Functional Differences of Two Forms of the Inhibitor of Caspase-activated DNase, ICAD-L, and ICAD-S*

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Caspase-activated DNase (CAD) is responsible for the DNA fragmentation that occurs during apoptosis. CAD is complexed with an inhibitor of CAD (ICAD) in non-apoptotic, growing cells. Here, we report that mouse WR19L and human Jurkat T lymphoma cells express two alternative forms of ICAD, ICAD-L and ICAD-S, at similar levels. CAD was predominantly associated with ICAD-L in these cell lines. When CAD was expressed alone in Sf9 cells, it was found in insoluble fractions. However, when CAD was co-expressed with ICAD-L and ICAD-S, it was recovered as a soluble protein complexed predominantly with ICAD-L. In vitro transcription and translation of CAD cDNA did not produce a functional protein. Addition of ICAD-L but not ICAD-S to the assay mixture resulted in the synthesis of functional CAD. These results indicated that ICAD-L but not ICAD-S works as a specific chaperone for CAD, facilitating its correct folding during synthesis. Recombinant CAD, as a complex with ICAD-L, was then produced in Sf9 cells. The complex was treated with caspase 3, and CAD was purified to homogeneity. The purified CAD had DNase activity with a high specific activity.

Apoptosis is a process by which cells can be eliminated in response to a wide range of stimuli, such as death factors (Fas ligand, tumor necrosis factor, and TRAIL), anti-cancer drugs, γ or UV radiation, and factor deprivation (1–4). This process is characterized morphologically by cell shrinkage accompanied by blebbing of the plasma membranes, and condensation and fragmentation of the nuclei (5–7). It is also characterized biochemically by internucleosomal degradation of chromosomal DNA (8, 9).

Apoptosis is driven by members of the caspase family, which are cysteine proteases (10). At least 14 caspase family members have been identified, and they are divided into 3 subgroups based on their substrate specificity (11, 12). Different apoptotic stimuli appear to sequentially activate different sets of caspases, which ultimately cleave various cellular substrates to cause the morphological changes in cells and nuclei (6). The DNA fragmentation that occurs during apoptosis also depends on caspase activation. We and others have recently identified a caspase-activated DNase (CAD),1 which is also called a caspase-activated nuclease or DNA fragmentation factor-40 (DFF-40) (13–16). CAD is complexed with its inhibitor (ICAD), also called DFF-45, in growing, non-apoptotic cells. Mouse CAD is a basic protein consisting of 344 amino acids, while ICAD is an acidic protein with two recognition sites for caspase 3. When caspase 3 is activated by apoptotic stimuli, ICAD is cleaved, resulting in the release of CAD. This released CAD appears to cause DNA fragmentation in nuclei.

Molecular cloning of mouse ICAD cDNA indicated that there are two forms of ICAD, designated ICAD-L and ICAD-S for the long and short forms, respectively. ICAD-L and ICAD-S are composed of 331 and 265 amino acids, respectively (13). They are translated from two different mRNAs produced by alternative splicing.2 Purification of the latent form of CAD from human HeLa cells indicated that the ICAD that is associated with CAD is ICAD-L (17). However, purification of mouse ICAD by following its activity led us to identify ICAD-S (13). Thus, it was not clear whether both ICAD-L and ICAD-S proteins were expressed in mouse and human cells, and whether they had any functional differences.

In this study, we showed that mouse and human lymphoma cell lines constitutively express both ICAD-L and ICAD-S at similar levels. Fractionation of the cell lysates from human Jurkat cells, and from Sf9 cells co-infected with a recombinant baculovirus containing ICAD and CAD indicated that CAD is complexed predominantly with ICAD-L. ICAD-L but not ICAD-S supported production of the functional CAD protein in vitro. These results indicated that ICAD-L but not ICAD-S has a chaperone-like function for CAD, and that it remains complexed with CAD. Recombinant CAD was produced in insect cells as a complex with ICAD-L, and was homogeneously purified after treatment with caspase 3. The purified CAD showed high DNase activity.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Recombinant Proteins

Mouse T-cell lymphoma WR19L and human Jurkat cells were maintained at 37 °C in RPMI medium containing 10% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD). Spodoptera frugiperda (Sf9) insect cells (Invitrogen, Carlsbad, CA) were cultured at 27 °C in TNM-FH medium (Invitrogen) supplemented with 10% fetal calf serum. Anti-human ICAD/DFF-45 (clone 6B8) and anti-FLAG (clone M2) monoclonal antibodies were purchased from MBL (Nagoya, Japan) and Kodak (Rochester, NY), respectively. Affinity purified, anti-murine ICAD polyclonal antibody was custom prepared at MBL by immunizing

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1 The abbreviations used are: CAD, caspase-activated DNase; ICAD, inhibitor of CAD; DFF, DNA fragmentation factor; GST, glutathione S-transferase; p-APMSF, p-amino-phenylmethylene sulfonyl fluoride hydrochloride; m.o.i., multiplicity of infection.

2 K. Kawane, H. Fukuyama, H. Sakahira, and S. Nagata, unpublished results.
rabbits with recombinant murine ICAD-L. Anti-murine CAD antiserum was custom prepared at the Peptide Institute (Osaka, Japan) by immunizing rabbits with a C-terminal peptide (RRKQPARKKRRPKRK, amino acids 330–344). The antiserum was affinity purified using AF-amino Toyopearl beads (Tosoh Co., Tokyo, Japan) to which the peptide had been attached using m-maleimidobenzoyl-N-hydroxysuccinimide esoter (Pierce, Rockford, IL). The horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from DAKO (Denmark) and Transduction Laboratories (Lexington, KY), respectively. The recombinant ICAD fused with glutathione S-transferase (GST), and the histidine-tagged recombinant human caspase 3 was prepared in Escherichia coli as described previously (18).

**Assay for CAD and ICAD**

The activities of CAD and ICAD were determined with mouse liver nuclei or plasmid DNA as described previously (13). For the ICAD assay, the S-100 fraction from Fas-activated mouse W4 cells (13) or purified recombinant CAD produced by SF9 cells (see below) was used as active CAD.

**Western Blotting and in Vitro Transcription/Translation**

Western blotting was performed as described previously (19). In brief, proteins were separated by electrophoresis in a 12.5 or 10–20% gradient polyacrylamide gel (Dai-iichi Chemical, Tokyo, Japan), and electrophoretically transferred to a polyvinylidene difluoride membrane (Hybond P, Amersham-Pharmaccia, Arlington Heights, IL) at 100 V for 1 h. After treatment with 25% Block Ace (Dai-Nihon Pharmaceutical, Osaka Japan) or 5% skim milk, the filters were incubated with primary antibodies against recombinant CAD, mouse CAD, mouse ICAD, or FLAG-epitope. Horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG was used as the secondary antibody, and proteins were visualized using the enhanced chemiluminescence system (Renaissance; NEN Life Science Products Inc., Boston, MA). The in vitro transcription/translation was carried out using a kit from Promega (Madison, WI) as described previously (13).

**Production and Purification of ICAD and CAD in SF9 Cells**

Production of ICAD and the ICAD-CAD Complex in SF9 Cells—DNA fragments coding for FLAG-tagged mouse ICAD-L and ICAD-S (F-ICAD-L and F-ICAD-S) were described previously (18). These fragments were cloned into the pBlueBac III vector (Invitrogen), and high titer recombinant baculoviruses were generated using the MaxBac baculovirus expression system according to the manufacturer’s instructions. To produce F-ICAD-L, F-ICAD-S, and the F-ICAD-CAD complex, SF9 cells were infected at a multiplicity of infection (m.o.i.) of 5.0 with the recombinant baculovirus. The cells were incubated at 27 °C for 3 days before harvest.

Preparation of Cell Lysates—Cell lysates from the infected SF9 cells were prepared essentially as described previously (13). In brief, cells were disrupted by three cycles of freezing and thawing in buffer A (10 mM HEPES-KOH, pH 7.2, 2 mM MgCl2, 5 mM EGTA, 50 mM NaCl, and 1 mM dithiothreitol) supplemented with protease inhibitors (1 mM p-APMSF [(p-amino-phenylmethanesulfonyl fluoride hydrochloride), 3 μg/ml aprotinin, 3 μg/ml leupeptin, and 3 μg/ml pepstatin A]. The lysates were spun for 30 min at 30,000 × g, and the supernatant fractions were used as S-30 fractions. The S-30 fractions were spun for 2 h at 100,000 × g to obtain the S-100 fraction. Glycerol was added to the S-30 and S-100 fractions at a final concentration of 10% (v/v).

Purification of the ICAD-CAD Complex—To purify the F-ICAD-L/CAD complex, the S-100 fraction from SF9 cells co-infected with the F-ICAD-L and CAD baculoviruses was applied to a DEAE-Sepharose FF column equilibrated with buffer B (10 mM HEPES-KOH, pH 7.2, 2 mM MgCl2, 5 mM EGTA, 10% glycerol, 0.1 mM dithiothreitol and 0.1 mM p-APMSF) containing 50 mM NaCl, and eluted with a 50–350 mM linear NaCl gradient. The active fractions were applied to an SP-Sepharose FF column, and eluted with a 50–600 mM linear NaCl gradient. The ICAD-CAD complex, which eluted at 350–500 mM NaCl, was then applied to a HiTrap Heparin column, and eluted with a 150–700 mM linear NaCl gradient. About 3 mg of F-ICAD-L/CAD complex was obtained from 1000 ml of SF9 culture.

Preparation of Active CAD—About 2.5 mg of purified F-ICAD-L/CAD complex was treated at 25 °C for 36 h with 25 μg of recombinant caspase 3 in buffer B supplemented with 2 mM dithiothreitol. The sample was applied to a HiTrap Heparin column (5 ml) equilibrated with buffer B (buffer B without glycerol) containing 300 mM NaCl, and eluted with a 300–1000 mM linear NaCl gradient. About 0.2 mg of active CAD was obtained.

**Fig. 1. Two forms of ICAD in mouse and human cells.** The S-100 fraction was prepared from mouse WR19L (A) and human Jurkat cells (B) as described previously (13). Twenty micrograms of protein before (lane 1) and after (lane 2) treatment with 0.2 μg of caspase 3 at 30 °C for 2 h were analyzed by Western blotting with rabbit anti-mouse ICAD antibody (A) for anti-human ICAD/DPFF-45 monoclonal antibody (B). As a control for anti-mouse ICAD antibody, the S-100 fraction from mouse WR19L cells was also analyzed by Western blotting with rabbit non-immune serum (lane 3). The sizes of molecular marker proteins are shown in kilodaltons on the left, and the bands for ICAD-L (L) and ICAD-S (S) are indicated by arrows on the right.

**Miscellaneous**—All purification steps were carried out using an fast protein liquid chromatography system (Amersham-Pharmacia) at 4 °C. Protein concentration was determined by the bicinchoninic acid method using a kit from Pierce. Quantification of homogeneous preparations of recombinant proteins was carried out by measuring A280 and using extinction coefficients calculated as described previously (20).

**RESULTS**

**Two Forms of ICAD and Predominant Association of CAD with ICAD-L**—We previously identified two forms of mouse ICAD cDNAs coding for ICAD-L and ICAD-S (13). To examine whether ICAD-L and ICAD-S proteins are indeed expressed, cell lysates were prepared from mouse WR19L and human Jurkat cells, and analyzed by Western blotting. As shown in Fig. 1A, the polyclonal antibody against mouse ICAD detected two bands at apparent Mr of 45,000 and 34,000. These two bands were not detected by rabbit non-immune serum. Sizes of the two proteins agreed with those detected in the lysates from SF9 cells infected with baculovirus carrying ICAD-L and ICAD-S, respectively. When the cell lysates were treated with caspase 3, both bands disappeared. These results indicated that the 45,000 and 34,000 bands represented ICAD-L and ICAD-S, respectively. ICAD-L appeared to be more abundant than ICAD-S in WR19L cells. The cell lysates from human Jurkat cells also showed two bands at Mr of 45,000 and 34,000 in Western blotting using an anti-human ICAD/DPFF-45 monoclonal antibody as the probe (Fig. 1B). Both bands were cleaved by caspase 3. In Jurkat cells, ICAD-S appeared to be more abundant than ICAD-L.

Mouse WR19L and human Jurkat cells carry a latent form of CAD (proCAD) that exists as a complex with ICAD, and can be activated by caspase 3 (13, 16, 18). When the S-100 fraction from Jurkat cells was loaded onto an SP-Sepharose column in buffer containing 50 mM NaCl, proCAD was retained in the column, and eluted with 600 mM NaCl (Fig. 2A). The S-100 fraction also contained the ICAD activity, which was recovered in the flow-through fractions (Fig. 2B). To determine which form of ICAD, ICAD-L or ICAD-S, is associated with CAD, the flow-through and bound fractions were analyzed by Western blotting with the anti-ICAD antibody. As shown in Fig. 2C, the ICAD in the flow-through fraction was predominantly ICAD-S, while ICAD-L was predominant in the bound fractions. Rechromatography of the bound fractions on SP-Sepharose showed co-elution of ICAD-L with the proCAD activity in a linear NaCl gradient. The ICAD activity in the flow-through fraction of SP-Sepharose was bound to a DEAE-Sepharose column, and co-eluted with ICAD-S in a linear NaCl gradient (data not shown). These results indicated that CAD is predominantly associated with ICAD-L. ICAD-S exists mainly in a free form. Similar data were obtained using the S-100 fraction from
mouse WR19L cells (data not shown).

**Predominant Incorporation of ICAD-L into the ICAD-CAD Complex**—To confirm the predominant association of CAD with ICAD-L, recombinant baculoviruses containing F-ICAD-L, F-ICAD-S, and CAD were constructed, and used to infect Sf9 cells. After culturing for 24 h, the cell lysates were spun at 30,000 \( \times g \) for 30 min, and the supernatant and precipitate fractions were designated as S-30 and P-30, respectively. As shown in Fig. 3A, the S-30 fraction from the parental Sf9 cells or the cells infected with F-ICAD-L, F-ICAD-S, or CAD baculovirus alone did not show CAD activity. However, when the cells were co-infected with baculoviruses containing CAD and F-ICADs, the CAD activity was detected in the S-30 fraction (Fig. 3A). The CAD activity in the S-30 fraction increased when the m.o.i. of the CAD baculovirus was increased. Proteins in the S-30 and P-30 fractions were then analyzed by Western blotting using the anti-FLAG antibody recognizing F-ICAD. As shown in Fig. 3B, two bands of an equal intensity at \( M_r \) of 45,000 and 34,000 were exclusively detected in the S-30 fraction, suggesting that both ICAD-L and ICAD-S are soluble proteins. In contrast, when Sf9 cells were infected with the CAD baculovirus alone, the CAD protein with \( M_r \) of 40,000 was exclusively found in the P-30 fraction, suggesting that CAD formed aggregates. However, co-expression of CAD with ICADs in Sf9 cells resulted in soluble CAD (Fig. 3C). At lower expression levels of CAD, all of the CAD was found in the S-30 fraction. When CAD was overexpressed, some of it was detected in the P-30 fraction. The expression levels of ICAD-S and ICAD-L, and their solubility, were not affected by the expression of CAD. These results suggest that the soluble and functional proCAD could be produced only in the presence of ICAD.

**To determine whether ICAD-L, ICAD-S, or both form a complex with CAD, the cell lysates from Sf9 cells infected with various dosages of CAD and ICAD baculovirus were loaded onto SP-Sepharose in 50 mM NaCl.** The proCAD activity was retained on the column, and eluted with 600 mM NaCl (Fig. 4B). ICAD-L and ICAD-S in the cell lysates from Sf9 cells infected with ICAD-L and ICAD-S baculovirus were found in the flow-through fractions (Fig. 4A). When CAD was co-expressed with ICAD-L and ICAD-S, ICAD-L but not ICAD-S bound to the column, and co-eluted with CAD. At the highest concentration of CAD, all of the ICAD-L protein was found in the bound fractions, while only a small portion of the ICAD-S bound to the column. These results indicated that CAD predominantly associates and forms a complex with ICAD-L during its synthesis in Sf9 cells.

**ICAD-L Supports Synthesis of the Functional CAD in Vitro**—Predominant association of ICAD-L with CAD during CAD synthesis suggested that ICAD-L, but not ICAD-S works as a chaperone to facilitate the correct folding of CAD during its synthesis. To examine this possibility, ICAD-L and ICAD-S were prepared in *Escherichia coli* as GST fusion proteins. At first, the inhibitory activity of ICAD was determined with the purified CAD (see below). As shown in Fig. 5A, both ICAD-L and ICAD-S efficiently inhibited CAD’s DNase activity at a molar ratio of 5–10 for ICAD to CAD, suggesting that ICAD-L and ICAD-S have comparable ability to bind intact CAD. When CAD cDNA was transcribed and translated in vitro, no functional CAD was produced (Fig. 5B), although CAD protein was synthesized (data not shown). The presence of ICAD-L in the reaction mixture stimulated synthesis of functional CAD in a dose-dependent manner. In contrast, almost no functional CAD was produced in the presence of ICAD-S.

**Production of Recombinant CAD**—To produce CAD in a large quantity, Sf9 cells were infected with baculoviruses of CAD and ICAD-L at m.o.i. of 5.0, and the ICAD-L-CAD complex was purified from the cell lysates by successive column chromatographies of DEAE-Sepharose, SP-Sepharose, and HiTrap Heparin column. As shown in Fig. 6A, the purified ICAD-CAD

![Image](https://example.com/image.png)
complex showed bands of $M_\text{r}$ of 45,000 and 40,000 on SDS-polyacrylamide gel electrophoresis, which correspond to ICAD-L and CAD, respectively. Treatment of the ICAD-CAD complex specifically cleaved ICAD into fragments of $M_\text{r}$ 18,000, 12,000, and 12,000. Although the cleaved ICAD-L fragments had some affinity to CAD, removal of glycerol from buffer reduced its affinity to CAD. Therefore, the ICAD-L-CAD complex after treatment with caspase 3 was loaded onto a heparin column, and the column was developed with a linear NaCl gradient in a buffer without glycerol. Under these conditions, CAD was recovered as a soluble protein preferentially complexed with ICAD-L. These results agree with previous reports that DFF (proCAD) is purified from human HeLa cells after culturing the cells for 24 h. The S-30 fractions (100 μg of protein) in 100 μl of buffer B containing 150 mM NaCl were incubated with 20 μl (bed volume) of SP-Sepharose at 4 °C for 1 h. The proteins that did not bind to the column were recovered as the flow-through fractions (A), while the proteins bound to the Sepharose beads were eluted with 100 μl of buffer B containing 600 mM NaCl (B). Top panels, aliquots (1 μl of the flow-through and bound fractions were treated with caspase 3, and the CAD activity was determined with plasmid DNA as the substrate. Middle and bottom panels, proteins (4-μl aliquots) in the flow-through and bound fractions were separated by electrophoresis on a 12.5% polyacrylamide gel, and analyzed by Western blotting using anti-FLAG (middle panels) or anti-CAD (bottom panels) antibodies. Sizes of the standard proteins are shown in kilodaltons on the left. The bands corresponding to ICAD-L (L), ICAD-S (S), and CAD are indicated by arrows on the right.

**DISCUSSION**

Apoptosis induced by various stimuli is mediated by caspases, which cleave various cellular substrates to promote the apoptotic process (10). One of these substrates is ICAD, also known as DFF-45 (13, 17, 18). Once ICAD/DFF-45 is cleaved by caspase-3, CAD (also known as caspase-activated nuclease or DFF-45) is released, is activated, and causes the characteristic fragmentation of chromosomal DNA into nucleosomal units. Characterization of mouse ICAD cDNA indicated that there are two different ICAD mRNAs (ICAD-L and ICAD-S), which can be generated by alternative splicing. Although both forms of ICAD are expressed at similar levels, CAD was predominantly associated with ICAD-L in mouse and human cells. This was confirmed by expressing CAD and ICAD in Sf9 cells, from which CAD was recovered as a soluble protein preferentially complexed with ICAD-L. These results agree with previous reports that DFF (proCAD) is purified from human HeLa cells after culturing the cells for 24 h. The S-30 fractions (100 μg of protein) in 100 μl of buffer B containing 150 mM NaCl were incubated with 20 μl (bed volume) of SP-Sepharose at 4 °C for 1 h. The proteins that did not bind to the column were recovered as the flow-through fractions (A), while the proteins bound to the Sepharose beads were eluted with 100 μl of buffer B containing 600 mM NaCl (B). Top panels, aliquots (1 μl of the flow-through and bound fractions were treated with caspase 3, and the CAD activity was determined with plasmid DNA as the substrate. Middle and bottom panels, proteins (4-μl aliquots) in the flow-through and bound fractions were separated by electrophoresis on a 12.5% polyacrylamide gel, and analyzed by Western blotting using anti-FLAG (middle panels) or anti-CAD (bottom panels) antibodies. Sizes of the standard proteins are shown in kilodaltons on the left. The bands corresponding to ICAD-L (L), ICAD-S (S), and CAD are indicated by arrows on the right.

**FIG. 4. Co-fractionation of CAD with ICAD-L.** Sf9 cells were infected with recombinant baculoviruses (ICAD-L, ICAD-S, and CAD) at m.o.i. as indicated above each lane, and S-30 fractions were prepared after culturing the cells for 24 h. The S-30 fractions (100 μg of protein) in 100 μl of buffer B containing 150 mM NaCl were incubated with 20 μl (bed volume) of SP-Sepharose at 4 °C for 1 h. The proteins that did not bind to the column were recovered as the flow-through fractions (A), while the proteins bound to the Sepharose beads were eluted with 100 μl of buffer B containing 600 mM NaCl (B). Top panels, aliquots (1 μl of the flow-through and bound fractions were treated with caspase 3, and the CAD activity was determined with plasmid DNA as the substrate. Middle and bottom panels, proteins (4-μl aliquots) in the flow-through and bound fractions were separated by electrophoresis on a 12.5% polyacrylamide gel, and analyzed by Western blotting using anti-FLAG (middle panels) or anti-CAD (bottom panels) antibodies. Sizes of the standard proteins are shown in kilodaltons on the left. The bands corresponding to ICAD-L (L), ICAD-S (S), and CAD are indicated by arrows on the right.

**FIG. 5. Chaperone-like activity of ICAD-L for CAD synthesis.** A, CAD-inhibitory activity of ICAD. Pure CAD (70 fmol) was incubated at 25 °C for 10 min without (lane 1) or with GST (lane 2, 1,500 fmol), GST-ICAD-L (lane 3, 75 fmol; lane 4, 375 fmol; lane 5, 1,500 fmol), or GST-ICAD-S (lane 6, 75 fmol; lane 7, 375 fmol; lane 8, 1,500 fmol). The CAD activities were then determined with plasmid DNA as the substrate. B, synthesis of functional CAD in a cell-free system. CAD cDNA was subjected to the in vitro transcription and translation coupled system in the presence of increasing amounts of GST (lanes 2–5), GST-ICAD-L (lanes 6–9), and GST-ICAD-S (lanes 10–13). Using 3-μl aliquots of the reaction mixtures, the CAD activity with plasmid DNA as a substrate was determined in the presence (upper panels) or absence (lower panels) of caspase-3. The amount of proteins added to the assay mixture were: lane 1, 0 pmol; lanes 2, 6, and 10, 0.75 pmol; lanes 3, 7, and 11, 1.5 pmol; lanes 4, 8, and 12, 3.75 pmol; and lanes 5, 9, and 13, 7.5 pmol.

**FIG. 6. Purification of the recombinant ICAD, ICAD-CAD complex, and CAD.** A, analysis of the purified proteins by SDS-polyacrylamide gel electrophoresis. About 1.5 μg of the purified recombinant F-ICAD-L-CAD complex (lane 1) and CAD (lane 2) were fractionated by electrophoresis on a 10–20% polyacrylamide gel, and stained with Coomassie Brilliant Blue. B, DNase activity of the purified CAD. Plasmid DNA (1 μg) was incubated at 30 °C for 2 h with the purified CAD, and analyzed by electrophoresis on a 1.5% agarose gel. The amounts of CAD used were: lane 1, 0 fmol; lane 2, 2.8 fmol; lane 3, 7.0 fmol; lane 4, 14 fmol; lane 5, 28 fmol; lane 6, 70 fmol; lane 7, 140 fmol.
as a complex with DFF-45 (ICAD-L) (17). The expression of CAD alone in Sf9 cells caused aggregation of the protein, and ICAD-L but not ICAD-S supported production of functional CAD in a cell-free system, suggesting a chaperone-like activity for ICAD-L but not ICAD-S. It is likely that during synthesis of CAD on the ribosome, ICAD-L binds to the nascent chain of CAD and facilitates its correct folding. Mice deficient in the ICAD gene express CAD, but it does not cleave chromosomal DNA during apoptosis (21), which agrees with our results indicating an absolute requirement for ICAD to produce functional CAD.

ICAD-L and -S are generated from a single gene with alternative splicing.² Alternative splicing often gives rise to products with different functions. It has been reported that alternative splicing can also affect the folding property of a chaperone-like protein (22). α-Crystallin is a member of the small heat shock protein family, and has a chaperone-like activity in suppressing nonspecific aggregation of denatured proteins in vitro (23). One of the subunits of α-crystallin (αA-subunits) has a minor product (αAins-crystallin) that carries an insertion of 23 amino acids, which is produced by alternative splicing. Both αA- and αAins-crystallin display similar heat-resistance, self-renaturation, and oligomerization properties, but αAins-crystallin has less chaperone-like activity than αA-crystallin. Similarly, both ICAD-L and ICAD-S are resistant to heat and easily renaturate (13).³ Although they interacted with intact CAD with similar efficiency, only ICAD-L was associated with CAD in the cells, and supported production of functional CAD in vitro. ICAD-L is 66 amino acids longer than ICAD-S at the C terminus. This extra region of ICAD-L is probably responsible for its interaction with the nascent chain of CAD. In addition to facilitating folding of nascent polypeptides, chaperone-like proteins can enhance renaturation of their denatured target proteins (24, 25). Whether or not ICAD-L has such a property remains to be studied.

Previously, we have expressed CAD in COS cells and in reticulocyte lysates, and suggested that it has a DNase activity (13). However, since the preparation contained many other proteins, the possibility that CAD needs other co-factors to show its DNase activity was not formally ruled out. Here, we showed that the recombinant homogeneous CAD has the DNase activity with a high specific activity. If the functional CAD containing a nuclear localization signal is produced in cells, it will digest chromosomal DNA, and kills the cells. Cells have developed a device to avoid such a deleterious situation. That is, the correctly folded CAD is synthesized only in the presence of its inhibitor, ICAD-L. ICAD-L remains as a complex with CAD in the cells until it is cleaved by action of caspase 3. There is a precedent for such a heteromeric structure for a chaperone and its target protein. Prolyl 4-hydroxylase, which catalyzes a post-translational modification of collagen, consists of two distinct polypeptides forming an αβ₄ heterotetramer (26). The β-subunit has protein disulfide isomerase activity. The α-subunit forms highly insoluble aggregates without β-subunits (27, 28). However, in the presence of β-subunits/protein disulfide isomerase, the α-subunit is correctly folded with intramolecular disulfide bonds, and becomes a soluble protein to form an αβ₄ heterotetramer. CAD is also rich in cysteine residues (14 residues in 344 amino acids), and correctly folded only in the presence of ICAD-L. To examine how ICAD-L promotes the correct folding of CAD, it would be necessary to analyze whether the cysteine residues of CAD are disulfide-bonded, and thus whether any free thiol is involved in the folding of the protein.

ICAD-L but not ICAD-S was necessary to produce functional CAD, and ICAD-L can inhibit the CAD's DNase. However, we found that both ICAD-L and -S are expressed in various human and mouse cell lines. Why is ICAD-S expressed in living cells? The analyses of the CAD and ICAD gene promoters indicated that their expression is controlled by constitutive promoter as found in many housekeeping genes.⁴ Since the expression level of CAD can be controlled by ICAD-L, it is possible that splicing for ICAD-L is prevalent in the tissues undergoing apoptosis at high frequency, to generate more CAD. Another possibility is that ICAD-S works as a dominant-negative form for the chaperone-like function of ICAD-L. Death signaling molecules such as caspases, Ced-4, and Bcl-x (29–32) have alternatively spliced forms with opposite functions. Similarly, if the amount of ICAD-S is increased, the complex formation between ICAD-L and CAD may be down-regulated. This may provide a fine-tuning mechanism for apoptotic DNA fragmentation.

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² H. Sakahira and S. Nagata, unpublished results.
³ H. Kawane and S. Nagata, unpublished observation.