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Characterization of splice variants of human caspase-activated DNase with CIDE-N structure and function

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Abstract Internucleosomal DNA fragmentation is an apoptotic event that depends on the activity of different nucleases. Among them, the DNA fragmentation factor B, better known as caspase-activated DNase (CAD), is mainly responsible for this DNA fragmentation in dying cells. CAD is an endonuclease that is chaperoned and inhibited by inhibitor of CAD (ICAD). Activation of CAD needs the cleavage of ICAD by activated caspase-3. During the characterization of the staurosporine-induced apoptotic process in human neuroblastoma cell lines, we have found three novel splice variants of CAD. In all three messengers, the open reading frame is truncated after the second exon of the CAD gene. This truncated open reading frame codifies the CAD protein amino terminal part corresponding to the cell death-inducing DFF45-like effector-N (CIDE-N) domain. We have detected these splicing variants in human tissues and in peripheral white blood cells from 10 unrelated individuals, and their products have been showed to be expressed in certain mouse tissues. We demonstrate that these truncated forms of CAD are soluble proteins that interact with ICAD. We also provided evidences that these CIDE-N forms of CAD promote apoptosis in a caspase-dependent manner.

Keywords: Apoptosis; DNA fragmentation factor; Caspase-activated DNase; Cell death-inducing DFF45-like effector; Alternative splicing; Neuroblastoma

1. Introduction

Apoptosis is a death process that plays an essential role in animal development and tissue homeostasis [1]. Chromatin condensation and internucleosomal DNA fragmentation are one of the main characteristic nuclear features and some of the best-recognized events in apoptotic cells [2]. These nuclear changes are mainly triggered by the DNA fragmentation factor (DFF) [3–8]. DFF complex is composed of two subunits, an endonuclease of 40 kDa named DFFB, also known as CPAN [5], caspase-activated DNase (CAD) [6] or DFF40 [7], and its inhibitor of 45 kDa named DFFA, also known as DFF45 [3] or inhibitor of CAD (ICAD) [8]. Here, we will refer to them as CAD and ICAD, respectively. ICAD is complexed with CAD in proliferating cells. When apoptosis is activated, executor caspases cleave ICAD at two Asp residues (D117 and D224) [6,8]. Cleaved ICAD is no longer able to inhibit CAD and nuclease release results in the cleavage of DNA at internucleosomal sites. There are two forms of ICAD that are generated by alternative splicing, ICAD-L and ICAD-S, for long and short forms, respectively [8]. Although both forms of ICAD can inhibit CAD nuclease activity, only ICAD-L together with Hsp70 and Hsp40 functions as a chaperone and is required for the production of functional CAD [9]. Moreover, experiments conducted with CAD and ICAD-null cells demonstrated that both CAD and ICAD-L mutually control their expression at the post-transcriptional level [10]. Thus, the DFF system ensures that a cell only undergoes DNA fragmentation when it receives apoptotic signals [11]. As both the human ICAD and CAD genes map to 1p36 [4,12], a region which is frequently deleted in neuroblastomas [13,14] and other tumours, a role for the DFF complex in malignal transformation has been suggested [15,16].

Although the contribution of other DNases, such as endonuclease G [17], AIF-activated DNase [18], and more recently Ape1 [19], to the apoptotic DNA fragmentation has also been proposed, CAD-null cells did not show DNA degradation upon apoptosis induction [20]. However, mice deficient in CAD showed no gross developmental abnormalities and were still able to undergo apoptotic chromosomal degradation. Disruption of both CAD and DNase II genes has been conducted in mice [20] and Drosophila [21]. The study of the double knockouts has led the authors to propose that the degradation of chromosomal DNA during apoptosis is mediated in vivo by two systems. One depending on the DFF complex and operating cell autonomously, and the other depending on the activity of DNase II inside the phagocytes [22].

CAD consists of two domains with differentiated functions. Its C-terminal domain displays DNA nuclease activity, whereas the N-terminal domain shows a regulatory role [23,24]. N-terminal domain of CAD is similar to N-terminal domain of ICAD and it has been demonstrated that the binding of the two proteins is due to homophilic interactions between the two N-terminal motifs [24–26]. The group of

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Abbreviations: CAD, caspase-activated DNase; CIDE, cell death-inducing DFF45-like effector; DFF, DNA fragmentation factor; ICAD, inhibitor of CAD; STP, staurosporine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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Nuñez described a novel family of cell death-inducing proteins structurally related to DFFs that they named CIDE (cell death-inducing DFF45-like effector). CIDE family is composed of three members, CIDE-A, CIDE-B and Fsp27 [27]. CIDE-3 has also been recently characterized as the human Fsp27 homologue [28]. Apoptosis-inducing effects of CIDE reside in its C-terminal domain. Both dimerization of the CIDE-C domain and mitochondrial localization of the CIDE dimers are essential for the induction of apoptosis [29]. Moreover, the different CIDEs share an N-terminal domain, the CIDE-N domain, homologous to the N-terminal domain of DFFs that mediates homophilic interactions with other CIDE-N domain-containing proteins. It has also been proposed that the CIDE-N of CIDE-B is able to sequester CIDE-N of ICAD from the DFF complex, thus releasing the nuclease function of CAD. These results also suggest that the different CIDE-N domains show displaceable binding [24].

While characterizing the cell death process induced by staurosporine (STP) in human neuroblastoma cell lines, we discovered three novel splice variants of CAD. Here, we report that these variants are produced from an aberrant splicing in the second intron of the CAD gene, which introduces a new stop codon and premature termination. These new CAD splice variants are not exclusive of neuroblastoma cells, since we have detected them in other human cell lines and in a number of human tissues, as well as in peripheral lymphocytes from 10 unrelated individuals. We have also demonstrated the expression of the corresponding truncated peptides in several mouse tissues. We finally provided evidences that these truncated proteins are structurally and functionally equivalent to the CIDE-N domain of the CIDE proteins and could promote apoptosis in a caspase-dependent manner.

2. Materials and methods

2.1. Amplification and sequencing of CAD

One microgram of total RNA was reverse transcribed with 10 pmols of the specific downstream primer DFFBR as described below. Five nanograms of cDNA was amplified by PCR in a Perkin–Elmer Thermal Cycler 2400 with 200 nM each of DFFBF (5′-TGCAATGCTCCAGAAGCCCAAGAGC-3′) and DFFBR (5′-TCACTGGCGTTTCCGCACAGGCTG-3′) primers. PCR conditions were 94 °C/20 s, 65 °C/20 s, 72 °C/1 min, for 35 cycles, in 50 mM Tris–HCl, pH 9.0, 2.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 1 U DyNAzime EXT DNA Polymerase (Finnzymes).

2.2. RT-PCR analysis

One microgram of RNA from either human neuroblastoma cell lines or lymphocytes was treated with 2 U DNase RNase free (Pharmacia) and reverse transcribed using 800 pmol of random hexamers (Roche Diagnostics), and 200 U MMLV-RT (Promega) for 1 h at 42 °C. To study the tissue distribution of the CAD splice variants, Human Multiple Tissue cDNA Panels I and II (Clontech, BD Biosciences) were analyzed.

Approximately 5 ng of cDNA was PCR amplified with the Herculase Hotstart DNA polymerase (Stratagene) as described for the CAD amplification, except for the reduction of the elongation time to 30 s. Primers used were: DFFBF (described above), CADIntR (5′-CCTGATGTCGCTCACATCTGCCAC-3′), which detect specifically the 7 and 50 bp insertions, and CADIntF (5′-CTGGCAGGGCTGT GAGTGGCA-3′), CADR3 (5′-TCTCAGATAGCCAGACTTGCT-CTG-3′), which amplify the 22 bp insertion, where CADIntF and CADIntR have been designed from the exon–intron boundary sequence [30] (see Fig. 1A). As a control, the RNA of the housekeeping L27 ribosomal protein was also amplified with 20 nM each primer in a multiplex reaction [31]. For the human cDNA panels, a set of commercial primers was used to amplify the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger.

2.3. Detection of CAD isoforms in mouse tissues

Mouse adult tissues were dissected and extracted in a 10-fold mass excess of ice-cold PD lysis buffer (40 mM Tris, pH 8.0, 500 mM sodium chloride, 6 mM EDTA, 6 mM EGTA, 0.1% Nonidet NP40, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM Na_3VO_4, 1 mM DTT, 2 μM PMSF, 1 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin). Twenty-five micrograms of protein was resolved in 15% SDS–PAGE and probed with the mouse Nt-CAD (Prosciences??) and the human CAD FL-338 (SantaCruz Biotechnology, sc-8342) antibody.

2.4. Cell lines and peripheral leukocytes

Human neuroblastoma cell lines IMR-5 [32] and SH-SY5Y [33] were kindly provided by Dr. Dionisio Martín-Zanca (Salamanca, Spain) and were cultured in DMEM containing 10% and 15% fetal calf serum, respectively. Human embryonic kidney 293T cells [34] were cultured in MEM containing 10% horse serum. Unless otherwise stated, all media and reagents were purchased from Sigma (St. Louis, MO). Apoptosis induction using STP was performed as described previously [35,36]. Human peripheral blood leukocyte samples from 10 healthy individuals were purified with an HistoPaque gradient (Sigma) following the manufacturer's instructions.

2.5. Expression and analysis of solubility of CAD fragments

A total of 1×10^6 human 293T cells were transiently transfected with 5 μg of either the wild type or the truncated forms of CAD plasmids using Lipofectamine^{TM}2000 (Gibco^{TM} Invitrogen Corp). After 24 h, cells were harvested and half of them were lysed in whole cell lysis buffer (50 mM Tris–Cl–2% SDS). The other half of harvested cells were lysed in soluble fraction buffer (50 mM HEPES, pH 6.8, 10 mM potassium chloride, 10% sucrose, 5 mM EGTA, 2 mM magnesium chloride and 1 mM dithiothreitol). These second fractions were then centrifuged at $13\,000 \times g$ for 10 min at 4 °C and supernatant (soluble fraction) and pellet (insoluble fraction) were recovered separately. Soluble fraction was directly processed, whereas insoluble fraction was resuspended in whole cell lysis buffer and further processed. Approximately 50 μg of protein from the different conditions was resolved in 15% SDS–PAGE. Western blotting using the anti-CAD (FL-338) antibody (Santa Cruz Biotechnology, sc-8342) was performed as described above.

A total of 1×10^6 human 293T cells were transiently transfected with 5 μg of the haemaglutinin (HA)-tagged constructs of the different forms of CAD either alone or in combination with FLAG-ICAD using Lipofectamine^{TM}2000 (Gibco^{TM} Invitrogen Corp). As a control of the Z-VAD treatment, anti-apoptotic HA-Bcl-X_L and pro-apoptotic HA-Bax plasmids were also included. Transfected cultures were then treated with 50 μM of Z-VAD-fmk or left untreated. After 24 h, cells were lysed in whole cell lysis buffer (50 mM Tris–HCl–2% SDS) and the level of expression of the different proteins analyzed by Western blot with the α-HA High Affinity antibody (Roche Diagnostics, clone 3F10). Membranes were stripped and re-blotted with the α-green fluorescent protein (Clontech BD Biosciences) and the α-panERK (Transduction Laboratories BD Biosciences) antibodies to control the transfection efficiency and the sample loading, respectively.

2.6. Immunoprecipitation assay

A total of 3×10^6 human 293T cells were transiently co-transfected with 25 μg of the different forms of CAD tagged with HA at their N-terminal region plus 15 μg of N-terminally FLAG-tagged full-length ICAD using the calcium phosphate method. After 24 h, cells were harvested in lysis buffer PD, lysates were clarified by centrifuging at $12\,000 \times g$ and 25 μg of protein of the supernatant was resolved in 15% SDS–PAGE. Western blotting using the α-HA High Affinity antibody (clone 3F10) (Roche Diagnostics) and the anti-FLAG M2 monoclonal antibody (Sigma) was performed. Alternatively, 1 mg of cleared supernatants was subjected to immunoprecipitation with 2 μg of the anti-FLAG antibody overnight at 4 °C. Antibodies were recovered with protein-G and samples were resolved in 15% SDS–PAGE. Western blotting was performed using the anti-HA antibody.

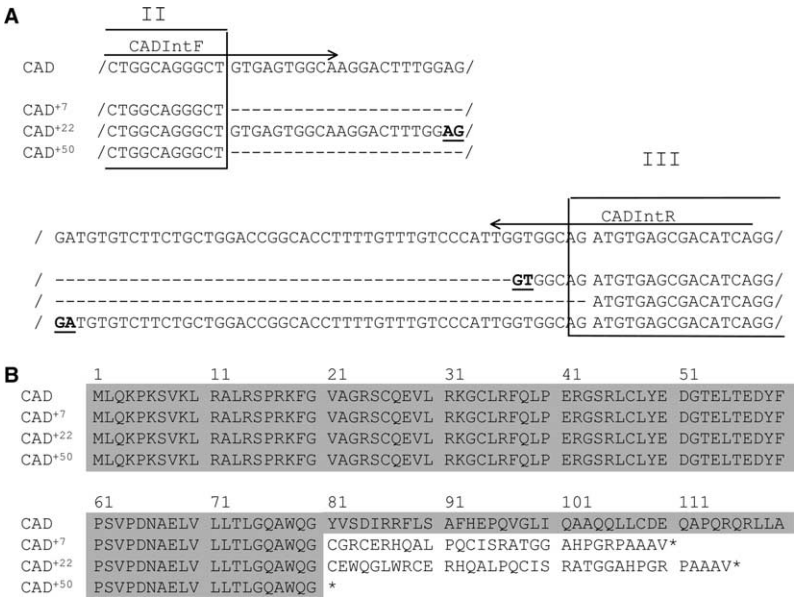

Fig. 1. Alternative spliced forms of the CAD gene are detected in neuroblastoma cell lines. (A) Sequence analysis of IMR-5 CAD RNAs population. Alignment of the alternative RNAs with the exon 2/intron 2 and the intron 2/exon 3 border sequences revealed an incorrect recognition of the donor site for the 22 bp insertion (CAD^{+22}), and a miss-recognition of the acceptor site for the 7 bp (CAD^{+7}) and 50 bp (CAD^{+50}) insertions. All the alternative splicing sites are in good agreement with the donor and acceptor consensus sites. The positions that are more conserved in the alternative transcripts compared with the CAD messenger are underlined. (B) Amino-acid alignment of the predicted truncated products with the full length CAD protein. Conceptual translation of these splicing variants give rise to truncated peptides of 110, 115 and 80 amino acids for the mRNAs with 7, 22 and 50 bp insertions, respectively, which contain the CIDE-N domain. Positions identical to the full length CAD protein [4] are shaded. Asterisks indicate stop codon. CAD^{+7}, CAD^{+22} and CAD^{+50} sequences have been deposited in the GenBank database under accession numbers AF409060, AF409061 and AF409062, respectively.

2.7. Assessment of apoptotic cell death

A total of 5×10^5 human 293T cells were co-transfected in triplicate with 2 μg of the adequate expression plasmids using Lipofectamine™2000 (Gibco™ Invitrogen Corp). The different forms of CAD were tagged with HA at their N-terminal region, whereas the ICAD was N-terminally FLAG-tagged. One microgram of enhanced green fluorescent protein (eGFP) was included systematically in the co-transfection to recognize transfected cells during counting. The total amount of plasmid transfected was adjusted with empty-pcDNA3 to be always the same in individual experiments. Twelve hours after transfection, cells were treated with 1 μM STP for 20 h or left untreated, and nuclei were stained for 30 min with 0.5 μg/ml of Hoechst 33258. The number of apoptotic versus normal nuclei in eGFP expressing cells was scored using an inverted Olympus microscope equipped with epifluorescence optics under UV illumination. Experiments were repeated four times and not less than 300 cells were counted per condition. Statistical significance was determined by Student's *t*-test. To control the level of expression of the different constructs, the same dishes used for the scoring were extracted in RIPA buffer and 50 μg of protein from different conditions was resolved in 15% SDS–PAGE. Western blotting using the anti-FLAG M2 antibody (Sigma), the Anti-HA antibody (Roche Diagnostics) and the anti-eGFP antibody (Clontech) was performed as described.

3. Results

3.1. Caspase-activated DNase gene shows alterations in the splicing of the second intron

STP is a powerful and non-specific inhibitor of protein kinases that has been established so far as an effective inductor of apoptotic cell death [35–39]. During the characterization of the STP-induced apoptotic process in neuroblastoma cells, we observed that IMR-5 cells did not show internucleosomal DNA fragmentation irrespective of the times and concentrations of STP used [35]. IMR-5 cells expressed normal levels of CAD but upon STP-induced cell death, CAD rapidly disappeared from cytosolic fractions. This fact explains the absence of oligonucleosomal degradation of DNA, since forced overexpression of CAD restored the laddering during apoptosis [36].

We further characterized the CAD gene by sequencing of the messenger. RT-PCR analysis was used to amplify the region corresponding to the open reading frame of the human CAD gene from human IMR-5 cells. Sequencing of several independent clones showed a major product with a sequence identical to that originally described by Mukae et al. [4]. Four out of 29 clones analyzed exhibited an insertion at the position corresponding to the exon 2–exon 3 junction (Fig. 1A). One of these clones holds a 7 bp insertion (CAD^{+7}), another a 22 bp one (CAD^{+22}), and the last two clones showed an insertion of 50 bp (CAD^{+50}). Sequence comparison of these extra fragments with the human genome sequence demonstrates that they were generated by miss-splicing of the CAD gene second intron, where the 7 and 50 bp insertions were produced by using novel splice acceptors, while the 22 bp insertion was produced by the use of a new donor site. These alternative-splicing sites showed an even better agreement with the consensus when compared with the canonical intron 2 splice sites (Fig. 1A). All these extra sequences resulted in a frameshift that generates a novel stop codon and premature termination (Fig. 1B).

Conceptual translation of the alternative sequences described above gives rise to truncated products that finish just at the exon–intron junction (CAD^{+50}, 80 amino acids in length) or a few amino acids later (110 and 115 amino acids in length for the CAD^{+7} and CAD^{+22} forms, respectively). These truncated peptides share a common feature; they correspond to the

CIDE-N domain of the CAD protein, a motive implicated in the protein–protein interaction with ICAD [23]. Moreover, it has been reported than the interaction between the CIDE-N domain of CAD and ICAD is increased when a part of the endonuclease domain is also implicated in this interaction [26]. The truncated products of 110 and 115 amino acids precisely fulfil that requirement (Fig. 1B).

3.2. Splicing variants of the CAD gene are detected in peripheral lymphocytes from normal individuals and are widely expressed in human and mouse tissues

We designed a RT-PCR-based analysis to explore for the presence of this kind of alternative RNAs in other cell types. We used primers spanning the exon–intron junction (see Fig. 1A) to avoid the amplification of the more abundant, full-length transcript and to detect the alternative ones. Fig. 2A shows the detection of these new messengers in IMR5 and SH-SY5Y neuroblastoma cells. Other cell lines such as HEK 293T, HeLa and Jurkat also express the different transcripts (data not shown). Moreover, in RNA samples from lymphocytes of 10 unrelated individuals, the three products were also amplified. In Fig. 2A, bands of 269 and 321 bp in upper panel correspond to the 7 and 50 bp insertion forms, respectively, and the single band of 263 bp in the lower panel corresponds to the 22 bp one. Bands were verified by sequencing. Co-amplification of the ribosomal gene L27 was carried out as a control for cDNA input in the amplification reaction.

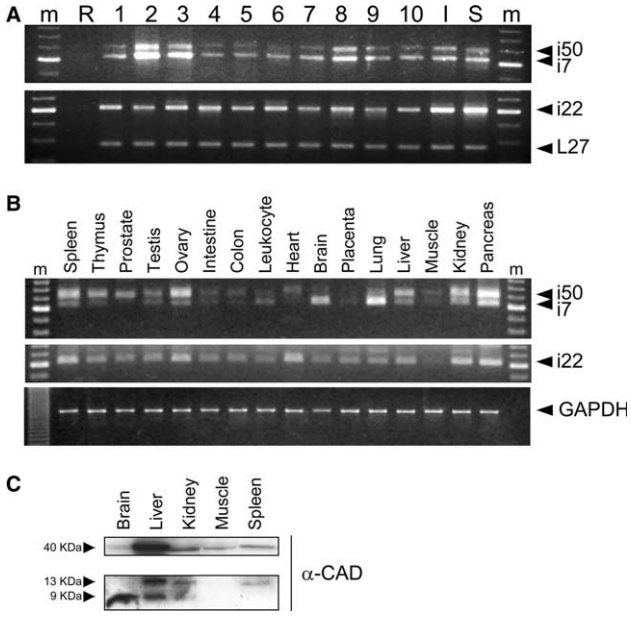

Fig. 2. Detection of the human CAD alternative transcripts in human cell lines and tissues. (A) RT-PCR analysis of lymphocytes of ten unrelated individuals (1–10), IMR-5 (I) and SH-SY5Y (S) neuroblastoma cell lines. (B) RT-PCR detection of the alternative messengers of CAD in a panel of human tissues (Clontech). Upper panels in A and B, detection of the 7 bp (i7) and 50 bp (i50) insertions. Lower panel in A and middle panel in B, amplification of the 22 bp insertion (i22). To control the amount of RNA in each sample, co-amplification of the L27 ribosomal protein (L27) and amplification of the GADPH messengers were performed in A and B, respectively. Controls without reverse transcriptase were performed to discard genomic amplification (R). m, 50 bp DNA ladder marker (Pharmacia). (C) Detection of the CAD truncated peptides in mouse tissues. Adult mouse tissues were analyzed for the presence of the different CAD isoforms by western blot with the CAD FL-338 antibody. Full length CAD is detected in the upper pannel as a single 40-kDa band in all tissues. A band of 13-kDa corresponding to the CAD^{+7} and CAD^{+22} products and a band of 9-kDa corresponding to the CAD^{+50} product, were detected in the lower panel after a longer exposure time.

We next used the same PCR strategy to analyze the expression profile of the CAD splicing variants on a commercial panel of human cDNAs. All three products were detected in a number of tissues in a more differential rather than ubiquitous pattern. Thus, some splice variants seem to be abundant in spleen, ovary, heart, brain, lung, kidney and pancreas, whereas all of them were barely detected in skeletal muscle (Fig. 2B). The GAPDH amplified product is shown in the lower panel as a control. Western blot analysis confirmed a differential expression pattern of the corresponding CAD truncated peptides in mouse adult tissues (Fig. 2C). Thus, two specific bands of 13- and 9-kDa were detected in liver and kidney, while single bands of 9- and 13-kDa were detected in brain and spleen extracts, respectively, and no bands were detected in skeletal muscle. As the predicted mass for the CAD^{+7}, CAD^{+22} and CAD^{+50} products are 12.1-, 12.6- and 8.8-kDa, respectively, the 13-kDa band might correspond to the first two products and the 9-kDa band to the third one. The protein expression profile match consistently with the RT-PCR data for these tissues.

Altogether, these results demonstrate that these splicing variants are generated in physiological conditions. Moreover, since the cells expressing these splice variants show the typical nuclear changes upon apoptotic induction, we were interested in analyzing what could be the function of these CAD short forms.

3.3. Truncated forms of CAD protein are soluble

It is well known that ICAD functions as a chaperone for CAD and is necessary both for the expression and the maintenance of CAD as a soluble protein [8,9,25]. Since it has been previously described that the insoluble character of CAD resides mainly in the endonuclease domain [26], we next explored the solubility of the truncated isoforms of CAD by Western blot analysis of different cellular fractions (Fig. 3A). To this end, the different CAD isoforms were over-expressed in HEK 293T cells. Full-length CAD was detected in the whole extract as a single 40-kDa band and was recovered mainly from the insoluble fraction. Moreover, a faint band in the soluble fraction was also detected. The same profile was obtained by expressing an HA-tagged CAD construct (data not shown), indicating that a fraction of the exogenous expressed protein could be maintained as a soluble protein probably by interacting with the endogenous ICAD. Both CAD^{+7} (not shown) and CAD^{+22} (Fig. 3A) constructs yielded a 13-kDa protein that was predominantly soluble, while the CAD^{+50} product gave rise to a shorter 9-kDa protein that was detected mainly in the soluble fraction (data not shown). When these products were co-expressed together with ICAD, the cellular levels of the truncated products clearly increased (Fig. 4A, middle panel). These results confirm that ICAD is also necessary for the proper production of the CAD short forms.

To identify the binding properties of the CAD truncated products, FLAG-tagged ICAD was co-expressed with HA-tagged CAD isoforms in 293T cells and immunoprecipitated with the anti-FLAG antibody. Immunoprecipitated extracts were analyzed by Western blot using the anti-HA antibody. Interaction of the control wild type CAD and the CAD^{+22}

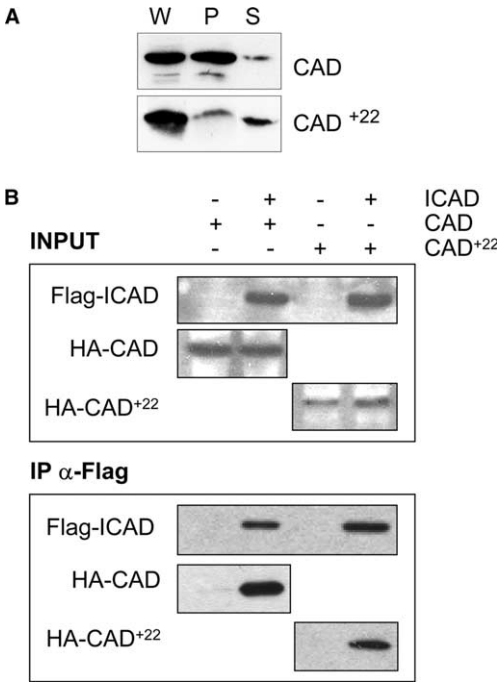

Fig. 3. CAD isoforms are soluble and interact with ICAD. (A) HEK 293T cells were transfected with the indicated constructs and whole cell extract (W), or soluble supernatant (S) and insoluble pellet (P) were analyzed by western blot using a N-terminal anti-CAD polyclonal antibody. A band of approximately 13 kDa is detected in both the soluble and pellet fractions of cells transfected with the CAD^{+22} splicing construct. Full-length CAD is detected as a 40 kDa band mainly in the insoluble fraction. (B) HEK 293T cells were co-transfected with the indicated expression plasmids and the resulting cellular lysates immunoprecipitated with the FLAG antibody. Upper panels (INPUT), total cellular lysates blotted with the indicated antibodies to assess equivalent expression of the different contructs. Lower panel (IP α-FLAG), immunoprecipitates blotted with the indicated antibodies. CAD and CAD^{+22} are clearly detected with the HA antibody in the FLAG immunoprecipitates.

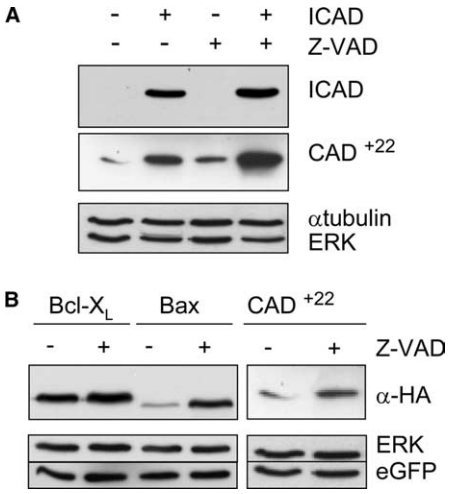

Fig. 4. ICAD enhances expression of truncated forms of CAD. Truncated forms of CAD induce a caspase-dependent cell death. (A) 293T cells expressing CAD^{+22} alone or in combination with ICAD were treated with 50 μM of Z-VAD or left untreated. Both the expression of ICAD and the treatment with Z-VAD dramatically increased the amount of CAD^{+22} protein as detected by western blot with the α-HA antibody. As a control, the same membranes were stripped and re-probed with α-tubulin or ERK antibodies. (B) Comparison of apoptosis induced with truncated forms of CAD with that observed expressing the proapoptotic protein Bax. HEK 293T cells co-transfected with the indicated HA-tagged proteins (Bcl-X_L, Bax and CAD^{+22}) plus the pEGFP plasmid were treated with Z-VAD or left untreated. Both CAD^{+22} and Bax protein expression are enhanced with Z-VAD treatment as detected by Western blot with the α-HA antibody. Western blot using the eGFP and ERK antibodies was performed to control the transfection efficiency and gel loading, respectively.

isoform with ICAD is illustrated in Fig. 3B. Wild type CAD and CAD^{+22} products were detected specifically in the corresponding immunoprecipitates of cells transfected with FLAG-ICAD, and were also detected specifically with the anti-HA antibody from the cellular lysates transfected with both plasmids. Same results were obtained for the CAD^{+7} and CAD^{+50} forms (not shown)

3.4. Truncated forms of CAD can induce a caspase-dependent cell death

When CAD fragments were transfected in HEK 293T cells, the amount of N-CAD protein detected by western blot was significantly increased if the cultures were simultaneously treated with the general caspase inhibitor Z-VAD (Fig. 4A and B). This observation might be interpreted as an enrichment of the culture on those cells expressing higher levels of the short proteins, which otherwise will die and disappear. This expression profile clearly resembles that obtained for Bax – a well characterized pro-apoptotic molecule – and might be considered as an indirect indication of a role of the CAD truncated forms in inducing cell death in a caspase-dependent manner.

To further understand the functionality of these alternative products, overexpression of the different isoforms of CAD either alone or in combination with ICAD was carried out in HEK 293T cells. STP-induced apoptosis, assessed by nuclear morphology with the Hoechst 33258 staining (Fig. 5C), was strongly inhibited by ICAD overexpression (Fig. 5A). When whole CAD was co-expressed together with ICAD, the percentage of apoptotic nuclei significantly increased, which indicate, a recovery in the endonuclease activity levels. Prevention of apoptosis by ICAD was partially reverted by co-expression of CAD^{+7}, CAD^{+22} or CAD^{+50}, thus suggesting that the CAD truncated forms might promote apoptosis. Although the apoptotic effect of short forms did not revert to the basal situation, the number of apoptotic cells significantly increased when compared with cells transfected with ICAD alone. Western blot analysis on the transfected cell lysates did not demonstrate any major interference in the levels of expression between the different constructs (Fig. 5B). Overexpression of each different form of truncated CAD alone did not significantly modify the percentage of STP-induced apoptotic nuclei when compared with the control cells (data not shown), a result which might be due to the requirement of ICAD to achieve functional cellular levels of the CAD short forms.

4. Discussion

We have described and characterized three novel splice variants of the CAD gene. In spite of their low level of expression, there are several evidences that support that these messengers are alternative variants rather than aberrant tran-

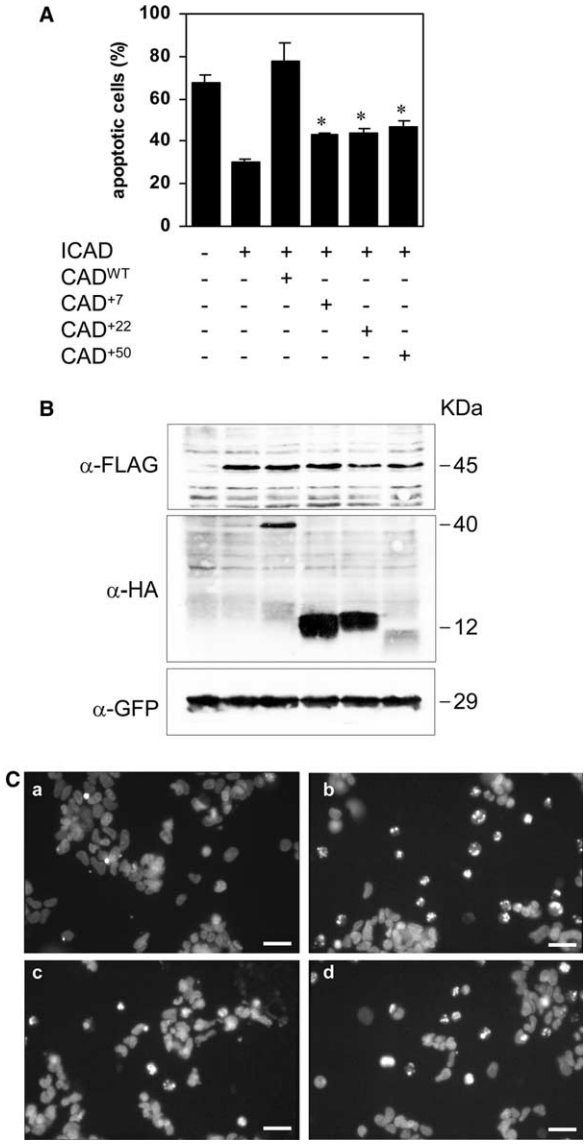

Fig. 5. Inhibition of apoptosis by ICAD is partially reverted by expressing isoforms of truncated CAD. (A) 293T cells were co-transfected with 2 μg of the indicated expression plasmids that contain N-terminally Flag-tagged ICAD and the different alternative forms of N-terminally HA-tagged CAD, together with 1 μg of a pEGFP-expressing reporter construct. The data (mean ± S.E.M.) represent the percentage of apoptotic nuclei, as revealed by the Hoechst staining, in the population of eGFP positive cells. Experiment was repeated three times. Asterisk indicates that the values are significantly different ($P < 0.01$) between the cultures transfected with ICAD alone and those co-transfected with ICAD and the CAD short isoforms. (B) Western blots of the corresponding transfected cell cultures were analyzed with anti-FLAG M2 antibody or anti-HA antibody. Numbers in the middle panel indicate the position of full length HA-tagged CAD (around 40 kDa) or the HA-tagged CAD fragments (around 12 kDa). An anti-eGFP antibody was used to control the comparable efficiencies in transfection or expression that were obtained in the different experimental conditions. (C) Representative photographs: a and b, cells transfected with the empty vector; c, cells transfected with ICAD; and d, cells transfected with ICAD and CAD^{+7}. In a, cells were untreated, whereas in b, c and d cells were treated with 1 μM STP for 20 h. Scale bars, 20 μM.

scripts. First, these forms of CAD are detected in primary lymphocytes from ten unrelated individuals and are widely expressed in a number of human tissues. Second, after extensive sequencing of several independent clones, no mutations in the splicing consensus sequences neither in exon 2 nor exon 3 have been detected. Third, it is worth noting that the cryptic splice donor and acceptor sites described for these splice variants are in fact more consistent than the normally used ones according to the consensus sequence [40]. And fourth, western blot analysis of mouse tissue extracts showed an expression profile for the different truncated isoforms which is in agreement with the RT-PCR data.

The ICAD/CAD system is mainly responsible for the internucleosomal DNA degradation and might be tightly regulated to prevent the release of a potentially harmful endonuclease in normal cells. Thus, ICAD binds to the nascent protein of CAD through their N-terminal domains to ensure the correct folding of a DNase which otherwise will result in a non-functional conformation, and at the same time ICAD remains complexed with CAD and inhibits CAD nuclease activity [22]. The truncated products of CAD described here lack the catalytic domain but retain the CIDE-N domain, a motive implicated in homophilic protein–protein interactions such as that between CAD and ICAD [23]. We demonstrated that these truncated CIDE-N containing molecules are soluble proteins which can interact with ICAD. Therefore, one possibility would be that these short forms could bind to ICAD to act as negative regulators by interfering in the correct folding of CAD. During the course of this work several abnormal CAD transcripts have been described in human hepatoma cells [16]. Although none of them match exactly with the ones described here, some transcripts specific of, and abundant in, poorly differentiated tumour cells codify for the CIDE-N domain of CAD. The authors speculate that these products may interfere with the normal CAD activity and in turn may award cells with survival advantages and carcinogenesis potential [16]. Conversely, the cell death assays conducted in the present study indicate that these short forms of CAD should induce apoptosis rather than interfering with the proper CAD activation.

It has been demonstrated that ICAD/CAD interaction is mediated by binding of three functional domains of ICAD (D1, D2 and D3) to two domains of CAD (CIDE and endonuclease), where D1 interact with the CIDE domain of CAD while D2 and D3 interacts with the catalytic domain of CAD [26]. Full-length ICAD or either D1–D2 or D2–D3 double domain fragments bind to CAD with higher affinity and are much more effective inhibitors than the isolated domains [25,26].

In STP-treated cultures, ICAD is degraded by active caspase-3 and dissociated from CAD. The endonuclease can still remain inhibited by the ICAD D1 domain, which interacts with the regulatory domain of CAD thus preventing its proper activation. It has been proposed that the residues of the EDG loop of the CAD CIDE-N domain are important for the nuclease activity, possibly by interacting directly with the CAD catalytic domain [23,24]. The fragments described here could displace the interaction between the ICAD D1 domain and CAD, allowing the regulatory domain of CAD available for the interaction with the catalytic domain. Moreover, the CAD isoforms described here contain the EDG loop and could act directly as trans-activators of the endonuclease activity. What could be the relevance of these products in a normal cell remains to be explained. In the model of endonuclease activation described above, either an excess of ICAD or a defect in CIDE

proteins could prevent proper activation of CAD once released from the DFF complex. The CAD isoforms described here are the first evidence of a CIDE-N only molecule produced physiologically. We favour the hypothesis that truncated forms of CAD could serve to modulate the endonuclease activity after apoptotic induction.

In conclusion, in this study we have identified three novel splice variants of the CAD gene in which the open reading frame is truncated after the second exon. These CIDE-N containing proteins are produced in physiological conditions and, unlike full-length CAD, these truncated forms are soluble proteins which can interact with ICAD and modulate the activity of the DFF complex. Altogether, our results suggest that CAD short isoforms can induce apoptosis in a caspase-dependent manner. Their function could be relevant during the apoptotic process of cellular systems in which high amounts of ICAD or absence of CIDE proteins could obstruct the proper activation of the endonuclease activity during apoptosis.

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References

[1] Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Cell 88, 347–354.
[2] Earnshaw, W.C. (1995) Curr. Opin. Cell. Biol. 7, 337–343.
[3] Liu, X.S., Zou, H., Slaughter, C. and Wang, X.D. (1997) Cell 89, 175–184.
[4] Mukae, N., Enari, M., Sakahira, H., Fukuda, Y., Inazawa, J., Toh, H. and Nagata, S. (1998) Proc. Natl. Acad. Sci. USA 95, 9123–9128.
[5] Halenbeck, R., MacDonald, H., Roulston, A., Chen, T.T., Conroy, L. and Williams, L.T. (1998) Curr. Biol. 8, 537–540.
[6] Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) Nature 391, 43–50.
[7] Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T. and Wang, X. (1998) Proc. Natl. Acad. Sci. USA 95, 8461–8466.
[8] Sakahira, H., Enari, M. and Nagata, S. (1998) Nature 391, 96–99.
[9] Sakahira, H. and Nagata, S. (2002) J. Biol. Chem. 277, 3364–3370.
[10] Nagase, H., Fukuyama, H., Tanaka, M., Kawane, K. and Nagata, S. (2003) Cell Death Differ. 10, 142–143.
[11] Nagata, S. (2000) Exp. Cell. Res. 256, 12–18.
[12] Leek, J.P., Carr, I.M., Bell, S.M., Markham, A.F. and Lench, N.J. (1997) Cytogenet. Cell. Genet. 79, 212–213.
[13] Abel, F., Sjoberg, R.M., Ejeskar, K., Krona, C. and Martinsson, T. (2002) Br. J. Cancer 86, 596–604.
[14] Judson, H., van Roy, N., Strain, L., Vandesompele, J., Van Gele, M., Speleman, F. and Bonthron, D.T. (2000) Hum. Genet. 106, 406–413.
[15] Konishi, S., Ishiguro, H., Shibata, Y., Kudo, J., Terashita, Y., Sugiura, H., Koyama, H., Kimura, M., Sato, A., Shinoda, N., Kuwabara, Y. and Fujii, Y. (2002) Cancer 95, 2473–2478.
[16] Hsieh, S.Y., Liaw, S.F., Lee, S.N., Hsieh, P.S., Lin, K.H., Chu, C.M. and Liaw, Y.F. (2003) Br. J. Cancer 88, 210–216.
[17] Li, L.Y., Luo, X. and Wang, X. (2001) Nature 412, 95–99.
[18] Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999) Nature 397, 441–446.
[19] Yoshida, A., Urasaki, Y., Waltham, M., Bergman, A.C., Pourquier, P., Rothwell, D.G., Inuzuba, M., Weinstein, J.N., Ueda, T., Appela, E., Hickson, I.D. and Pommier, Y. (2003) J. Biol. Chem. 278, 37768–37776.
[20] Kawane, K., Fukuyama, H., Yoshida, H., Nagase, H., Ohsawa, Y., Uchiyama, Y., Okada, K., Iida, T. and Nagata, S. (2003) Nat. Immunol. 4, 138–144.
[21] Mukae, N., Yokoyama, H., Yokokura, T., Sakoyama, Y. and Nagata, S. (2002) Genes Dev. 16, 2662–2671.
[22] Nagata, S., Nagase, H., Kawane, K., Mukae, N. and Fukuyama, H. (2003) Cell Death Differ. 10, 108–116.
[23] Inohara, N., Koseki, T., Chen, S., Benedict, M.A. and Núñez, G. (1999) J. Biol. Chem. 274, 270–274.
[24] Lugovskoy, A.A., Zhou, P., Chou, J.J., McCarty, J.S., Li, P. and Wagner, G. (1999) Cell 23, 747–755.
[25] McCarty, J.S., Toh, S.Y. and Li, P. (1999) Biochem. Biophys. Res. Commun. 264, 176–180.
[26] McCarty, J.S., Toh, S.Y. and Li, P. (1999) Biochem. Biophys. Res. Commun. 264, 181–185.
[27] Inohara, N., Koseki, T., Chen, S., Wu, X. and Nunez, G. (1998) EMBO J. 17, 2526–2533.
[28] Liang, L., Zhao, M., Xu, Z., Yokoyama, K.K. and Li, T. (2003) Biochem. J. 370, 195–203.
[29] Chen, Z., Guo, K., Toh, S.Y., Zhou, Z. and Li, P. (2000) J. Biol. Chem. 275, 22619–22622.
[30] Sugimoto, N., Fukuda, Y., Saito-Ohara, F., Kamiyama, R., Nakagawara, A., Mukae, N., Nagata, S. and Inazawa, J. (1999) J. Hum. Genet. 446, 408–411.
[31] Soler, R.M., Dolcet, X., Encinas, M., Egea, J., Bayascas, J.R. and Comella, J.X. (1999) J. Neurosci. 19, 9160–9169.
[32] Reed, J.C., Meister, L., Tanaka, S., Cuddy, M., Yum, S., Geyer, C. and Pleasure, D. (1991) Cancer Chemoth. Pharm. 51, 6529–6538.
[33] Biedler, J.L., Roffler-Tarlov, S., Schachner, M. and Freedman, L.S. (1978) Cancer Res. 38, 3751–3757.
[34] Numa, F., Hirabayashi, K., Tsunaga, N., Kato, H., O'Rourke, K., Shao, H., Stechmann-Lebakken, C., Varani, J., Rapraeger, A. and Dixit, V.M. (1995) Cancer Res. 55, 4676–4680.
[35] Boix, J., Llecha, N., Yuste, V.J. and Comella, J.X. (1997) Neuropharmacology 36, 811–821.
[36] Yuste, V.J., Bayascas, J.R., Llecha, N., Sanchez-Lopez, I., Boix, J. and Comella, J.X. (2001) J. Biol. Chem. 276, 22323–22331.
[37] Bertrand, R., Solary, E., O'Connor, P., Kohn, K.W. and Pommier, Y. (1994) Exp. Cell. Res. 211, 314–321.
[38] Jarvis, W.D., Turner, A.J., Povirk, L.F., Traylor, R.S. and Grant, S. (1994) Cancer Res. 54, 1707–1714.
[39] Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millán, J.A. and Reed, J.C. (1995) Cell 80, 279–284.
[40] Watson, J.D., Gilman, M., Witkoswki, J. and Zoller, M. (1992) Recombinant DNA, second ed. Scientific American Books, New York.