Co-translational Folding of Caspase-activated DNase with Hsp70, Hsp40, and Inhibitor of Caspase-activated DNase*

Hideki Sakahira‡ and Shigekazu Nagata§

From the Department of Genetics, Osaka University Medical School, and Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, 2-2 Yamada-oka, Suta, Osaka 565-0871, Japan

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Co-translational Folding of Caspase-activated DNase (CAD) that causes chromosomal DNA fragmentation during apoptosis exists as a complex with ICAD (inhibitor of CAD) in proliferating cells. Here, we report that denatured CAD is functionally refolded with Hsc70-Hsp40 and ICAD. Hsc70-Hsp40 suppresses the aggregation of the denatured CAD, but cannot restore its enzymatic activity. In contrast, ICAD could not suppress the aggregation of CAD, but supported the CAD’s renaturation with Hsc70-Hsp40, indicating that ICAD recognizes the quasi-native folding state of CAD that is conferred by Hsc70-Hsp40. Using an in vitro translation system, we then showed that during CAD translation, Hsc70-Hsp40 as well as ICAD bind to the nascent CAD polypeptide, while on ribosomes. These results indicate that ICAD together with Hsc70-Hsp40 assists the folding of CAD during its synthesis, and that the CAD/ICAD heterodimer is formed co-translationally.

The amino acid sequence of a polypeptide chain carries information that determines the three-dimensional structure of the protein (1). However, in many cases, proper protein folding requires the assistance of molecular chaperones that bind to proteins in their non-native states (2–7). Two major chaperone systems for the folding of cytosolic proteins in eukaryotes have been well studied. In one system, Hsc70/Hsp70 assists the folding of a large variety of proteins together with its co-chaperone, Hsp40. In the other system, the hetero-oligomeric chaperonin TCP1-ring complex (TRiC, also called CCT) together with its co-chaperone, prefoldin/GimC, assists the folding of a more limited set of proteins that includes actin and tubulin. In both chaperone systems, substrate proteins achieve their native states through cycles of ATP-dependent binding and release of chaperone proteins.

It is well established that the nascent polypeptides of cytosolic proteins can fold co-translationally (8–10), and co-translational, domain-wise folding is the basis for the efficient folding of a large number of eukaryotic multidomain proteins (11). In contrast to monomeric proteins, little is known about the molecular mechanism of the folding and complex formation of proteins that have a subunit structure.

CAD (caspase-activated DNase), also called DFF40 (DNA fragmentation factor 40), is a protein responsible for chromosomal DNA fragmentation during apoptosis (12–15). In proliferating cells, CAD exists as a complex with ICAD (inhibitor of CAD), which suppresses its DNase activity (12, 13). When ICAD is cleaved by caspases (cysteine proteases that are activated during apoptosis), CAD is released from ICAD and degrades the chromosomal DNA (16). ICAD is not only an inhibitor, but is also essential for generating properly folded CAD; functional CAD is synthesized only in the presence of ICAD both in vitro and in vivo (17–19). CAD and ICAD share a homologous domain (the CAD/CIDE domain) of about 80 amino acids at the N terminus (20, 21). These domains interact with each other to form the heterodimeric complex, and this interaction has been suggested to be essential for the correct folding of CAD (22). However, it remains unknown whether any other molecular chaperones in addition to ICAD are required for producing the functional CAD in CAD/ICAD complex and how these chaperones assist the folding of the CAD polypeptide during its synthesis.

In this study, we found that in addition to ICAD, Hsc70 and Hsp40 are essential for the refolding of chemically denatured CAD. Hsc70 and Hsp40 could co-operatively suppress the aggregation of CAD in an ATP-dependent manner in the absence of ICAD, but could not restore the CAD DNase activity. The results from time-order addition experiments suggested that the denatured CAD is partially refolded by Hsp70 and Hsp40, followed by the binding of ICAD to produce a functional CAD/ICAD complex. Furthermore, in vitro translation experiments using truncated CAD mRNAs indicated that ICAD co-translationally assisted the folding of CAD by binding to the CAD/CIDE domain of the nascent CAD polypeptide on ribosomes.

EXPERIMENTAL PROCEDURES

Protein Purification—Production of recombinant mouse CAD was performed as described previously (17). FLAG-tagged mouse ICAD-L fused with glutathione S-transferase (GST-ICAD-L) and hexahistidine-tagged human caspase 3 were prepared as described previously (16). The CAD/CIDE domain of CAD (CAD-CD: residues 3–87) or CAD/CIDE domain-deleted CAD (CAD-ΔCD: residues 87–344) was tagged with an HA (influenza hemagglutinin protein) epitope and hexahistidines (EFAGYPYDVPDYAGRSHHHHH) at the C terminus, expressed in Escherichia coli with a pET expression vector, and purified with an Ni2⁺-nitrilotriacetic acid-agarose column. Hexahistidine-tagged human Hsp40 (HDJ1) was expressed in E. coli SG13009 and purified to homogeneity as described previously (23). Hsc70 was purified to homogeneity.

* To whom correspondence should be addressed: Dept. of Genetics, Osaka University Medical School, B-3, 2-2 Yamada-oaka, Suta, Osaka 565-0871, Japan. Tel.: 81-6-6879-3310; Fax: 81-6-6879-3319; E-mail: nagata@genetic.med.osaka-u.ac.jp.

† Supported by Research Fellowships of the Japan Society for the Promotion of Science. Present address: Max-Planck-Institute for Biochemistry, Dept. of Cellular Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany.

§ To whom correspondence should be addressed: Dept. of Genetics, Osaka University Medical School, B-3, 2-2 Yamada-oaka, Suta, Osaka 565-0871, Japan. Tel.: 81-6-6879-3310; Fax: 81-6-6879-3319; E-mail: nagata@genetic.med.osaka-u.ac.jp.

The abbreviations used are: CAD, caspase-activated DNase; ICAD, inhibitor of CAD; GST, glutathione S-transferase; HA, hemagglutinin; DFF, DNA fragmentation factor; BSA, bovine serum albumin; ATPγS, adenosine 5’-O-(thiotriphosphate).
FIG. 1. Refolding of CAD with reticulocyte lysates and ICAD. A, ATP-dependent renaturation of CAD with reticulocyte lysates and ICAD. Denatured CAD (lanes 1–7, 12.5 μg; lane 8, 0 μg) was diluted 100-fold into 50 μl of buffer A containing 40 μl of reticulocyte lysates (lanes 3–8), 0.3 μM GST-ICAD-L (lanes 2 and 5–8), and 5 mM ATP (lanes 4, 6, and 8), 5 mM ATP/S (lane 7), or 20 units/ml apyrase (lanes 3 and 5). After incubation at 25 °C for 2 h, CAD DNase activity was determined with 1 μl of the reaction mixture in the presence of caspase 3, B, involvement of Hsc70/Hsp70 in the refolding of denatured CAD. After preclearing with 60 μl of protein G-Sepharose, the reticulocyte lysates (300 μl) were incubated at 4 °C overnight with 100 μl of protein G-Sepharose (lanes 1 and 3), anti-TCP1/CCTα antibody-coupled protein G-Sepharose (lane 2), or with anti-Hsc70/Hsp70 antibody-coupled protein G-Sepharose (lane 4), and the beads were removed by centrifugation. The denatured CAD (12.5 μg) was diluted 100-fold into 50 μl of buffer A containing 40 μl of the immunodepleted reticulocyte lysates, 0.3 μM GST-ICAD-L, and 5 mM ATP and incubated at 25 °C for 2 h. The CAD DNase activity was then determined with 1 μl of the reaction mixture in the presence of caspase 3 (upper panels). The changes in the supernatants and the immunodepleted reticulocyte lysates (4 μl) was analyzed by Western blotting (lower panels) using anti-TCP1/CCTα (clone 23c) or anti-Hsc70/Hsp70 (clone BB70) antibodies.

from bovine brain as described (24). Protein concentration was determined by measuring A280.

Antibodies and Rabbit Reticulocyte Lysates—Rabbit anti-CAD antibody recognizing a C-terminal peptide of mouse CAD was described previously (17). Anti-FLAG antibody (clone M2) and anti-HA antibody (clone 16B12 or rabbit polyclonal antibody) were purchased from Sigma-Aldrich. Anti-FLAG antibody (clone 23c) was covalently cross-linked to 100 μl of protein G-Sepharose (Amersham Biosciences, Inc.) using dimethyl pimelimidate (Pierce). Rabbit reticulocyte lysates were purchased from Promega. For use in the refolding assay, the reticulocyte lysates were spun for 60 min at 500,000 × g, and the supernatants were passed through PD10 column (Amersham Biosciences, Inc.) equilibrated with buffer A (10 mM HEPES-KOH (pH 7.2), 5 mM MgCl2, 0.5 mM EGTA, 50 mM KCl, and 10 mM dithiothreitol).

Assay for CAD DNase—The DNase activity of CAD was determined as described previously (17). In brief, DNA (1.0 μg) was incubated with the CAD-ICAD complex at 30 °C for 2 h in 20 μl of buffer B (10 mM HEPES-KOH (pH 7.2), 2 mM MgCl2, 5 mM EGTA, 20% glycerol, 50 mM NaCl, and 10 mM dithiothreitol) supplemented with 1 mM p-amidino-phenyl methanesulfonyl fluoride hydrochloride, 1 mg/ml BSA, and 2.4 pmol of human caspase 3. After incubation, the DNA was analyzed by electrophoresis on a 1.5% agarose gel.

Refolding of CAD and Light Scattering Assay—CAD was precipitated with acetone and denatured by incubation at a concentration of 12.5 μM for 1 h at 25 °C in buffer A containing 6 μg guanidine hydrochloride, as described previously (18). The denatured CAD was diluted 100-fold with buffer A containing Hsc70, Hsp40, GST-ICAD-L, and ATP and incubated at 25 °C for 2 h. To monitor CAD aggregation, CAD was denatured at a concentration of 50 μM, then diluted 100-fold with buffer A containing chaperones and ATP. ATP/S or apyrase. Aggregation was followed at 25 °C by measuring the turbidity at 320 nm as described previously (23).

Assay of Binding to Denatured Proteins—To detect the binding of Hsc70 to the denatured proteins, CAD, CAD-ICD, or CAD-CD was denatured at a concentration of 12.5 μM, as described above. The denatured protein was diluted 100-fold into buffer A containing Hsc70, Hsp40, and ATP and incubated at 25 °C for 15 min. One unit of apyrase (Sigma) was added to the mixture to stop the reaction, followed by incubation at 25 °C for 5 min. The samples were diluted with 500 μl of 10 mM HEPES-KOH buffer (pH 7.2) containing 5 mM MgCl2, 0.5 mM EGTA, 50 mM KCl, 0.1% Tween 20, 25% glycerol, 1 mM ADP, and 10 mg/ml BSA. Fifteen micrograms of anti-Hsc70/Hsp70 antibody (clone BB70) and 20 μl of protein G-Sepharose were added to the mixture, followed by incubation at 4 °C for 1 h. The beads were washed thoroughly, and suspended in 50 μl of SDS-sample buffer. After heating at 95 °C for 5 min, the eluates were subjected to SDS-PAGE. Proteins were transferred to a membrane and analyzed by Western blotting. To pull-down the CAD-ICAD complex, denatured proteins were diluted 100-fold into 50 μl of buffer A containing Hsc70, Hsp40, GST-ICAD-L, and ATP and incubated at 25 °C for 2 h. The samples were diluted with 500 μl of buffer A supplemented with 0.1% Tween 20, 25% glycerol, and 10 mg/ml BSA. After the addition of 20 μl of glutathione-Sepharose 4B (Amersham Biosciences, Inc.), the mixture was incubated at 4 °C for 1 h, and proteins bound to the beads were analyzed by Western blotting.

In Vitro Translation—Full-length mouse CAD and its deletion mutants were fused with an HA epitope at their C termini and ligated into pBluescript II (SK−). The plasmids were linearized after the C terminus of the HA tag and transcribed with T7 RNA polymerase using Ribomax Large Scale RNA Production Systems (Promega). The in vitro translation was performed using Flexi Rabbit Reticulocyte Lysate System (Promega) at 30 °C for 25 min with 4 μg of mRNA in the presence of 6 pmol of GST-ICAD-L, 10 mM dithiothreitol, and 40 units of RNase In Vitro (Promega) in a final volume of 50 μl.

To isolate the ribosome-nascent chain complexes, 2 mM cycloheximide (Wako) and 20 units/ml apyrase were added to the lysates, followed by incubation at 25 °C for 5 min. The mixture was diluted 3-fold with buffer C (buffer A containing 0.5 mM cycloheximide, 0.3 unit/ml RNase, and 1 mM ADP) and spun for 10 min at 15,000 × g to remove aggregates. The supernatant was spun for 20 min at 100,000 rpm (Beckman TL100.2 rotor) at 4 °C through 800 μl of buffer C containing 500 mM sucrose. Ribosomal pellets were washed in buffer C, resuspended in 100 μl of SDS-sample buffer, and subjected to Western blotting. To analyze the ribosomes on a sucrose density gradient, the lysates were diluted 3-fold, layered onto a 12-ml linear 20–40% (w/v) sucrose gradient in buffer C, and spun at 4 °C for 4 h at 39,500 rpm using a Beckman SW40Ti rotor. One-milliliter fractions were collected from the bottom of the tube, and the absorbance at 254 nm was measured. Proteins in each fractions were precipitated by 5% trichloroacetic acid, washed with diethyl ether, and resuspended in 50 μl of SDS-urea-sample buffer for Western blotting analysis.

RESULTS

ATP-dependent Refolding of CAD with Hsc70, Hsp40, and ICAD—We have previously shown that chemically denatured CAD can be renatured in the presence of ICAD and reticulocyte lysates (18). Reticulocyte lysates are known to catalyze the refolding of a variety of proteins, by a process that requires ATP hydrolysis (25, 26). We first examined the requirement of ATP for the renaturation of CAD by reticulocyte lysates and
ICAD. As shown in Fig. 1A, CAD denatured by guanidine HCl was efficiently renatured in the presence of reticulocyte lysates, ICAD, and ATP. When apyrase or ATPγS was added to the reaction mixture instead of ATP, the denatured CAD was not renatured. This renaturation process required ICAD, but ICAD alone (without reticulocyte lysates) had little effect on the renaturation of CAD under these conditions. These results indicated that the reticulocyte lysates contained a factor(s) that enhances the correct folding of CAD in an ATP- and ICAD-dependent manner.

Reticulocyte lysates carry the Hsc70 (the constitutively expressed Hsp70 homolog)-Hsp40 and TRiC-prefoldin chaperone systems, both of which require ATP hydrolysis to catalyze protein folding (25, 26). To examine which of these systems is responsible for the renaturation of CAD, TRiC or Hsc70 was immunodepleted from the lysates. As shown in Fig. 1B, lysates in which TRiC was immunodepleted to less than 10% of its original level still promoted the renaturation of CAD as efficiently as the original lysates. On the other hand, Hsc70-depleted lysates could not promote the renaturation of CAD. These results suggested that Hsc70-Hsp40, but not TRiC-prefoldin, was involved in the refolding of CAD.

To confirm this finding, we next tried to reconstitute the renaturation of CAD using purified components. Recombinant human Hsp40 (HDJ1) and native bovine Hsc70 were purified to homogeneity (Fig. 2A) and used for the refolding of CAD. As shown in Fig. 2B, the denatured CAD (12.5 μM) was diluted 100-fold into 50 μl of buffer A containing 2 μM Hsc70, 1 μM Hsp40, 0.3 μM GST-ICAD-L, and 5 mM ATP. After incubation at 25 °C for 2 h, the CAD DNase activity was determined with 1 μl of the reaction mixture in the presence of caspase 3. A, time course of refolding of CAD. The denatured CAD (12.5 μM) was diluted 100-fold into 50 μl of buffer A containing 2 μM Hsc70, 1 μM Hsp40, 0.3 μM GST-ICAD-L, and 5 mM ATP. After incubation at 25 °C for 0 min (lane 1), 10 min (lane 2), 30 min (lane 3), 60 min (lane 4), and 120 min (lane 5), the CAD DNase activity was determined with 1 μl of the reaction mixture.
shown in Fig. 2B, Hsc70 or Hsp40 alone could not promote the renaturation of denatured CAD, even in the presence of ICAD. However, the addition of both Hsc70 and Hsp40 stimulated the renaturation of CAD in a dose-dependent manner. CAD was not renatured in the absence of ATP or in the presence of ATPγS, indicating that ATP hydrolysis is required (Fig. 2B).

This process was time-dependent and was completed within 1 h (Fig. 2C). These results indicated that Hsc70/Hsp70 and Hsp40 together with ICAD could promote the refolding of CAD in an ATP-dependent manner.

**Two-step Refolding of CAD with the Hsc70-Hsp40 System and ICAD**—The Hsc70-Hsp40 system enhances the folding of proteins by suppressing aggregation of the denatured proteins (23). To determine how CAD is refolded with Hsc70, Hsp40, and ICAD, we first examined whether CAD aggregation was suppressed by these chaperones. As shown in Fig. 3A, when chemically denatured CAD was diluted 100-fold, the protein was aggregated within 5 min. BSA or ICAD had no effect on the aggregation of CAD. In contrast, Hsc70 and Hsp40 suppressed the aggregation of CAD in the presence of ATP in a dose-dependent manner (Fig. 3B). When added individually, Hsc70 or Hsp40 alone could not prevent CAD aggregation. Furthermore, ATPγS could not substitute for ATP, indicating that this process was accompanied by ATP hydrolysis.

Hsc70-Hsp40 prevented CAD aggregation, but could not produce functional protein from denatured CAD. The addition of ICAD to Hsc70 and Hsp40 was required to complete the process, suggesting that ICAD recognized an intermediate folding state of CAD conferred by Hsc70-Hsp40. To confirm this possibility, time-order addition experiments were carried out using each chaperone. As shown in Fig. 4, when denatured CAD was diluted in a buffer containing Hsc70, Hsp40, and ICAD, functional CAD was regenerated. A similarly efficient refolding of CAD was observed when the denatured CAD was diluted into a buffer containing Hsc70 and Hsp40, and ICAD was added to the mixture 20 min after dilution. The addition of apyrase together with ICAD prevented the renaturation of CAD, which is consistent with the previous observations that the release of Hsc70/Hsp70 from substrate proteins is ATP-dependent (23). When the denatured CAD was first diluted into a buffer containing ICAD alone, the addition of Hsc70 and Hsp40 at 20 min after dilution had little effect on the renaturation of

![Image](http://www.jbc.org/)
CAD. This result supported the idea that ICAD could not prevent the aggregation of denatured CAD and suggested that previously aggregated CAD could not be refolded with Hsc70-Hsp40.

**Essential Role of the CAD/CIDE Domain for the Refolding of CAD**—The above data suggested that CAD refolding occurs in an ordered manner: Hsc70 and Hsp40 partially refold the denatured CAD in the first step, and ICAD recognizes the quasinative state of CAD to complete the folding of CAD in the second step. We have previously reported that an interaction between the CAD/CIDE domain of CAD (CAD-CD) and that of ICAD (ICAD-CD) is an essential step for the production of functional CAD (22). To investigate the role of the CAD/CIDE domain in the Hsc70-, Hsp40-, and ICAD-dependent folding of CAD, CAD-CD and CAD/CIDE domain-deleted CAD (CAD-ΔCD) were produced in *E. coli* and purified to homogeneity (Fig. 5A). The wild-type CAD, CAD-CD, and CAD-ΔCD were denatured by treatment with 6 M guanidine HCl, diluted into a buffer containing Hsc70 and Hsp40, and incubated for 15 min. As shown in Fig. 5B, immunoprecipitation with the anti-Hsc70 antibody showed that CAD-ΔCD and wild-type CAD were associated with Hsc70, while CAD-CD was not. The interaction of CAD-ΔCD with Hsc70 was confirmed by the aggregation assay. That is, when the denatured CAD-ΔCD was diluted into a buffer, it quickly aggregated, and Hsc70/Hsp40 prevented this process (Fig. 5C). In contrast, the denatured CAD-CD did not undergo aggregation upon dilution, suggesting that the CAD-CD domain refolds spontaneously.

We then examined the interaction of the denatured CAD, CAD-ΔCD, and CAD-CD with ICAD during the refolding process (Fig. 5D). In this experiment, each of the denatured proteins was diluted into a solution containing Hsc70, Hsp40, and ICAD (GST-ICAD-L) and incubated for 2 h. When ICAD-L was pulled down with glutathione-Sepharose, CAD-CD and the wild-type CAD were found to be associated with ICAD. On the other hand, very little CAD-ΔCD was associated with ICAD. These results suggested that CAD-ΔCD, which might have been partially refolded by the Hsc70-Hsp40 system, could not be recognized by ICAD, and the CAD/CIDE domain worked as a scaffold to transfer CAD from Hsc70-Hsp40 to ICAD during its refolding.

**Co-translational Binding of ICAD to the Ribosome-associated Nascent CAD Polypeptide**—We next examined whether Hsc70-Hsp40 and ICAD were involved in the *de novo* protein folding that must be accomplished in the context of the vectorial synthesis of polypeptide chains on ribosomes (5, 27). For this set of experiments, CAD mRNA lacking a stop codon was prepared and translated in reticulocyte lysates (9, 28, 29). As shown in Fig. 6A, CAD was detected in the lysates when CAD mRNA was translated either in the presence or absence of ICAD. Upon centrifugation of the lysates through a sucrose cushion, at least 50% of the CAD polypeptides were recovered in the ribosomal fractions. When the CAD mRNA was translated in the absence of ICAD, both Hsc70 and Hsp40 were found to be associated with ribosomes, and when CAD mRNA was translated in the presence of ICAD, Hsc70, Hsp40, and ICAD were all found in the ribosomal fraction. In contrast, when firefly luciferase mRNA lacking a stop codon was translated in the reticulocyte lysates in the presence of ICAD, Hsc70 and Hsp40, but not ICAD, were found in the ribosomal fraction (data not shown). To confirm the binding of ICAD to ribosomes, the CAD-translating reticulocyte lysates were analyzed by centrifugation on a
polymerase. The in vitro "through a sucrose cushion as described under CAD/CIDE domain (residues 5–87). The four histidine residues (His242, His263, His308, and His313) in the C-terminal region are essential for the in vitro of CAD used for

M1[1–156], M2[1–202], M3[1–278], and ΔCD[87–344] cDNAs are indicated by lines. The coding region of each cDNA was fused with an HA tag at its C terminus and lacked a stop codon.

M2[1–202] catalytic activity of CAD DNase (31). The CAD coding regions of the wild-type (wt) and four mutant (M1[1–156], M2[1–202], M3[1–278], and ΔCD[87–344]) cDNAs were transcribed in vitro using T7 RNA polymerase. The in vitro translation was carried out in the presence of GST-ICAD-L, and ribosomal fractions were collected by centrifugation through a sucrose cushion as described under “Experimental Procedures.” Aliquots containing 1/10th of the input for sucrose cushions (total) and 1/30th of the ribosomal fraction (ribosomal) were analyzed by Western blotting using anti-HA antibody for CAD (top panels) or anti-FLAG antibody for ICAD (bottom panels). In lanes 1 and 7, the in vitro translation was carried out in the absence of mRNA.

linear sucrose gradient. As shown in Fig. 6B, ribosomes, CAD, and ICAD co-sedimented. These results suggested that ICAD specifically bound to the nascent polypeptide of CAD on ribosomes.

To examine which part of the CAD polypeptide is responsible for the binding of ICAD, mRNA coding for truncated HA-tagged CAD containing a series of deletions was prepared (Fig. 7A) and translated in reticulocyte lysates in the presence of ICAD. As shown in Fig. 7B, each mRNA produced a protein of the expected sizes. After centrifugation of the lysates through a linear sucrose gradient. As shown in Fig. 6B, each mRNA produced a protein of the expected sizes. After centrifugation of the lysates through a linear sucrose gradient, most of the mutant CAD polypeptides were found in the ribosomal fraction, indicating that the nascent CAD polypeptides were still on the ribosomes. ICAD was found in the ribosomal fraction when the mRNAs for the CAD mutants M1, M2, and M3 (defined in the legend for Fig. 7A) as well as the wild-type CAD were translated. In contrast, ICAD was not detected in the ribosomal fraction when CAD-ΔCD was translated. These results indicated that the CAD/CIDE domain at the N terminus of CAD is necessary and sufficient to recruit ICAD to the nascent CAD polypeptide on ribosomes.

DISCUSSION

Co-translational, domain-wise folding is proposed to be important for the proper formation of multidomain proteins (30). Although the tertiary structure of CAD has not yet been determined, it seems to be composed of two domains: the N-terminal CAD domain (CAD-CD) and the C-terminal DNase domain (31). In support of this notion, the limited digestion of CAD with proteinase K produced two distinct fragments (data not shown), and the active Drosophila CAD is composed of two subunits (32). CAD-CD seems to fold spontaneously, because the denatured CAD-CD does not undergo aggregation upon dilution, and it does not bind Hsc70. ICAD binds CAD-CD that has been spontaneously folded (22), suggesting that the spontaneously folded CAD-CD had the proper tertiary structure to be recognized by ICAD. On the other hand, Hsc70 and Hsp40 interacted with CAD-ΔCD and prevented its aggregation. Furthermore, ICAD was capable of producing functional CAD from the Hsc70-Hsp40-treated denatured CAD. Because Hsc70, Hsp40, and ICAD were also found to be associated with the CAD nascent polypeptide on ribosomes, we concluded that CAD is co-translationally folded by Hsc70-Hsp40 and ICAD. It is possible that as the N-terminal part of CAD is synthesized and emerges from the ribosome, CAD-CD spontaneously folds. ICAD may bind to the CAD nascent polypeptide by recognizing the folded CAD-CD. As the translation proceeds, Hsc70 and Hsp40 bind to the elongating C-terminal part of CAD, assist its folding, and generate a "molten globule"-like status for CAD (33, 34). The ICAD associated with CAD-CD then recognizes the partially folded C-terminal part of CAD and produces the correctly folded CAD polypeptide. The CAD-ICAD complex thus releases from the ribosomes. Because neither Hsc70 nor Hsp40 is associated with the purified CAD-ICAD (DIFF) complex (13), it is likely that Hsc70 and Hsp40 dissociate from CAD when CAD is completely folded as a complex with ICAD.

Netzer and Hartl (30) proposed that the co-translational folding of proteins is an important mechanism to reduce the possibility of intramolecular misfolding that may lead to aggregation. When CAD is expressed alone in mammalian cells, insect cells, or E. coli, it undergoes aggregation (18). However, the co-expression of CAD with ICAD generates soluble, functional CAD as a complex with ICAD, indicating that the ICAD-assisted co-translational folding of CAD occurs in cells. Furthermore, no functional CAD is produced in ICAD-knockout mice (19), also supporting the essential role of ICAD for CAD synthesis. By immunohistochemical analysis with anti-ICAD antibody or by following the localization of an ICAD-GFP fusion protein, several groups have reported that ICAD is located mainly in nuclei (35, 36). However, the essential role of ICAD for the co-translational folding of CAD indicates that some ICAD must be in the cytoplasm. How ICAD shuttles between the nucleus and the cytoplasm remains to be studied. Misfolded, aggregated proteins are often cytotoxic and lead to cell death (37). However, cells deficient in the ICAD gene grow and are healthy and contain no detectable CAD protein, suggesting that misfolded CAD is rapidly removed from the cells. It will be interesting to study how this process is regulated.

How hetero-oligomeric proteins are folded and formed into complexes is not well understood. Here we showed that ICAD co-translationally binds to CAD during its folding and is released from ribosomes as a heterocomplex with CAD. There are

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a few reports of the co-translational assembly of homophilic protein complexes (38, 39). For example, the reovirus cell attachment protein σ1 is a trimeric protein carrying two domains and seems to assemble co-translationally. The N-terminal domain trimerizes in an ATP-independent manner, followed by the trimerization of the C-terminal domain. Because the trimerization (and/or folding) of the C-terminal domain requires ATP hydrolysis, Gilmore et al. (38) postulated the involvement of a chaperone system. Similarly, the N-terminal CAD-CD domain may fold spontaneously, with ICAD then binding to the prefolded N-terminal CAD-CD domain. The folding of the C-terminal part of CAD then continues with the help of Hsc70-Hsp40 and ICAD. Such co-translational and domain-wise folding coupled with the formation of the protein complex is probably a general mechanism for oligomeric proteins and may be facilitated by ribosome stacking and elongation arrest, as suggested previously (39). Taken together, a basic mechanism for the proper folding of both monomeric and oligomeric multidomain proteins may involve the spontaneous or easy folding of the N-terminal domain, with the prefolded N-terminal domain then promoting the proper folding of the C-terminal domain. Our demonstration that ICAD and general chaperones function collaboratively to produce a functional protein complex will contribute to the understanding of the folding and complex formation of hetero-oligomeric proteins.

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Hideki Sakahira and Shigekazu Nagata

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