Specific Chaperone-like Activity of Inhibitor of Caspase-activated DNase for Caspase-activated DNase*

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Caspase-activated DNase (CAD) is the enzyme that causes DNA fragmentation during apoptosis. CAD forms aggregates when it is synthesized in the absence of an inhibitor of CAD (ICAD). Here, using renaturation systems of chemically denatured CAD, we report that ICAD-L, a long form of ICAD, has a chaperone-like activity specific for CAD. Murine CAD carries 14 cysteines, most of which were found to be in reduced form. Reducing agents enhanced the production of the functional CAD in an in vitro translation system. The denatured CAD could be efficiently renatured under highly reducing conditions only in the presence of ICAD-L. This process was ATP-independent. In contrast, reticulocyte lysates stimulated ICAD-L- and ATP-dependent renaturation of denatured CAD without requiring a high concentration of reducing agents. These results indicate that ICAD-L works not only as a specific inhibitor but also as a specific chaperone for CAD.

Apoptosis is a process by which cells can be eliminated in response to a wide range of stimuli such as cytokines, anticancer drugs, γ or UV irradiation, and factor deprivation (1–4). This process is characterized morphologically by the cell shrinkage with blebbing of the plasma membranes and nuclear condensation (5–7). It is also biochemically characterized by the internucleosomal degradation of chromosomal DNA (8, 9).

The apoptosis induced by most stimuli is mediated by members of the caspase family of cysteine proteases (10). The DNA fragmentation that occurs during apoptosis also depends on caspase activation. We and others (11–16) previously identified a caspase-activated DNase (CAD)1 of 40 kDa, also called a caspase-activated nuclease or DNA fragmentation factor 40 (DFF40). CAD is complexed with its inhibitor (ICAD-L), also called DFF45, as a latent form in growing non-apoptotic cells. When caspase 3 is activated by apoptotic stimuli, it cleaves ICAD-L at two sites. This cleavage causes the release of CAD, which degrades chromosomal DNA in nuclei. We previously found that when CAD is synthesized alone in insect cells, the protein forms aggregates and has no detectable DNase activity (17). However, active, soluble CAD protein was recovered as a complex with ICAD-L when CAD was co-expressed with ICAD-L, suggesting that ICAD-L is necessary for production of the functional CAD.

One of characteristics of CAD is a high content of cysteine residues (11). Mouse CAD consists of 344 amino acids, 14 of which are cysteines. In this report, we found that most, if not all, of the cysteine residues in mouse CAD exist as thiol form.

The ICAD-L-dependent production of functional CAD in reticulocyte lysates was enhanced by reducing agents and inhibited by oxidized glutathione, suggesting that the formation of disulfide bonds causes CAD to aggregate. Furthermore, ICAD-L enhanced the renaturation of the chemically denatured CAD in an ATP-independent manner under highly reducing conditions. In contrast, the reticulocyte lysates stimulated the renaturation of denatured CAD in the absence of a high concentration of reducing agents. This process was dependent not only on ICAD-L but also on ATP. These results suggest that reticulocyte lysates contain a factor(s) that keeps CAD at a reduced and folding competent state, in an ATP-dependent manner. They further suggest that this form of CAD is recognized specifically by ICAD-L, which helps it to acquire its final, appropriately folded form.

EXPERIMENTAL PROCEDURES

Enzymes and in Vitro Transcription and Translation—Recombinant mouse ICAD-L or ICAD-S fused with glutathione S-transferase (GST-ICAD-L or GST-ICAD-S) and hexahistidine-tagged recombinant human caspase 3 were prepared in Escherichia coli as described (11, 16). Recombinant FLAG-tagged mouse ICAD-L (F-ICAD-L) and the F-ICAD-L-CAD complex were produced in Sf9 cells and purified to homogeneity as described previously (17). Active CAD, free from ICAD-L, was prepared from the F-ICAD-L-CAD complex by treatment with caspase 3, and purified on a Hi-Trap heparin-agarose column as described (17). Potato aprotase was purchased from Sigma. Rabbit reticulocyte lysates were obtained from Promega.

The coupled transcription and translation were carried out using a TNT in vitro transcription and translation kit (Promega) as described previously (11). In brief, plasmid DNA (1 μg) carrying mouse CAD in a pBluescript vector was incubated at 30 °C for 2 h with 40 μl of the TNT reagents.

Assays for CAD, DNase I, and Luciferase—The CAD activity was determined by measuring DNase activity as described previously (11). In brief, plasmid DNA (1.0 μg) was incubated at 37 °C for 2 h with samples in 20 μl of buffer A (10 mM HEPES-KOH, pH 7.2, 50 mM NaCl, 20% (v/v) glycerol, 2 mM MgCl2, 5 mM EGTA, 0.1 mM (p-aminoethyl) methanesulfonfonyl fluoride (p-APMSF), 5 mM DTT, and 1 mg/ml bovine serum albumin) containing 150 ng of caspase 3. After the reaction, the DNA was extracted and analyzed by electrophoresis on a 1.5% agarose gel. The DNase activity was quantified by determining the concentra-

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tion of CAD that causes the complete disappearance of the intact plasmid DNA (17).

Two-step S-Alkylation of Mouse CAD and Mass Spectrometry Analysis—Mouse CAD was immobilized on a PVDF membrane and subjected to S-alkylation and protease digestion as described previously (18). In brief, the recombinant mouse CAD (1.0 μg) was spotted onto a PVDF membrane (Applied Biosystem), incubated at 25 °C for 30 min in the dark with 2 mg of 4-vinylpyridine (Wako Pure Chemicals) in 0.2 mM sodium acetate (pH 6.0), 0.1% SDS, 5% acetonitrile, and 8 mM guanidine hydrochloride. After successive washings with H2O and 2% acetonitrile, the filter was incubated at 25 °C for 1 h with 1 mg of DTT in 0.5 mM Tris- HCl, pH 8.5, 0.1% EDTA, 5% acetonitrile, and 8 mM guanidine hydrochloride. The reduced cysteine residues were then carboxymethylated at 25 °C for 15 min with 3 mg of iodoacetic acid in 1 N NaOH. The filter was washed with 2% acetonitrile and then with 0.1% SDS. The immobilized, alkylated CAD was digested with *Achromobacter protease I* as described (19), and the peptides released from the filter were analyzed by matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) mass spectrometry using a Perceptive Biosystem Voyager-DE/ RP as described (20).

Renaturation of CAD—Recombinant CAD (10 μg) was concentrated by acetone precipitation, dissolved in 20 μl of buffer B (10 mM HEPES- KOH, pH 7.2, 5 mM MgCl2, 5 mM EGTA, 50 mM KCl, 20% (v/v) glycerol) containing 10 mM DTT and 6 mM guanidine hydrochloride, and incubated at 30 °C for 60 min to denature the protein. The 0.5-μl aliquots of the reaction mixture were diluted 100-fold with buffer C (buffer B supplemented with 500 mM NaCl) containing 0.5 μM GST-ICAD-L and 100 μM DTT and incubated at 30 °C for 120 min.

To renature CAD in the presence of reticulocyte lysates, 10 μg of the recombinant mouse CAD was denatured in 20 μl of buffer D (10 mM HEPES-KOH, pH 7.2, 5 mM MgCl2, 0.5 mM EGTA, 50 mM KCl, and 10 mM DTT) containing 6 mM guanidine hydrochloride as described above. The denatured protein (0.5 μl of the reaction mixture) was diluted with 50 μl of buffer D containing reticulocyte lysates and 0.3 μM GST-ICAD-L and incubated at 30 °C for 120 min.

Preparation of Recombinant ICAD-CAD Complex in *E. coli*—The 1.0-kilobase pair coding sequence of mouse CAD cDNA (11) was inserted under the T7 promoter of pET expression vector, and the resultant plasmid was introduced into *E. coli* strain BL21 (DE3) (21). The cells were grown in 400 ml of 2x YT medium at 37 °C until A600 reached 0.8 and were pelleted, and the cells were resuspended in 40 ml of buffer E (10 mM HEPES-KOH buffer, pH 7.2, containing 2 mM MgCl2, 5 mM EGTA, 10% (v/v) glycerol, 0.1 mM DTT, and 0.1 mM p-APMSF) and disrupted by sonication with 3 cycles of freezing and thawing. The lysates were spun for 30 min at 30,000 × g, and the precipitates were resuspended in 6 ml of buffer B containing 10 mM DTT and 7 mM guanidine hydrochloride. After incubation at 30 °C for 2 h, the suspensions were spun for 30 min!000 × g, and the supernatant fractions were diluted 100-fold with buffer C containing 0.3 μM GST-ICAD-L. The mixture was incubated at 30 °C for 120 min and applied to a 6-ml glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). The GST-ICAD-L-CAD complex was eluted with 20 mM glutathione in 100 mM Tris-HCl buffer, pH 9.6, containing 10% glycerol, 0.1 mM DTT, and 0.1 mM p-APMSF and further purified on a Mono S HR5/5 column (Amersham Pharmacia Biotech) with a 150–650 mM linear NaCl gradient in buffer E. Approximately, 2 mg of GST-ICAD-L-CAD complex was obtained.

**RESULTS**

**Effect of Reducing Agents on the Activity and Synthesis of CAD**—Mouse and human CADs carry 14 and 11 cysteine residues, respectively, 10 of which are conserved between them (15). The cytosol of mammalian cells is in a highly reduced state (22), where CAD is localized, suggesting that all cysteine residues of CAD can be in reduced form. To examine whether these cysteine residues form disulfide bonds or exist as thiols, the cysteine residues of mouse CAD were alkylated in two steps. In the first step, the denatured mouse CAD was immobilized on a PVDF membrane, and the free thiols were alkylated with 4-vinylpyridine under nonreducing conditions. Any disulfide bonds in CAD were then reduced by treatment with a high concentration of DTT and were alkylated with iodoacetic acid. The alkylated CAD was then digested with *Achromobacter protease I* and released several peptides. Molecular masses of the peptides were determined by MALDI-MS (Fig. 1) and compared with those expected for pyridylethylated or carboxymethylated peptides. As shown in Table I, molecular masses of 5 cysteine-containing peptides agreed well with the masses expected for S-pyridylethylated peptides, indicating that Cys-2, -20, -29, -30, and -317 do not form disulfide bonds. Double digestion of the alkylated CAD with *Achromobacter protease I* and Asp-N released additional peptides. Analysis of these peptides with MALDI-MS indicated that Cys-37, -50, -197, -221, and -238 are also pyridylethylated, and no carboxymethylated peptides were detected (data not shown). Although the status of the other four cysteine residues (Cys-10, -62, -163, and -229) was not confirmed, these results indicated that most of cysteine residues in mouse CAD exist as free thiols.

Free SH groups often form part of the active site of enzymes. To examine the involvement of cysteine residues in the enzymatic activity of CAD, CAD's DNase activity was assayed in the presence of reducing or oxidizing agents. As shown in Fig. 2A, the DNase activity of CAD was barely affected by these agents. Similarly, no inhibition of DNase activity was seen with other cysteine-modifying reagents such as 5 mM iodoacetamide (data not shown), suggesting that cysteine residues are not actively involved in the enzymatic activity of CAD. On the other hand, a high concentration of DTT greatly enhanced the

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**Fig. 1. Mass spectrum of the peptides derived from alkylated mouse CAD.** Mouse CAD (1.0 μg) was immobilized on a PVDF membrane, and its cysteine residues were successively alkylated by S-pyridylethylatation under non-reducing conditions, and by S-carboxymethylation under reducing conditions. The S-alkylated CAD was digested in situ with *Achromobacter protease I*, and the peptide fragments released from the membrane were analyzed by MALDI-TOF mass spectrometry.
production of functional CAD in a cell-free system. As shown in Fig. 2B, the addition of 30 mM DTT to the reaction mixture produced about 4-fold more functional CAD in the presence of ICAD-L, whereas the presence of 3 mM oxidized glutathione (GSSG) completely inhibited the production of active CAD. The other reducing agent, 2-mercaptoethanol, also showed an enhancing effect although its effect at 30 mM was weaker than that of DTT (data not shown). The enhancing and inhibitory effects of these agents were not due to effects on the transcription or translational machinery, because Western blotting analysis with anti-mouse CAD antibody detected similar amounts of 40-kDa CAD protein under both reducing and oxidizing conditions (data not shown).

**ICAD-L-dependent Refolding of Denatured CAD under Reducing Conditions**—The above results suggested that most of the cysteine residues of CAD should be in reduced form upon its synthesis. The requirement of ICAD-L for the production of functional CAD (17) suggests that ICAD-L works as a chaperone for CAD. Chaperones are known to enhance the renaturation of denatured proteins (23–25). To examine whether ICAD-L in fact works as a chaperone, we established an assay system for the refolding of denatured CAD. Purified CAD was denatured at a concentration of 12.5 μM with 6 M guanidine hydrochloride and diluted 100-fold with a buffer containing 20% glycerol and 0.5 M NaCl. Under these conditions, CAD did not renature, and the addition of 0.3 μM ICAD-L had little effect. However, ICAD-L-dependent renaturation of CAD was observed in the presence of reducing agents. As shown in Fig. 3A, when the denatured CAD was renatured in the presence of 100 mM DTT and 0.3 μM ICAD-L, about 60% of the original activity of CAD was recovered within 2 h. Similarly, 100 mM 2-mercaptoethanol stimulated renaturation of CAD, although its effect was weaker than that of 100 mM DTT (data not shown), whereas 3 mM oxidized glutathione completely inhibited the renaturation of CAD (Fig. 3A). Many chaperones require ATP to fold the target protein (23–25). In contrast, the renaturation of CAD under reducing conditions with ICAD-L was not affected by the addition of ATP or apyrase.

ICAD has two different forms (ICAD-L and ICAD-S), which are generated by alternative splicing (26). We and others (17, 27) have previously shown that ICAD-L but not ICAD-S can enhance synthesis of functional CAD in an in vitro transcription and translation coupled system. Accordingly, the recombinant ICAD-L but not ICAD-S could efficiently stimulate the renaturation of CAD (Fig. 3B). Maximal renaturation of CAD was obtained in the presence of near equimolar ICAD-L and CAD concentrations, suggesting that CAD forms a one-to-one complex with ICAD-L during its renaturation. The renaturation of CAD is a rather slow process; it took about 30 min for the reaction to be completed at 30 °C (Fig. 3C). Other components in the reaction mixture such as 20% glycerol and 0.5 M NaCl were also required for the ICAD-dependent renaturation of CAD, because their removal from the renaturation buffer severely slowed the reaction (data not shown). ICAD inhibits the DNase of CAD but not DNase I (11). Similarly, ICAD-L had no effect on the renaturation of denatured DNase I (data not shown).

**Production of the Functional ICAD-L-CAD Complex from the Inclusion Bodies**—The above results indicated that functional CAD can be produced from denatured CAD by refolding in the presence of ICAD-L. This procedure was used to produce functional CAD from protein synthesized in E. coli. Mouse CAD was synthesized in E. coli using the pET expression system (21) (Fig. 4A). Most of the 40-kDa mouse CAD was recovered as
was determined with 0.3-m M C containing with 100 mM DTT, and its activity is shown in ICAD-L-dependent refolding of CAD. The denatured CAD (0.25 m M; lane 3, lane 1) or supplemented with DTT (lane 2) were treated at 90 °C in 0.5 ml of buffer E. Five-microliter aliquots of the supernatant fraction (lane 1 were disrupted in 0.5 ml of buffer E, and the cell lysates were spun as described under “Experimental Procedures.” The precipitates were suspended diluted with buffer C containing 100 mM DTT alone (lane 4, lane 3), or supplemented with GST (lane 2, lane 1) or supplemented with GST-ICAD-L (lanes 3–6), or GST-ICAD-S (lanes 7–10). The concentrations of GST, GST-ICAD-L, and ICAD-S added to the reaction mixture were 0.03 m M GST-ICAD-L and 0.3 m M GST-ICAD-L. After incubation at 30 °C for 120 min, the DNase activity was determined with 0.3-μl aliquots of the reaction mixture. C, time course of ICAD-L-dependent refolding of CAD. The denatured CAD was diluted with buffer C containing 100 mM DTT and 0.3 m M GST-ICAD-L. After incubation at 30 °C for 0 (lane 1), 10 (lane 2), 30 (lane 3), 60 (lane 4), and 120 (lane 5) min, the DNase activity was determined with 0.3-μl aliquots.

Fig. 3. ICAD-L-dependent refolding of denatured CAD under reducing conditions. A, the effect of reducing and oxidizing agents on the ICAD-L-dependent refolding of CAD. The denatured CAD (0.25 μg) was diluted 100-fold with 50 μl of buffer C containing 0.3 μM GST-ICAD-L alone (lane 1) or supplemented with DTT (lane 2, 10 mM; lane 3, 100 mM), GSH (lane 4, 3 mM; lane 5, 10 mM), GSSG (lane 6, 3 mM; lane 7, 10 mM), 100 mM DTT, and 5 mM ATP (lane 8) or 100 mM DTT and 20 units/ml of apyrase (lane 9). After incubation at 30 °C for 120 min, the DNase activity of CAD was determined using 0.5-μl aliquots of the reaction mixture. The native CAD (0.125 μg) was subjected to the same procedure with buffer C containing with 100 mM DTT, and its activity is shown in lane 10. B, refolding of CAD by ICAD-L, but not by ICAD-S. The denatured CAD was diluted with buffer C containing 100 mM DTT alone (lane 1) or supplemented with GST (lane 2), GST-ICAD-L (lanes 3–6), or GST-ICAD-S (lanes 7–10). The concentrations of GST, GST-ICAD-L, and ICAD-S added to the reaction mixture were 0.03 μM (lanes 3 and 7), 0.1 μM (lanes 4 and 8), 0.3 μM (lanes 5 and 9), or 1 μM (lanes 2, 6, and 10). After incubation at 30 °C for 120 min, the DNase activity was determined with 0.3-μl aliquots of the reaction mixture.

Fig. 4. Production of the functional ICAD-L-CAD complex from the CAD inclusion bodies. A, the formation of inclusion bodies of mouse CAD expressed in E. coli. E. coli (5 ml of culture) harboring the CAD expression plasmid was treated at 37 °C for 2.5 h with 1 mM IPTG. The cells were disrupted in 0.5 ml of buffer E, and the cell lysates were spun as described under “Experimental Procedures.” The precipitates were suspended in 0.5 ml of buffer E. Five-microliter aliquots of the supernatant fraction (lane 1) and the resuspended precipitate (lane 2) were treated at 90 °C for 5 min in Laemmli’s sample buffer, separated by electrophoresis on a 10–20% SDS-polyacrylamide gel (Dai-ichi Pure Chemicals), and stained with Coomassie Brilliant Blue. B, recovery of the functional ICAD-CAD complex from the CAD inclusion bodies. E. coli (400 ml of culture) harboring the CAD expression plasmid were treated with IPTG, and the cell lysates were prepared as described above. The CAD inclusion bodies were recovered by centrifugation, dissolved in 6 ml of buffer B containing 7 M guanidine hydrochloride, and incubated at 30 °C for 2 h. The solution was then diluted 100-fold with buffer C containing 100 mM DTT in the presence of increasing amounts of GST-ICAD-L (lane 1, 0 μM; lane 2, 0.06 μM; lane 3, 0.15 μM; lane 4, 0.3 μM; lane 5, 0.6 μM). The CAD activity was determined using 0.5-μl aliquots of the mixture. C, purification of the GST-ICAD-L-CAD complex. The CAD inclusion bodies were prepared from a 400-ml culture of E. coli, denatured with guanidine-hydrochloride, and renatured as described under “Experimental Procedures.” The GST-ICAD-L-CAD complex was then purified on a glutathione-Sepharose affinity column, and a Mono S ion exchange column. The purified GST-ICAD-L-CAD complex (3 μg) was separated by electrophoresis on a 10–20% SDS-polyacrylamide gel, and stained with Coomassie Brilliant Blue. The bands corresponding to CAD and GST-ICAD-L are indicated by closed and open arrows, respectively, on the right.

inclusion bodies. The purity of the protein was about 50%, but it had no DNase activity. This CAD protein, when denatured with 7 M guanidine hydrochloride, could then be renatured in an ICAD-L-dependent manner, and a large amount of the DNase activity was recovered in the soluble fraction (Fig. 4B). The GST-ICAD-L-CAD complex was then purified by chromatography on glutathione-Sepharose and Mono S column. As shown in Fig. 4C, the purified protein existed as a complex of the 62-kDa GST-ICAD-L and the 40-kDa CAD as indicated by its mobility on an SDS-polyacrylamide gel. This complex had DNase activity when it was assayed in the presence of caspase 3, and its specific activity was similar to that obtained with the
A dilution buffer contained 20 units/ml apyrase \((\text{o r} 5 \text{m M ATP})\) in the presence \((\text{o r} \text{ absence}) \) and amounts of reticulocyte lysates \((\text{aliquots were diluted 100-fold with buffer D containing increased mM ATP, and 0.3 \text{ l aliquots were withdrawn from the reaction mixture, and the DNase activity of the renatured CAD was determined. The weak CAD activity seen without}

The above results indicate that the denatured CAD may quickly aggregate, even in the high salt and glycerol content of the reaction mixture. This form was recognized by ICAD-L, which facilitated the further folding of CAD. In agreement with the properties of other chaperones, but unlike the recently identified Hsp104 \((34)\), if ICAD-L was added to the refolding mixture after the denatured CAD had been diluted, no functional refolding of CAD was observed (data not shown). These results indicate that the denatured CAD may quickly aggregate, even in the presence of 0.5 M NaCl, and 100 mM DTT, and that ICAD-L can prevent this aggregation. Mouse ICAD-S, 66 amino acids shorter at the C terminus than ICAD-L, can inhibit the DNase activity of CAD by forming a complex with CAD \((16)\). However, ICAD-S could not support the folding of CAD, suggesting that the C-terminal 66 amino acid region of ICAD-L is indispensable for recognizing the denatured CAD.

In the second refolding system, which does not contain 0.5 M NaCl and 20% glycerol, reticulocyte lysates enhanced folding of the denatured CAD in the presence of ICAD-L. In contrast to the first assay system, this reaction required ATP, suggesting that general chaperone systems such as those involving Hsp70 (Hsc70) or TRiC may be involved in the folding of CAD. This situation is similar to that found for the folding of nuclear receptors with Hsp90 \((24, 35)\). Hsp90 can refold denatured proteins in an ATP-independent manner \((36)\). However, reconstitution experiments with purified glucocorticoid receptor and Hsp90 showed that Hsp70 and other chaperones transiently bind to the unfolded glucocorticoid receptor in an ATP-dependent manner and keep the molecule in the correct state for further folding \((37-39)\). This form of the receptor is transferred to Hsp90 which facilitates its final folding, and thus the newly

**Discussion**

Chaperones are proteins that aid in the correct folding of the nascent polypeptides of various target proteins during synthesis or that help transport proteins into mitochondria or plastids by keeping them in an unfolded state \((23, 25, 30)\). Two major chaperone systems have been well characterized for the folding of cytosolic proteins. In one system, heat-shock protein 70 (Hsp70), a eukaryotic homolog of \(E. coli\) DnaK, together with Hsp40 (a homolog of \(E. coli\) DnaJ) helps to fold a set of proteins through the expenditure of ATP. In the other system, the chaperonin TCP1-ring complex (TRiC, also called CCT, a homolog of \(E. coli\) GroEL) cooperates with another hetero-oligomeric complex called GIM or prefoldin to mediate the folding of polypeptide chains in an ATP-dependent manner \((31, 32)\). These two systems work as general chaperones, that is they facilitate the folding of many different proteins, although recent analysis indicates that only about 20% of newly synthesized polypeptides transiently bind Hsp70 (Hsc70) and only 15% associate with TRiC \((33)\). These results suggest that there are other chaperone systems or that different polypeptides have different and specific mechanisms for folding during their synthesis.

Since the functional CAD was not synthesized in reticulocyte lysates, or Sf9 cells without ICAD, we proposed that general chaperons cannot fold CAD, and ICAD works as a specific chaperone for CAD \((11, 17)\). The key properties that characterize a protein as a chaperone are as follows: 1) suppression of aggregation of the target protein during protein folding; 2) suppression of aggregation during protein unfolding; 3) influence on the yield and kinetics of folding; and 4) effects exerted on near-stoichiometric levels \((24)\). In this report, we showed in two different assay systems that ICAD-L is indispensable for the refolding of chemically unfolded CAD. In the first assay system \((0.5 \text{ M NaCl and 20% glycerol})\), functional CAD could be folded in the presence of a near-stoichiometric concentration of ICAD-L, if the environment contained large amounts of reducing agent. This refolding was inhibited by oxidized glutathione, which may cause disulfide bond formation. The successful folding in the presence of 100 mM DTT confirms that all 14 cysteine residues in mouse CAD are in the reduced thiol form and are probably buried inside the molecule. During the folding of CAD, the stretches of peptides carrying thiol residues were probably partially refolded due to the high salt and glycerol content of the reaction mixture. This form was recognized by ICAD-L, which facilitated the further folding of CAD. In agreement with the properties of other chaperones, but unlike the recently identified Hsp104 \((34)\), if ICAD-L was added to the refolding mixture after the denatured CAD had been diluted, no functional refolding of CAD was observed (data not shown). These results indicate that the denatured CAD may quickly aggregate, even in the presence of 20% glycerol, 0.5 M NaCl, and 100 mM DTT, and that ICAD-L can prevent this aggregation. Mouse ICAD-S, 66 amino acids shorter at the C terminus than ICAD-L, can inhibit the DNase activity of CAD by forming a complex with CAD \((16)\). However, ICAD-S could not support the folding of CAD, suggesting that the C-terminal 66 amino acid region of ICAD-L is indispensable for recognizing the denatured CAD.

**Fig. 5.** Renaturation of CAD using reticulocyte lysates. A, mouse CAD \((10 \mu \text{g})\) was denatured by incubation at 30 °C for 60 min with 6 M guanidine hydrochloride in 20 µl of buffer D. The 0.5-µl aliquots were diluted 100-fold with buffer D containing increased amounts of reticulocyte lysates \((\text{lanes 1 and 5, 0% (v/v); lanes 2 and 6, 20% (v/v); lanes 3 and 7, 40% (v/v); lanes 4 and 8, 80% (v/v)})\). The dilution buffer contained 20 units/ml apyrase \((\text{lanes 1–4})\) or 5 mM ATP \((\text{lanes 5–8})\) in the presence \((\text{upper panel})\) or absence \((\text{lower panel})\) of 0.3 mM GST-ICAD-L. After incubation at 30 °C for 2 h, 1-µl aliquots were withdrawn from the reaction mixture, and the DNase activity of the renatured CAD was determined. The weak CAD activity seen without ICAD-L in lane 8 is likely to be due to the endogenous ICAD-L in the reticulocyte lysates. B, mouse CAD was denatured and diluted as described above with buffer D containing 80% \((\text{v/v})\) reticulocyte lysates, 5 mM ATP, and 0.3 mM GST-ICAD-L. After incubation at 30 °C for the indicated times, 0.5-µl aliquots were withdrawn from the reaction mixture, and the DNase activity was determined.
translated glucocorticoid receptor is recovered as a complex with Hsp90 (40, 41). A similar scenario may apply to the folding of CAD, that is when CAD is synthesized on ribosomes, its nascent chain may be recognized by general chaperones and kept in a soluble and aggregation-resistant form. When translation is complete, the folding of CAD could be completed by ICAD-L. If the concentration of ICAD-L added to the re-folding mixture largely exceeded that of CAD, the yield of the functional CAD-ICAD complex decreased, suggesting that excess ICAD-L can bind to inappropriate places in the unfolded or partially folded CAD, inhabiting its correct folding. These results and the lack of ATP dependence for the ICAD-L-mediated folding, suggest that once ICAD-L binds to the unfolded CAD, it remains complexed. In any case, the development of an ATP-dependent CAD-refolding system with the reticulocyte lysates will facilitate the identification of molecules involved in its folding.

Hsp90 works as a chaperone not only for a family of nuclear receptors but also for other molecules such as oncogeno tyrosine kinases and cellular serine-threonine kinases (24). It also enhances the folding of citrate synthase and the Fab fragment of antibodies. Mice deficient in the ICAD gene lack CAD activity, but ICAD-L could not enhance the refolding of DNase I or luciferase. It is also involved in the processing and activation of pro-caspase 3, a key enzyme in the promotion of apoptosis, is regulated by Hsp60 in mitochondria (45, 46). BAG-1, a negative regulator of the Hsp70 chaperone (47, 48), binds to Bcl-2 and enhances its antiapoptotic activity (49). The death receptor, tumor necrosis factor type I receptor is associated with SODD, a member of the BAG-1 family (50, 51). Further studies on the molecular mechanisms of how chaperones regulate the apoptotic machinery will increase our understanding of the apoptotic mechanism.

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