, 16). Like several other apoptosis-related genes (21, 22, 36),
the expression of the dICAD gene seems to be regulated maternally, and its mRNA and protein could be found during early embryogenesis. The dICAD mRNA level decreased later in embryogenesis, and the gene was reactivated when flies entered metamorphosis. The metamorphosis of flies is regulated by a steroid hormone, ecdysone. In the promoter region of the dICAD gene (a 1.5-kb DNA fragment between the RNA polymerase III gene and the first exon of the dICAD gene), there are several potential binding sites for the ecdysone-responsive elements, the E74A protein or the Broad complex (37, 38). It will be interesting to examine whether the ICAD gene is in fact regulated by ecdysone and whether the elements in the promoter region are responsible for its expression. We have recently found, in the mouse system, that the apoptotic DNA fragmentation is mediated not only cell autonomously by the CAD-ICAD system but also by phagocytes (39). Mutational analyses of the dICAD and/or dCAD gene in Drosophila will answer whether a similar auxiliary system for the apoptotic DNA fragmentation also exists in Drosophila or not and may reveal physiological roles for apoptotic DNA fragmentation during animal development.

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FIG. 9. Alignment of the CAD/CIDE domains. The N-terminal amino acid sequences of Drosophila ICAD (dICAD), Drosophila CAD (dCAD), mouse ICAD (mICAD), and mouse CAD (mCAD) are aligned to give maximum homology by introducing several gaps (−). The identical residues identical between dICAD and dCAD, and between mICAD and mCAD are shown in bold, and residues identical among all four proteins are underlined.

dICAD: METAMGDSKPKPFDVTRNKIKVASSLEIEISKVAESK-----ECDNL---P (1−50)
dCAD: SKRGTGAPWNRQKTISRTKDYGANSMLAVKAKSKW----PLLEE--- (37−84)
mICAD: SAPPDDVRLFPRCLRRHNRDDQGVASSLEISLASCALLIKITPI (8−59)
mCAD: MCAVRQPCCKVLKALHSACKFVAQPSQCELRCVRF------QLPMPSG (47−85)

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