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The effect of ICAD-S on the formation and intracellular distribution of a nucleolytically active caspase-activated DNase

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ABSTRACT

We show here that co-expression of murine CAD with either ICAD-L or ICAD-S in *Escherichia coli* as well as mammalian cells leads to a functional DFF complex, which after caspase-3 activation releases a nucleolytically active DNase. The chaperone activity of ICAD-S is between one and two orders of magnitude less effective than that of ICAD-L, as deduced from cleavage experiments with different activated recombinant DFF complexes produced in *E.coli*. With nucleolytically active EGFP fusion proteins of CAD it is demonstrated that co-expression of ICAD-S, which lacks the C-terminal domain of ICAD-L, including the NLS, leads to a homogeneous intracellular distribution of the DNase in transfected cells, whereas co-expression of human or murine ICAD-L variants lacking the NLS leads to exclusion of EGFP–CAD from the nuclei in ~50% of cells. These results attribute a particular importance of the NLS in the long isoform of the inhibitor of CAD for nuclear accumulation of the DFF complex in living cells. It is concluded that ICAD-L and ICAD-S *in vivo* might function as tissue-specific modulators in the regulation of apoptotic DNA degradation by controlling not only the enzymatic activity but also the amount of CAD available in the nuclei of mammalian cells.

INTRODUCTION

The caspase-activated DNase CAD (synonym DFF40) plays an important role in apoptotic DNA fragmentation (1–4). It is expressed as a heterodimeric complex (DNA fragmentation factor or DFF) with ICAD-L (synonym DFF45), which not only serves as the specific inhibitor of CAD but is also required for the proper folding and formation of a catalytically competent nuclease (2,3,5,6). ICAD-L co-translationally binds to the nascent CAD polypeptide chain involving a homophilic interaction of the homologous N-terminal domains (7–11). Upon apoptotic stimuli the inhibitory subunit of the DFF complex is proteolytically processed by caspase-3 or caspase-7, leading to the release of the nucleolytic subunit, which in turn catalyses the fragmentation of chromosomal DNA in apoptotic cells (5,12,13).

CAD and ICAD-L both have nuclear localisation sequences (NLSs) at their C-termini, which contribute to the nuclear accumulation of the CAD/ICAD-L complex (14). In addition to ICAD-L there exists a shorter splice variant termed ICAD-S (synonym DFF35) that differs from ICAD-L in the absence of the C-terminal domain, including the NLS (15). The function of this short form of the inhibitor and chaperone ICAD-L is not clear, since only an inhibitory but no chaperone activity for this protein has been demonstrated so far (16,17). On the other hand, it has been reported that in many tissues in which a caspase-3-dependent nucleolytic activity can be detected ICAD-S, and not ICAD-L, is the only form of the inhibitor expressed (18,19), suggesting that in these tissues ICAD-S acts as a chaperone and inhibitor of CAD. This prompted us to compare the effect of ICAD-L and ICAD-S on the formation and intracellular distribution of a nucleolytically active caspase-activated DNase in living cells. Our results with recombinant DFF complexes produced in *Escherichia coli* and fluorescent fusion proteins expressed in mammalian cells demonstrate that not only ICAD-L but also ICAD-S supports the formation of a catalytically competent nuclease and that ICAD-S and ICAD-LΔNLS lead to a similar, however not identical, intracellular distribution of EGFP–CAD, which in the case of ICAD-LΔNLS, in contrast to ICAD-S, is excluded from the nucleus in ~50% of transfected cells, suggesting an important role for the NLS in ICAD-L in the nuclear accumulation of CAD in the CAD/ICAD-L complex.

MATERIALS AND METHODS

Mammalian expression constructs

pCI-GST-DFF45 and pCS2-MT-CAD have been described by Korn *et al*. (20). pCS2-MT-DFF40 was constructed by

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inserting the DFF40 (i.e. human CAD) open reading frame amplified from cDNA isolated from HeLa cells by standard RT–PCR as a *Nco*I–*Stu*I/*Hpa*I fragment into pCS2-MT. pCMV-GST-ICAD-S was obtained by inserting the murine ICAD-S gene as a *Bam*HI–*Sma*I/*Bst*1107I fragment into the plasmid pCMV-GST (21). EGFP fusion constructs of murine CAD and CADΔNLS were produced by inserting the respective open reading frames as *Bam*HI/*Bgl*II–*Sal*I/*Xho*I fragments into a modified pcDNA3.1 vector allowing the expression of fusion proteins with an N-terminal EGFP tag. EGFP fusion constructs of DFF40 were produced by inserting the open reading frame as a *Kas*I–*Not*I fragment into a modified pCI vector allowing the expression of fusion proteins with an N-terminal EGFP tag. DFF40 with a C-terminal EGFP tag was produced by inserting DFF40 as a *Nhe*I–*Kpn*I fragment into a modified pCI vector allowing the expression of target genes with a C-terminal EGFP tag. The pEF-BOS-FlagICAD-L vector was a kind gift of Dr S. Nagata (Department of Genetics, Osaka University Medical School, Osaka, Japan) (3,5) (GenBank accession no. AB009375). DFF45 (i.e. human ICAD-L), ICAD-LΔNLS and DFF45ΔNLS variants were expressed using a modified pCI vector allowing the expression of fusion proteins with an N-terminal c-*myc* tag.

In vitro mutagenesis

For the analysis of the effects of ICAD-L, DFF45 and ICAD-S on the cellular distribution of full-length CAD and a CADΔNLS variant, we have generated variants of CAD as well as ICAD-L and DFF45 devoid of their C-terminally located NLSs. To this end the coding regions for these proteins were amplified by standard PCR procedures using reverse primers introducing the intended deletions and inserted into an appropriate expression vector as described above.

Cell culture and transfection

For the production of different GST-tagged DFF complexes in mammalian cells, HEK-293 cells (3 maxi-dishes/transfection) cultured at 37°C in a humidified atmosphere of 5% CO_2 in DMEM with 10% fetal calf serum, 100 U penicillin and 100 µg/ml streptomycin were transfected with 8 µg of each appropriate expression construct using Transfast transfection reagent (Promega) according to the supplier's recommendations. The complexes were purified by GST affinity chromatography as described by Korn *et al*. (20).

To study the cellular distribution of the variant EGFP-tagged DFF complexes, NIH 3T3 or CV1 cells, cultured in 6-well dishes under the same conditions as described above for HEK-293 cells, were co-transfected with 0.5–2 µg of each expression construct (3 µg total DNA) using Transfast transfection reagent (Promega) according to the supplier's recommendations.

For live cell imaging, transfected cells cultured in 6-well dishes were transferred to and directly analysed under a microscope (see below) 24–48 h after transfection.

Immunoblotting

Protein extraction from cultured cells and affinity purification of DFF complexes containing GST-tagged DFF45 or ICAD-S were performed as described earlier (20). After SDS–PAGE, the proteins were electroblotted onto PVDF membranes and GST-tagged DFF45 and ICAD-S were detected using a goat polyclonal anti-GST antibody (α-GST) (Pharmacia), CAD and DFF40 carrying an N-terminal *myc* tag using a monoclonal anti-*myc* antibody (α-*myc*) (Invitrogen). α-GST and α-*myc* were identified on the immunoblots using a horseradish peroxidase-conjugated anti-mouse antibody (Pierce) and a horseradish peroxidase-conjugated anti-goat antibody (Roche), respectively, in combination with enhanced chemiluminescence detection reagents (ECL; Amersham/Pharmacia).

Microscopy

Fluorescence microscopy was performed with a Leica DMLB microscope equipped with a Leica HCX APO L40X/0.80 W U-V-1 objective. An attached JVC TK-C1360 video camera was connected to a Pinnacle Systems Miro Video Capture Card to store digital images on a desktop computer. EGFP fluorescence was observed using Leica Filterset I3. Nuclear counterstaining of living cells was achieved using Hoechst 33342 dye, whose fluorescence was observed using Leica Filterset A. Transfected cells were also examined with a Leica TCS4D confocal laser scanning microscope using a Leica HCX APO L40X/0.80 W U-V-1 objective. Excitation of EGFP was achieved at 488 nm by a 75 mW Omnichrome argon/krypton laser. Emission was observed using a 580 nm beam splitter and a 510 nm longpass filter. Individual images were pseudo-coloured.

Bacterial expression of the DFF complexes

pGEX-2T-CAD was produced as described by Meiss *et al*. (22). pGEX-2T-DFF40 was generated by inserting the DFF40 open reading frame as a *Bam*HI/*Bgl*II–*Sma*I/*Ehe*I fragment into pGEX-2T. To set up a two plasmid system for independent induction of the two DFF subunits, the open reading frame of DFF45 was amplified by PCR and inserted as a *Bam*HI–*Nhe*I fragment into the P_L promoter-controlled expression vector pHisNuiA (23). To produce a vector compatible with pGEX-2T-CAD, the whole P_L promoter expression cassette including the DFF45 open reading frame was amplified by PCR and inserted as a *Nhe*I–*Sma*I fragment into pREP4 (Qiagen), providing resistance to kanamycin and the pA15 origin of replication, giving plasmid pLK-HisDFF45. pLK-HisFlagICAD-S was constructed by inserting the murine ICAD-S open reading frame including an N-terminal Flag tag as a *Bam*HI–*Eco*RI/*Mfe*I fragment into pLK-HisDFF45. Hamster caspase-3 cDNA was a kind gift of Dr X. Wang (Howard Hughes Medical Center and University of Texas, TX) and recombinant caspase-3 was produced as described by Meiss *et al*. (22). Production of the different DFF complexes was achieved by growing cultures of *E.coli* TGE900 [F^-, *su-1*, *ilv-1*, *bio*(Δ*cIts*857Δ*Bam*HI)], transfomed with the appropriate plasmids described above, at 28°C to an OD_{600} of 0.5 and then shifting the temperature to 42°C for 45 min. After 45 min, IPTG was added to a final concentration of 1 mM and after a further 45 min the temperature was lowered to 28°C again and the cells allowed to grow overnight. After cell lysis the complexes were purified by affinity chromatography using glutathione–Sepharose 4B beads as described by Korn *et al*. (20).

In vitro DNA cleavage assay

Plasmid DNA cleavage was performed by incubating CAD at 37°C in a buffer consisting of 20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol, 0.01% Triton X-100 and 5 mM $MgCl_2$. Cleavage products were analysed by electrophoresis on 0.8% TBE–agarose gels containing 0.05 μg/ml ethidium bromide. For the quantification of relative specific activities of CAD produced with either DFF45 or ICAD-S as the chaperone, different amounts of CAD released from the respective complexes by caspase-3 cleavage were incubated for 1, 3, 9, 27 and 60 min with plasmid DNA as described above. The disappearance of the supercoiled plasmid DNA band as measured by the analysis of ethidium bromide stained agarose gels was then used to determine the rate of DNA cleavage.

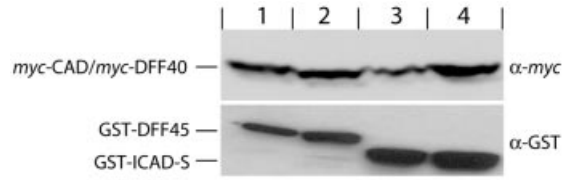

Figure 1. Production of DFF complexes in mammalian cells. HEK-293 cells were co-transfected with plasmids pCI-GST-DFF45 and pCS2-MT-DFF40 (lane 1), pCI-GST-DFF45 and pCS2-MT-CAD (lane 2), pCMV-GST-ICAD-S and pCS2-MT-DFF40 (lane 3) or pCMV-GST-ICAD-S and pCS2-MT-CAD (lane 4). The DFF complexes consisting of GST-tagged inhibitory subunits and *myc*-tagged CAD or DFF40 were purified from transfected cells by affinity chromatography using glutathione–Sepharose 4B beads. The presence of *myc*-tagged CAD or DFF40 was detected by immunoblotting after SDS–PAGE using anti-*myc* antibody (α-*myc*) and the presence of GST–DFF45 or GST–ICAD-S by immunoblotting using anti-GST antibody (α-GST). Both isoforms of the inhibitor of CAD, DFF45 and ICAD-S, support the production of CAD and DFF40.

RESULTS

Formation of CAD/ICAD-S complexes in mammalian cells

Data obtained with ICAD-S from *Rattus norvegicus* have shown that this isoform is the predominant form of the inhibitor of the caspase-3-activated DNase in rat brain and many other tissues (18). Since a chaperone activity for ICAD-S has not yet been demonstrated (6,7,16,17), the questions arise whether ICAD-S is able to function as a specific chaperone for CAD at all and what the differences in the chaperone activity between the long and the short isoforms of ICAD might be. In order to find out whether ICAD-S supports the formation of a nucleolytically competent caspase-activated DNase in mammalian cells we transfected HEK-293 cells with plasmids encoding GST-tagged DFF45 or ICAD-S and *myc*-tagged DFF40 or CAD, respectively. Forty-eight hours after transfection the cells were harvested and the cell lysates purified by affinity chromatography using glutathione–Sepharose 4B beads. As can be seen from Figure 1, we were able to detect ectopically expressed DFF40 and CAD by immunoblotting with an anti-*myc* antibody not only when co-expressed with DFF45 but also with ICAD-S, demonstrating that the short splice variant of the inhibitor of CAD has the ability to function as a specific chaperone for CAD and DFF40. It should be mentioned here that overexpression of CAD alone in mammalian cells or *E.coli* does not lead to a functional enzyme but rather to the formation of insoluble aggregates or inclusion bodies, respectively (6,7; G.Meiss, unpublished results). Results of activity assays with the *myc*-tagged nucleases demonstrate that nucleolytically active murine and human CAD can be obtained after co-expression with either DFF45 or ICAD-S (data not shown). The nucleolytic activity of the DNases produced with ICAD-S as the inhibitory subunit in the DFF complex is, however, lower than that produced with DFF45 (see below).

Production of functional CAD/ICAD-S complexes in *E.coli*

Since the nucleolytic activity of CAD produced in the presence of ICAD-S as the chaperone in mammalian cells was lower than that of CAD produced with DFF45 we wanted to set up a bacterial expression system that would allow the production of high amounts of the respective DFF complexes, which are needed for a more quantitative measurement of the relative activities of CAD preparations produced with either DFF45 or ICAD-S as the chaperone. Our idea was to generate an expression system that allows proper folding of the chaperone-dependent CAD in the presence of sufficiently high amounts of a given chaperone. To this end we established a two plasmid system which allows the independent and successive expression of heterologous genes in transformed *E.coli* cells. To produce recombinant DFF complexes with either DFF45 or ICAD-S as the chaperone, *E.coli* TGE900 cells, transformed with pLK-HisDFF45 or pLK-HisFlagICAD-S and pGEX-2T-CAD or pGEX-2T-DFF40, were first heat induced by raising the temperature from 28 to 42°C for 45 min to allow the expression of HisDFF45 or HisFlagICAD-S, followed by the addition of IPTG to induce the expression of GST-tagged CAD or DFF40. This procedure leads to the production of the different DFF complexes, which were subsequently purified by affinity chromatography using glutathione–Sepharose 4B beads, as demonstrated in Figure 2A for DFF complexes containing murine CAD. Upon activation of the purified complexes by caspase-3, the inhibitory subunits were cleaved (Fig. 2A), releasing nucleolytically active murine CAD (Fig. 2B). Similarly as observed for the CAD/ICAD-S complex formed in mammalian cells, the nucleolytic activity of CAD produced with ICAD-S as the chaperone and inhibitory subunit in the DFF complex was much lower than with DFF45 as the chaperone. Quantitative measurements show that the relative specific nucleolytic activity of CAD produced with ICAD-S is ~40-fold lower than that of CAD produced with DFF45, as determined with a plasmid DNA cleavage assay in which the disappearance of the supercoiled plasmid DNA band reflects the nucleolytic activity of the DNase (Fig. 2C). For the human counterpart of CAD, DFF40, we have measured an ~50-fold lower activity when produced with ICAD-S instead of DFF45 (data not shown).

Intracellular distribution of the CAD/ICAD-S complex in transfected cells

Given that in *E.coli* as well as mammalian cells ICAD-S is able to support the formation of catalytically active CAD,

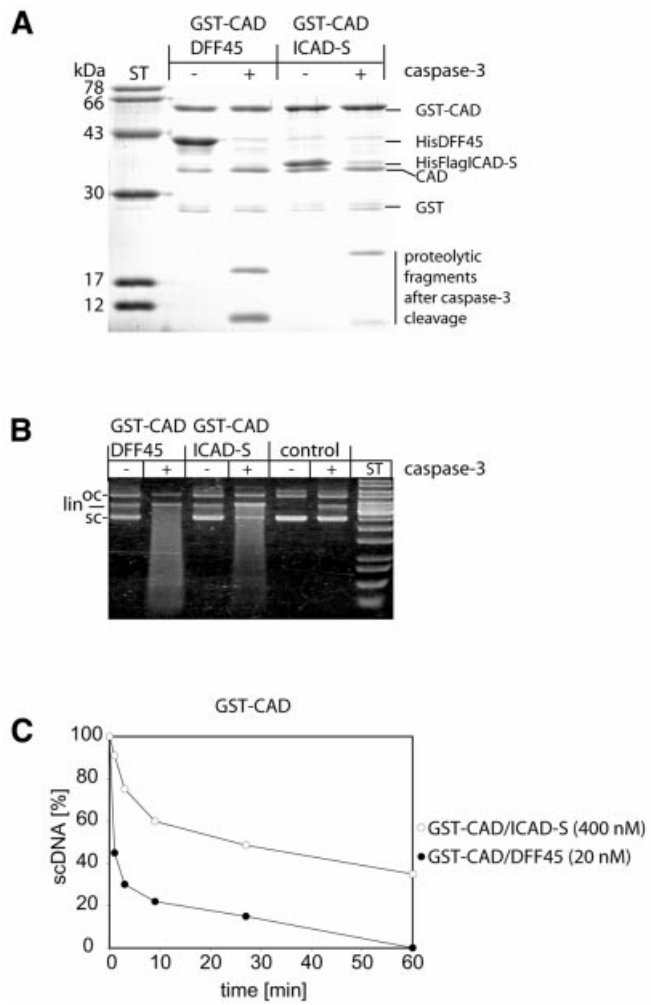

Figure 2. Formation of a functional CAD/ICAD-S complex in *E.coli*. (**A**) DFF complexes of GST–CAD and DFF45 or ICAD-S as the partner were produced in *E.coli* as described in Materials and Methods. SDS–PAGE analysis of the activation of GST–CAD in complex with DFF45 or ICAD-S by recombinant caspase-3 demonstrates the proteolytic processing of the inhibitory subunits. (**B**) Free GST–CAD, released from the purified GST–CAD/DFF45 or GST–CAD/ICAD-S complexes after activation with recombinant caspase-3, is nucleolytically active, as demonstrated by a plasmid DNA cleavage assay. (**C**) The activity of GST–CAD produced in the presence of ICAD-S (open circle, 400 nM GST–CAD in the assay) is lower by a factor of approximately 40 than that of GST–CAD produced in the presence of DFF45 (human ICAD-L) (filled circle, 20 nM GST–CAD in the assay). The different relative specific activities of CAD preparations produced with either ICAD-S or DFF45 as the chaperone were determined by a kinetic analysis of the disappearance of supercoiled (sc) plasmid DNA upon incubation with GST–CAD released from the complexes with ICAD-S or DFF45, respectively.

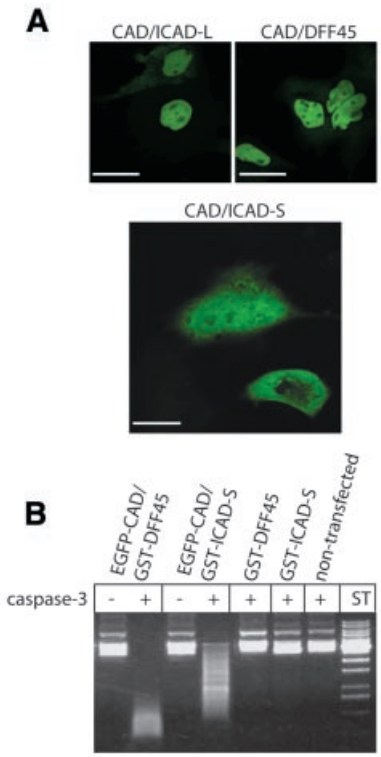

Figure 3. Effect of ICAD-S and ICAD-L on the distribution and formation of a nucleolytically active EGFP–CAD fusion protein. (**A**) To visualise the effect of the isoforms of ICAD on the distribution of an EGFP–CAD fusion protein in mammalian cells we have co-transfected CV1 cells with pcDNA-EGFP-CAD and pEF-BosFlagICAD-L, pcDNA-EGFP-CAD and pCI-c-*myc*-DFF45 as well as pcDNA-EGFP-CAD and pCMV-GST-ICAD-S, as described in Materials and Methods. Twenty-four hours after transfection, living cells were examined with a Leica TCS4D confocal laser scanning microscope. In the presence of ICAD-L or DFF45 (human ICAD-L), EGFP–CAD is found in the nuclei of transfected CV1-cells (top), whereas in the presence of ICAD-S, EGFP–CAD is predominantly found equally distributed throughout the cells (bottom). The bar corresponds to 10 µm. (**B**) To investigate whether the DFF complexes observed after co-transfection contain a catalytically competent DNase, HEK-293 cells were co-transfected with pcDNA-EGFP-CAD and pCI-GST-DFF45 or pCMV-GST-ICAD-S, respectively, and, as a control, transfected with pCI-GST-DFF45 or pCMV-GST-ICAD-S alone. The purified EGFP fusion proteins of CAD produced in the presence of either DFF45 or ICAD-S as the chaperone and inhibitory subunit in the DFF complexes are nucleolytically active after activation with caspase-3 *in vitro*.

albeit not as effectively as DFF45, we were interested to determine the intracellular distribution of a functional CAD/ICAD-S complex in living cells. For this purpose we have generated plasmids encoding EGFP fusion proteins of the murine and human caspase-activated DNases and co-transfected CV1 and NIH 3T3 cells with these plasmids and a plasmid coding for ICAD-L, DFF45 or ICAD-S. In contrast to the CAD/ICAD-L and CAD/DFF45 complexes, which accumulate in the nuclei of transfected cells (12,14,15), the CAD/ICAD-S complex was found evenly distributed throughout the cytoplasm and the nuclei in >90% of the transfectants (Figs 3A and 4C). In order to verify that the CAD/ICAD-S complex observed in the cells was a functional complex we have purified GST–ICAD-S/EGFP–CAD and, for comparison, GST–DFF45/EGFP–CAD complexes after co-transfection of HEK-293 cells with the respective plasmids. Subsequently, plasmid DNA cleavage assays were performed in order to see if a nucleolytically active DNase could be released from the purified complexes. As can be seen from Figure 3B, after caspase-3 activation of the complexes, produced either with GST–DFF45 or GST–ICAD-S as the chaperone and inhibitory subunit, a nucleolytically active EGFP–CAD fusion protein is released. In the case of ICAD-S, similarly as observed for GST–CAD in *E.coli*, the nucleolytic activity of EGFP–CAD is lower than that of the complex with DFF45 as the chaperone.

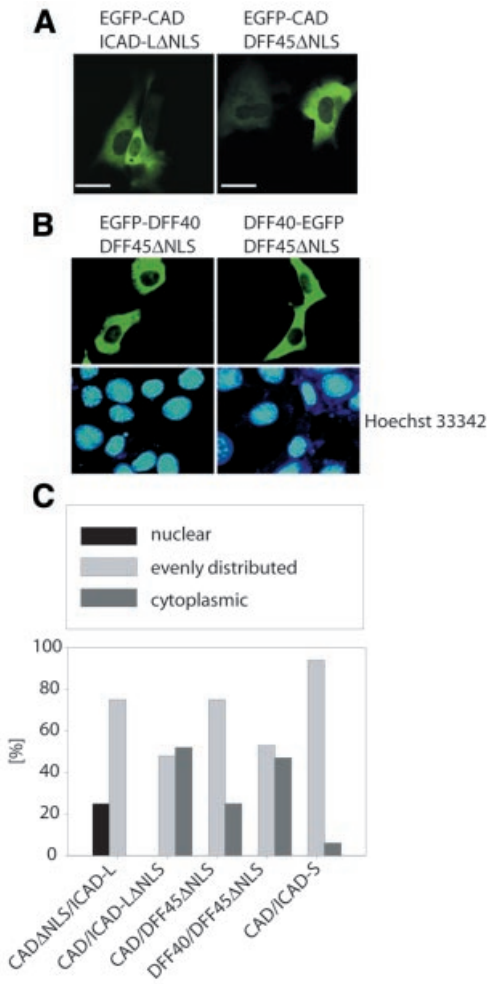

Figure 4. Effect of ΔNLS variants of the long isoforms of ICAD on the intracellular distribution of murine and human CAD. (**A**) Whereas in the presence of ICAD-S, EGFP–CAD was found predominantly equally distributed throughout transfected CV1 cells and only in a few cases excluded from the nuclei (see Fig. 3A), we find a considerable number of cells showing nuclear exclusion of EGFP–CAD in the presence of ICAD-LΔNLS (left) or its human counterpart DFF45ΔNLS (right), suggesting that in the CAD–ICAD-L complex the NLS of the long isoform of ICAD is particularly important for the nuclear accumulation of the apoptotic DNase. The cells were transfected with pcDNA-EGFP-CAD and pCI-c-*myc*-ICAD-LΔNLS or pCI-c-*myc*-DFF45ΔNLS and examined 24 h after transfection by confocal laser scanning microscopy as described in Materials and Methods. The bar corresponds to 10 μm. (**B**) The pure cytoplasmic distribution of two different EGFP-tagged fusion proteins of DFF40 (human CAD), carrying the EGFP moiety either at the N- (EGFP–DFF40) or the C-terminus (DFF40–EGFP), with DFF45ΔNLS as the partner in the DFF complex, was confirmed in NIH 3T3 cells transfected with pCI-c-*myc*-DFF45ΔNLS and pCI-EGFP-DFF40 or pCI-DFF40-EGFP (top). Nuclear counterstaining of living cells was done using Hoechst 33342 dye (bottom). Twenty-four hours after transfection the cells were examined by fluoresence microscopy as described in Materials and Methods. (**C**) For a statistical analysis of the effect of the ΔNLS variants of the long isoforms of ICAD and ICAD-S on the intracellular distribution of EGFP–CAD and DFF40 at least 150 cells were scored in every single transfection experiment. It is noteworthy that in the case of a CADΔNLS–EGFP fusion protein co-expressed together with ICAD-L, nuclear exclusion can only be observed in a very few cases; in fact 25% of the cells still accumulate EGFP–CADΔNLS in the nuclei of transfected cells, demonstrating that the NLS of ICAD-L can to a certain extent substitute for the NLS of CAD in targeting the enzyme to the nucleus.

These results clearly demonstrate that ICAD-S acts as a chaperone supporting the formation of a functional CAD/ICAD-S complex, which upon activation by caspase-3 releases nucleolytically active CAD that can be found in the cytoplasm and the nuclei of transfected cells.

Comparison of the effects of ICAD variants on the intracellular distribution of CAD in living cells

In order to compare the influence of the two isoforms of ICAD as well as an ICAD-L variant lacking the NLS on the intracellular distribution of CAD in living cells we performed co-transfection experiments with plasmids encoding EGFP–CAD and wild-type or NLS-deficient variants (ΔNLS) of ICAD-L and DFF45 as well as ICAD-S. Whereas in the case of the CAD/ICAD-L and CAD/DFF45 complexes, as expected, the majority of the cells showed a pure nuclear localisation, the CAD/ICAD-S complex is found evenly distributed throughout the whole cell (94%) and only in a few cases excluded from the nuclei (6%), similarly as observed earlier for DFF (14). However, in the case of the CAD/ICAD-LΔNLS complex we find an even distribution of the complex in 48% of the transfected cells and a purely cytoplasmic distribution with the nuclei excluded in 52% of the transfected cells (Fig. 4A and C). Similar results were obtained with the DFF40/DFF45ΔNLS complex: 53% of the transfected cells with an even distribution, 47% being excluded from the nuclei (Fig. 4B and C). Whereas in the presence of the ΔNLS variants of the long isoforms of murine and also human ICAD nuclear exclusion of different CAD and DFF40 fusion proteins, carrying the EGFP moiety either at the N- (EGFP–DFF40) or C-terminus (DFF40–EGFP), can be observed in many cases (Fig. 4B and C), there is only a minor fraction of cells showing nuclear exclusion of CADΔNLS–EGFP or DFF40ΔNLS–EGFP fusion proteins in the presence of ICAD-L or DFF45. In fact, in the case of EGFP–CADΔNLS in complex with ICAD-L we find 25% of the transfectants still accumulating EGFP–CADΔNLS in the nucleus (Fig. 4C), suggesting that the NLS of the long isoform of ICAD is particularly important for the nuclear accumulation of CAD.

DISCUSSION

The biological function of the short form of the inhibitor of the caspase-3-activated DNase, ICAD-S, has largely remained unclear, since a chaperone and inhibitory function has only been demonstrated so far with the long form of the inhibitor, ICAD-L (16,17). As it has been shown *in vitro* that ICAD-S, in spite of its presumptive inability to act as a chaperone, is a potent inhibitor of CAD, it was tempting to assume that ICAD-S acts as an inhibitory back-up system in the control of the nucleolytic activity of CAD through ICAD-L (5,16,17). On the other hand, it has been reported that in many tissues in which a caspase-3-dependent nucleolytic activity can be detected ICAD-S, and not ICAD-L, is the only form of the inhibitor present (18,19). Western analyses demonstrated that, for example, the brain, lung and kidney of the rat and mouse do not contain the long isoform of ICAD and, likewise, only ICAD-S is present in the human brain (18). These results, which established that ICAD-S is the endogenous inhibitory subunit of the caspase-3-activated DNase in rat neuronal and also some other tissues, suggested that in these tissues ICAD-S must also act as a chaperone for CAD (18). However, a

chaperone activity for ICAD-S has not yet been demonstrated in experiments using reticulocyte transcription/translation procedures or bacterial expression systems (6,7,16,17), which could mean that in those tissues lacking ICAD-L other chaperones might support the folding of CAD. Indeed, it has been shown using the reticulocyte transcription/translation system that the folding process of CAD requires the cooperation of general chaperones, like Hsc70 and Hsp40. These general chaperones, however, are only required to suppress aggregation of nascent CAD but are unable to restore its enzymatic function in the absence of ICAD-L (7). The results reported here with recombinant DFF complexes produced in *E.coli* and mammalian cells clearly show that ICAD-S indeed functions as a chaperone for CAD, supporting the formation of a catalytically competent caspase-3-activated DNase. The specific activity of CAD preparations obtained with ICAD-S is ~40- to 50-fold lower than that of CAD preparations obtained with DFF45. We attribute this difference in activity to the less effective chaperone activity of the short isoform ICAD-S as compared to that of the long isoform. Results obtained with a C-terminal deletion mutant of DFF45 that roughly corresponds to the shorter splice variant (Fig. 5) support our finding that ICAD-S acts as a specific chaperone for CAD (24,25). It has been shown previously that the homologous N-terminal domains (CAD or CIDE-N domain) of CAD and ICAD-L bind to each other co-translationally and that this binding is a crucial step in complex formation between CAD and ICAD-L, giving ICAD-L the possibility to fulfil its chaperone function (7,24–26). Since the splice variant ICAD-S only differs from ICAD-L in the absence of the C-terminal domain, it could well be that the two domains common to ICAD-S and ICAD-L have a basic chaperone activity that is enhanced in the case of the long isoform by its C-terminal domain (Fig. 5). It must be emphasised here that the three isolated domains of DFF45 did not show any detectable chaperone function when used in bacterial or mammalian co-expression experiments with DFF40 (24,25).

Ectopic expression of EGFP–CAD in the presence of ICAD-S in mammalian cells demonstrates that ICAD-S supports a moderate nuclear uptake of CAD. When EGFP-tagged CAD was expressed in the presence of GST fusion proteins of DFF45 or ICAD-S we were able to purify GST–DFF45/EGFP–CAD or GST–ICAD-S/EGFP–CAD complexes by affinity chromatography using glutathione–Sepharose 4B beads. After caspase-3 treatment these complexes release a nucleolytically active CAD, demonstrating directly that after co-expression in mammalian cells ICAD-S serves as a chaperone for CAD and that the complexes with EGFP–CAD observed after transfection of the cells contain a functional DNase. Biochemical fractionation of neuronal cells demonstrated that the endogenous CAD/ICAD-S complex is predominantly found in the nuclear fraction whereas ICAD-S alone can also be found in high amounts in the cytosol (18). Our transfection experiments show an even distribution of the complex in living cells, with equal amounts of the fusion protein in the cytosol and the nuclei of the transfectants, which confirms that EGFP–CAD in the presence of ICAD-S is able to enter the nucleus.

A comparison of the effect of ICAD-S and ICAD-L on the cellular distribution of the caspase-3-activated DNase demonstrates that in the presence of ICAD-S almost all cells show

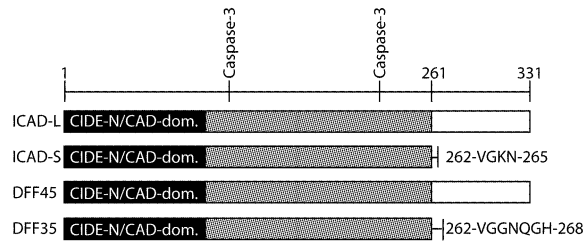

Figure 5. Structure of the splice variants of the inhibitors of CAD. The short isoforms of the inhibitors of CAD, ICAD-S and DFF35 (human ICAD-S) differ from the full-length isoform ICAD-L (DFF45) only in the absence of the C-terminal domain (white bars), including the NLSs. ICAD-S and DFF35 share the N-terminal domain (CAD or CIDE) (8,9,25) and the second domain of the full-length inhibitors, with short stretches of additional amino acid residues at their C-termini (ICAD-S, $_{262}$VGKN$_{265}$; DFF35, $_{262}$VGGNQGH$_{268}$). The CAD-N or CIDE-N domain of CAD (DFF40) and the inhibitors of CAD (amino acids 1–100) have been shown to be particularly important for the co-translational binding of the nascent CAD polypeptide chain to its chaperone ICAD-L (6,7,9,10). On the basis of our results, a basic chaperone activity can be attributed to ICAD-S and DFF35, which roughly resemble the first two domains of ICAD-L and DFF45 (amino acids 1–261), whereas the C-terminal domains of ICAD-L and DFF45 (amino acids 261–331) seem to enhance the chaperone activity of these isoforms.

an even distribution of EGFP–CAD throughout the cell, whereas ICAD-L leads to an almost exclusive nuclear localisation of CAD, as demonstrated earlier (14). It could well be that the relatively low amounts of CAD found in the nuclei of the cells in the presence of ICAD-S, compared to the high amounts of CAD accumulating in the nuclei in the presence of ICAD-L, are part of a regulatory mechanism to control apoptotic DNA degradation in those tissues having only ICAD-S and not ICAD-L as the complex-forming partner for CAD.

ICAD-LΔNLS, in contrast to ICAD-S, leads to the nuclear exclusion of EGFP–CAD in many cells, indicating that the NLS of ICAD-L in the DFF complex plays a decisive role in the nuclear transport of the apoptotic DNase. Interestingly, we find EGFP–CADΔNLS, co-expressed together with ICAD-L, excluded from the nuclei in only a very few cases, whereas the majority of the cells display an even distribution throughout the transfected cells or even a pure nuclear localisation of EGFP–CAD, suggesting differential effects of the NLSs of CAD and ICAD-L on the nuclear transport of CAD. Such differential effects could arise from masking of the NLS of CAD in the DFF complex with ICAD-L, similarly as has been observed for the interdependence of IκB and NFκB in nuclear transport (27).

In conclusion, we show here that not only the long isoform of the inhibitor of the caspase-activated DNase (ICAD-L) has a specific chaperone activity but also the short isoform (ICAD-S). This result and our studies on the intracellular distribution of a nucleolytically active apoptotic DNase in living mammalian cells with wild-type and NLS-deficient variants of ICAD-L as well as ICAD-S as the partner in the DFF complex demonstrate differential effects of the isoforms of ICAD on the relative specific activities and the amount of CAD available in the nuclei of mammalian cells. Together with data published by Chen *et al.* (18) our results support the hypothesis that ICAD-L and ICAD-S might function *in vivo* as

tissue-specifc modulators of an overall CAD activity available in the nuclei of cells, by controlling both the specific enzymatic activity and the amount of CAD in the nuclei.

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