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Letter to the Editor

Mutually regulated expression of caspase-activated DNase and its inhibitor for apoptotic DNA fragmentation

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Dear Editor,

One of the hallmarks of apoptosis is the degradation of chromosomal DNA into nucleosomal units.[1–3] The apoptotic DNA fragmentation depends on caspase activation in most cases. We and others have identified a caspase-activated DNase (CAD), which is also called a caspase-activated nuclease (CPAN) or DNA fragmentation factor-40 (DFF 40).[4–6] The CAD's DNase activity is controlled by its inhibitor, ICAD (inhibitor of CAD), which is also called DFF45. CAD is complexed with ICAD in proliferating cells,[5] and when caspases, particularly caspase 3, are activated by apoptotic stimuli, ICAD is cleaved, resulting in the release of CAD, which fragments the cellular DNA.[3,4] There are two forms of ICAD that are coded by alternatively spliced ICAD mRNAs, and they are designated ICAD-L and ICAD-S for the long and short forms, respectively.[7] We have previously shown that both ICAD-L and ICAD-S are ubiquitously expressed at the mRNA and protein levels in mouse tissues.[8,9] Since ICAD-L, but not ICAD-S, supported the functional production of CAD in an *in vitro* translation system with reticulocyte lysates,[4,10] we concluded that ICAD-L works as a chaperone for CAD during its synthesis. Meanwhile, Chen *et al.*[11] reported that most mouse and rat tissues express only ICAD-S (DFF35) and that ICAD-S is complexed with the functional CAD. We have recently established lines of ICAD-null or CAD-null mice by gene-targeting (ref. 19, and KK, HF, and SN, unpublished results). Using primary splenocytes and thymocytes, and the established mouse embryonal fibroblasts (MEF) from them, we show here that ICAD-L, but not ICAD-S, is responsible for the functional production of CAD, and that CAD and ICAD-L mutually regulate their expression at the post-transcriptional level.

The spleens and thymus of ICAD$^{-/-}$ mice did not express CAD (Figure 1a), and stimulation of the ICAD$^{-/-}$ lymphocytes with Fas ligand (FasL) did not cause DNA fragmentation (data not shown), which agree with the previous report by Zhang *et al.*[12] Northern hybridization of ICAD$^{-/-}$ mouse spleen and thymus mRNA indicated that these ICAD$^{-/-}$ tissues expressed CAD mRNA as abundantly as the wild-type tissues (Figure 1a), indicating that the expression of CAD is post-transcriptionally regulated by ICAD. The MEFs established from the wild-type embryos expressed CAD at a very low level, but a similar result – no CAD protein in the ICAD-deficient cells – was observed in the ICAD$^{-/-}$ MEFs (Figure 1b). To examine which ICAD isoforms, ICAD-L or ICAD-S, is responsible for CAD expression, the ICAD$^{-/-}$ MEF was transformed with an ICAD-L or ICAD-S expression plasmid, and two independent clones for each were established (Figure 1b). As shown in the lower panel of Figure 1b, both ICAD-L transformed clones expressed a significant level of CAD, while ICAD-S did not support the CAD expression. Accordingly, the ICAD-L transformants underwent DNA fragmentation upon the stimulation with FasL, while no DNA fragmentation was observed in the ICAD$^{-/-}$ MEF or its ICAD-S transformants (Figure 1c). These results indicated that ICAD-L is necessary for the functional production of CAD.

In proliferating cells, CAD is complexed with ICAD-L.[5] The CAD protein was expressed abundantly in lymphocytes of the spleen and thymus, while MEFs expressed little CAD protein (Figure 1a and b). Western blotting analysis with anti-ICAD protein indicated that ICAD was also expressed abundantly in lymphocytes, but weakly in MEFs (Figure 1d). In particular, the expression level of ICAD-L differed significantly among tissues. That is, ICAD-L was more abundant than ICAD-S in the spleen and thymus, which abundantly express CAD, while ICAD-S was more abundant than ICAD-L in MEFs, which express little CAD (Figure 1d), suggesting that the expression of ICAD-L is regulated by CAD. In fact, the expression level of ICAD-L, but not ICAD-S, was greatly diminished in the spleen, thymus, and MEFs in the CAD$^{-/-}$ background. This effect was at the post-transcriptional level, because the expression levels of the 2.3-kb ICAD-L mRNA and the 3.8-kb ICAD-S mRNA[8] were not reduced by the CAD deficiency (Figure 1d). To confirm the effect of CAD on ICAD-L expression, the CAD$^{-/-}$ MEF was transformed with the CAD expression vector and two clones were obtained. As shown in Figure 1e, the ICAD-L protein level was greatly upregulated in the two transformed clones, although the expression level for ICAD-L and ICAD-S mRNA was similar to that found in the CAD$^{-/-}$ parental cells.

Here, we showed that ICAD-L must be present for the functional production of CAD. These results do not agree with the results obtained by Chen *et al.*,[11] but do agree with the fact that ICAD-L, but not ICAD-S has the ability to facilitate the folding of CAD although both forms of ICAD can inhibit the CAD's DNase with similar efficiency.[10] In the absence of ICAD-L, no CAD protein was detected, although CAD mRNA in the ICAD$^{-/-}$ cells was as abundant as in the ICAD$^{+/+}$ cells. ICAD-L binds to the nascent polypeptide of CAD while it is on ribosomes, and CAD is produced as a complex with ICAD-L.[13] It seems that if the nascent polypeptide of CAD is misfolded without ICAD-L, it is immediately degraded as proposed for many other proteins.[14] Examining how the misfolded CAD is recognized by cells as a target for degradation may help us understand the molecular mechanism for the quality control of protein synthesis. CAD expression level differs significantly among different tissues. By comparing the expression of

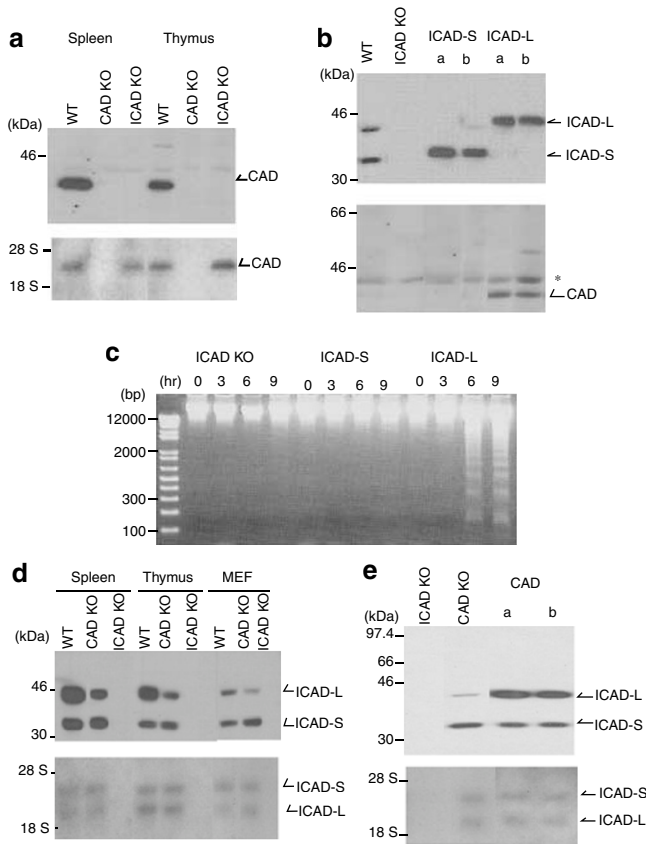

Figure 1 (**a**) No expression of CAD in ICAD$^{-/-}$ cells. A hamster monoclonal antibody (mAb) against mouse CAD was established by immunizing Armenian hamsters with the recombinant active CAD produced in Sf9 cells.[10] The S-30 fractions (20 μg protein each) from the spleen and thymus of the wild-type (WT), CAD$^{-/-}$ (CAD-KO), and ICAD$^{-/-}$ (ICAD-KO) genotypes were analyzed by Western blotting using the anti-mouse CAD mAb. The relative molecular weights of the marker proteins are shown in kDa at left. In the lower panel, poly(A) RNA (3 μg) from the spleen and thymus of the wild-type, CAD$^{-/-}$, and ICAD$^{-/-}$ mice were analyzed by Northern hybridization with mouse CAD cDNA[4] as the probe. The positions of 28S and 18S rRNA are indicated at left. (**b**) Establishment of MEFs expressing ICAD-L or ICAD-S. The wild-type, CAD$^{-/-}$, and ICAD$^{-/-}$ MEFs were established as described[17] using embryos at day 13.5 of gestation as the source of the primary fibroblasts. The expression plasmid for ICAD-S or ICAD-L was introduced into the ICAD$^{-/-}$ MEF, and two transformant clones (a and b) were established for each. The S-30 fractions (20 μg protein each) from wild-type and ICAD$^{-/-}$ MEFs and the two ICAD-S-expressing and two ICAD-L-expressing transformant MEF clones were analyzed by Western blotting using a rabbit anti-mouse ICAD antibody[10] (upper panel) or the anti-CAD mAb (lower panel). Bands indicated by * are probably nonspecific. (**c**) Apoptotic DNA fragmentation in ICAD transformants. The ICAD$^{-/-}$, ICAD-S- or ICAD-L-expressing MEFs were incubated for the indicated periods of time with 5000 U/ml of leucine-zippered human FasL[18] in the presence of 1.5 μg/ml cycloheximide. The chromosomal DNA was then analyzed by electrophoresis on an agarose gel as described.[4] (**d**) The reduced expression of the ICAD-L protein in CAD-deficient cells. The S-30 fractions (20 μg protein each) from the spleen and thymus of the wild-type, CAD$^{-/-}$, and ICAD$^{-/-}$ mice, and their MEFs were analyzed by Western blotting using a rabbit anti-ICAD antibody. The bands for ICAD-L and ICAD-S are indicated by arrows at right. In the lower panel, poly(A) RNA (3 μg) from the corresponding tissues and the MEFs were analyzed by Northern hybridization using mouse ICAD cDNA[4] as the probe. The upper and lower ICAD mRNAs code for the ICAD-S and ICAD-L proteins, respectively,[8] and are indicated by arrows at right. (**e**) Upregulation of the ICAD-L expression by CAD. The expression plasmid for CAD[10] was introduced into the CAD$^{-/-}$ MEF, and two transformed clones were established. The S-30 fractions (20 μg protein each) from ICAD$^{-/-}$, CAD$^{-/-}$, and the two CAD-expressing MEF clones (clones a and b) were analyzed by Western blotting using the rabbit anti-ICAD antibody. In the lower panel, poly(A) RNA (3 μg) was analyzed by Northern hybridization with mouse ICAD cDNA as the probe

ICAD among these tissues, we unexpectedly found that the expression of ICAD-L is regulated by CAD. This regulation was not at the transcriptional level. It may not be at the translational level either, because, unlike CAD, functional ICAD-L can be synthesized without CAD in *E. coli* or mammalian cells, and it can be refolded from the denatured protein without CAD.[7] There are a few precedents for the post-translational regulation of the regulatory subunit by the catalytic subunit. For example, the cAMP-dependent protein kinase is composed of regulatory and catalytic subunits, and the regulatory subunit is rapidly degraded in cells when it is not associated with the catalytic subunit.[15,16] Since ICAD-L is complexed with CAD,[5] it is possible that the ICAD-L that is associated with CAD is more stable than is uncomplexed ICAD-L. This may explain the lack of effect of CAD on the expression of ICAD-S, which is not complexed with CAD.[4] In any event, the autoregulatory loop between ICAD and CAD indicates that the CAD–ICAD system is tightly regulated for the production of the functional CAD, a potentially harmful protein for cells.

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1. Earnshaw WC (1995) Curr. Biol. 7: 337 – 343
2. Wyllie AH (1980) Nature 284: 555 – 556
3. Nagata S (2000) Exp. Cell Res. 256: 12 – 18
4. Enari M *et al.* (1998) Nature 391: 43 – 50
5. Liu X *et al.* (1997) Cell 89: 175 – 184
6. Halenbeck R *et al.* (1998) Curr. Biol. 8: 537 – 540
7. Sakahira H *et al.* (1998) Nature 391: 96 – 99.
8. Kawane K *et al.* (1999) Cell Death Differ. 6: 745 – 752
9. McIlroy D *et al.* (2000) Genes Dev. 14: 549 – 558
10. Sakahira H *et al.* (1999) J. Biol. Chem. 274: 15 740 – 15 744.
11. Chen D *et al.* (2000) J. Biol. Chem. 275: 38 508 – 38 517.
12. Zhang J *et al.* (1998) Proc. Natl. Acad. Sci. USA 95: 12 480 – 12 485.
13. Sakahira H and Nagata S (2002) J. Biol. Chem. 277: 3364 – 3370.
14. Schubert U *et al.* (2000) Nature 404: 770 – 774.
15. Steinberg RA and Agard DA (1981) J. Biol. Chem. 256: 10 731 – 10 734.
16. Amieux PS *et al.* (1997) J. Biol. Chem. 272: 3993 – 3998
17. Todaro GJ and Green H (1963) J. Cell Biol. 17: 299 – 313
18. Matsumura H *et al.* (2000) J. Cell Biol. 151: 1247 – 1255.
19. Kawane K *et al.* Nature Immunol., (in press).