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FLICE is activated by association with the CD95 death-inducing signaling complex (DISC)

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Upon activation, the apoptosis-inducing cell membrane receptor CD95 (APO-1/Fas) recruits a set of intracellular signaling proteins (CAP1-4) into a death-inducing signaling complex (DISC). In the DISC, CAP1 and CAP2 represent FADD/MORT1. CAP4 was identified recently as an ICE-like protease, FLICE, with two death effector domains (DED). Here we show that FLICE binds to FADD through its N-terminal DED. This is an obligatory step in CD95 signaling detected in the DISC of all CD95-sensitive cells tested. Upon prolonged triggering of CD95 with agonistic antibodies all cytosolic FLICE gets proteolytically activated. Physiological FLICE cleavage requires association with the DISC and occurs by a two-step mechanism. Initial cleavage generates a p43 and a p12 fragment further processed to a p10 fragment. Subsequent cleavage of the receptor-bound p43 results in formation of the prodomain p26 and the release of the active site-containing fragment p18. Activation of FLICE is blocked by the peptide inhibitors zVAD-fmk, zDEVD-fmk and zIETD-fmk, but not by crmA or Ac-YVAD-CHO. Taken together, our data indicate that FLICE is the first in a cascade of ICE-like proteases activated by CD95 and that this activation requires a functional CD95 DISC.

Keywords: apoptosis/CAP1-4/CD95(APO-1/Fas)/DISC/ICE-proteases

Introduction

The apoptosis-inducing receptor CD95 (APO-1/Fas) belongs to the TNF receptor superfamily (Smith *et al.*, 1990). Activation of CD95 by crosslinking, either with its natural ligand CD95L (Suda *et al.*, 1993) or with agonistic antibodies such as anti-APO-1 (Trauth *et al.*, 1989), induces apoptosis in sensitive cells. Recently, a number of proteins have been identified that bind to the intracellular death domain (DD) of the stimulated CD95 receptor (for a review see Peter *et al.*, 1996). These proteins, which have been designated cytotoxicity-dependent APO-1-associated proteins (CAP1-4) form the death-inducing signaling complex (DISC) together with CD95 (Kischkel *et al.*, 1995). CAP1 and CAP2 were identified as the DD-containing signaling molecule FADD (also called MORT1) (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995). CAP4 has recently been identified as a member of the interleukin-1β converting enzyme (ICE) family (Muzio *et al.*, 1996) and was named FLICE (for FADD-like ICE; Muzio *et al.*, 1996) or MACHα1 (Boldin *et al.*, 1996). FLICE shows homology to the ICE-like proteases in the C-terminus of the protein. In its activated form FLICE was shown to cleave poly(ADP)-ribose polymerase (PARP) or the fluorogenic substrate Ac-DEVD-AMC. The N-terminus of FLICE (prodomain) contains two regions which share substantial homology with the death effector domain (DED) of FADD. These domains are essential for binding of FLICE to FADD and therefore for recruitment of FLICE to the DISC (Boldin *et al.*, 1996; Muzio *et al.*, 1996). ICE-like proteases play a key role in the induction of most forms of apoptosis. They fall into three classes: (i) ICE (Cerreti *et al.*, 1992; Thornberry *et al.*, 1992), ICErelII/TX/Ich-2 (Faucheu *et al.*, 1995; Kamens *et al.*, 1995; Munday *et al.*, 1995) and ICErelIII/TY (Munday *et al.*, 1995; Faucheu *et al.*, 1996); (ii) ICE-like proteases similar to Yama/CPP32/apopain (Tewari *et al.*, 1995; Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995) such as ICE-LAP3/Mch3/CMH-1 (Fernandes-Alnemri *et al.*, 1995a; Duan *et al.*, 1996a; Lippke *et al.*, 1996), Mch2 (Fernandes-Alnemri *et al.*, 1995b), ICE-LAP6 (Duan *et al.*, 1996b), Mch4 (Fernandes-Alnemri *et al.*, 1996); (iii) Ich-1/Nedd2 (Kumar *et al.*, 1994; Wang *et al.*, 1994). FLICE belongs to the group of Yama-like proteases believed to execute the death signal. We now present data on the mechanism of activation of FLICE, the most receptor-proximal ICE-like protease. We show that in the CD95 pathway FLICE can only be processed and activated when recruited to the CD95 DISC. This initial step of CD95-mediated apoptosis results in cleavage of all cytosolic FLICE at the receptor level. Active FLICE is released into the cytosol, where it can activate a cascade of ICE-like proteases.

Results

The DISC is essential for induction of apoptosis by CD95

To test whether binding of all CAP proteins to the CD95 DISC is a general feature of CD95-induced apoptosis, a number of CD95-sensitive cell lines of different origins were analyzed. In all tested cell lines the complete DISC (CAP1-4) was detected (Figure 1). We therefore conclude that DISC formation is crucial for initiation of a death signal by CD95.

FLICE has been shown to exist in various isoforms

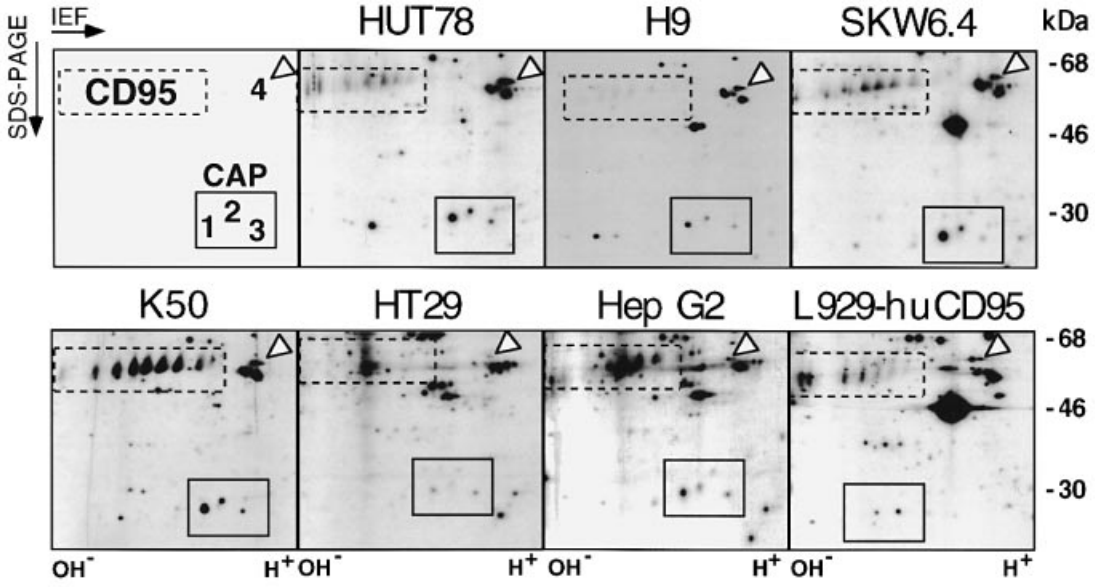

Fig. 1. DISC formation is the essential first step during CD95-induced apoptosis. Analysis of the CD95 DISC in different metabolically labeled cells using a two-dimensional gel system. In the upper left panel the migration positions of CD95 and CAP1-4 are indicated. Note that the DISC shown in the bottom right panel was derived from a murine cell line (L929 cells transfected with human CD95) and therefore the CAP proteins showed a slightly different running behavior as compared with the human cell lines.

(presumably representing different splice variants), which were named MACHα2–3/MACHβ1–4 (Boldin *et al.*, 1996) or Mch5 (Fernandes-Alnemri *et al.*, 1996). In addition, a FLICE-like protease, Mch4, has recently been found that is derived from a different gene (Fernandes-Alnemri *et al.*, 1996). Since CAP4/FLICE migrated as a doublet or triplet spot in all cell lines tested, we determined whether all CAP4 spots represent the same FLICE protease or alternatively different isoforms of FLICE. The CAP4 spot originally sequenced by nanoelectrospray tandem mass spectrometry (Muzio *et al.*, 1996) was identical with spot 1 in Figure 2D. To test whether the other prominent CAP4 spot (spot 2 in Figure 2D) might represent a FLICE-like molecule such as Mch4 co-migrating with FLICE at a similar position, both spots were subjected to MALDI mass spectrometry (MS) (Figure 2A and B). In the spectrum in Figure 2A, nine peptides match the FLICE sequence with a mass accuracy better than 0.1 Da covering 22% of the sequence. The same peptide pool is observed in the spectrum of CAP4/2 (Figure 2B), showing that these two spots originate from the same gene. Additionally, three peptides from each of the digests were sequenced by nanoelectrospray tandem MS (data not shown), confirming this conclusion.

To determine whether both CAP4 spots might represent differentially spliced FLICE proteins, migration of *in vitro*-translated ^{35}S-labeled FLICE was compared with DISC-associated CAP4 (Figure 2C and D). The *in vitro* labeled protein (Figure 2C) migrated as a triplet spot at exactly the same position as the DISC-bound CAP4 (Figure 2D). The inhomogeneity in the CAP4 spot must therefore be the result of either a post-translational modification or of different structural conformations during isoelectric focussing (IEF).

We conclude that all CAP4 spots represent one protein only, full-length FLICE. In addition, no other protein that would correspond to an alternatively spliced FLICE molecule was detected as part of the DISC in any of the cell lines tested (data not shown).

Prolonged stimulation of CD95 converts CAP4 into CAP5 and CAP6

The CD95 DISC forms within seconds of receptor engagement (Kischkel *et al.*, 1995). To test whether the composition of the DISC changes after prolonged stimulation, Raji B cells were treated with the agonistic anti-APO-1 monoclonal antibody (mAb) for 30 min. In addition to CAP1-4, two new proteins, designated CAP5 and CAP6, were identified (insert b in Figure 3A). Detailed kinetics showed that DISC formation was detectable after 10 s by increased binding of CAP1/FADD and CAP4/FLICE (Figure 3B). The CAP2 and CAP3 spots were very weak in these cells. Binding of the CAP proteins peaked at 1–5 min. At 20 min the intensity of CAP4/FLICE was reduced. At this time CAP5 and CAP6 appeared. After 40 min no CAP4/FLICE was detectable in the DISC. However, CAP5 and CAP6 were very prominent. At 60 min, when cells started to disintegrate, the intensities of all spots were reduced. Notably, during the entire kinetics the intensity of CAP3, which was recently identified as containing at least part of the FLICE N-terminus, did not change. To test whether CAP5 and CAP6 could represent the FLICE prodomain, a polyclonal antibody was raised against the linker region between the second DED and the ICE protease domain. This antibody (anti-FLICE-N) therefore recognizes both full-length FLICE and the FLICE prodomain. Re-immunoprecipitation of DISC-bound CAP5 and CAP6 with this antibody confirmed that CAP5 and CAP6 are FLICE N-terminal fragments (data not shown). We used the anti-FLICE-N antibody to immunoprecipitate cytosolic FLICE (Figure 4A) after clearing the lysates of CD95 DISC (Figure 3). Uncleaved FLICE (CAP4) was detected in the cytosol from cells unstimulated or stimulated for up to 5 min. At 20 min the amount of

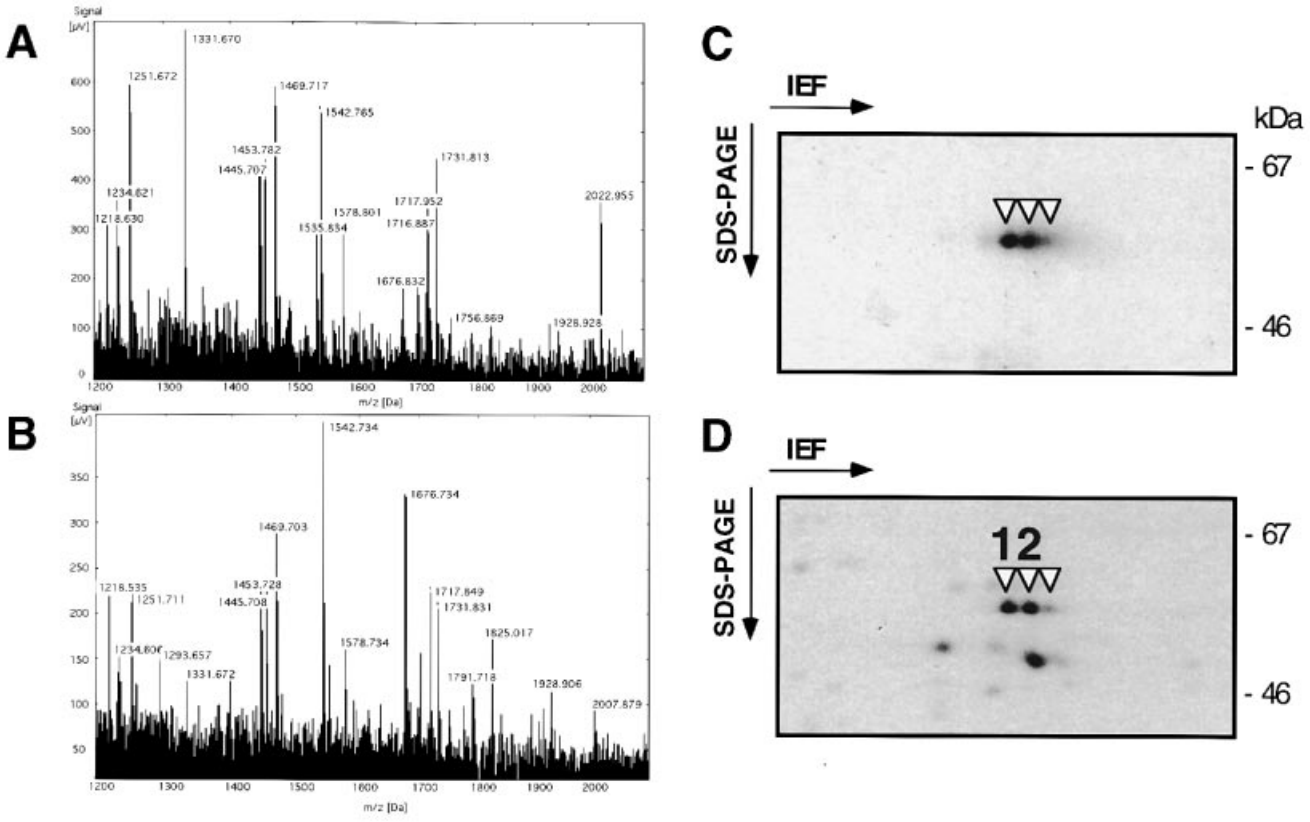

Fig. 2. The different CAP4 spots represent FLICE. (**A** and **B**) Comparison of MALDI peptide maps of CAP4/1 (A) [corresponding to spot 1 in (D)] and CAP 4/2 (B) [corresponding to spot 2 in (D)]. Silver stainable amounts of CAP4 were prepared as previously described (Muzio *et al.*, 1996). (**C**) *In vitro* translated ^{35}S-labeled FLICE and (**D**) *in vivo* ^{35}S-labeled, CD95-co-immunoprecipitated CAP4 forms, analyzed by two-dimensional IEF/SDS–PAGE. Only the parts of the gels covering the CAP4 migration area are shown. Arrowheads point to the three different spots which run at identical positions in both gels.

FLICE in the cytosol was reduced and it was almost absent after 60 min stimulation. Starting at 20 min stimulation anti-FLICE-N immunoprecipitated CAP5 and CAP6 in the cytoplasm. CAP5 and CAP6 therefore represent different forms of the FLICE prodomain, perhaps resulting from cleavage at two different sites. Interestingly, cleavage of DISC-bound FLICE (Figure 4B) slightly preceded the appearance of cleaved cytosolic FLICE (Figure 4C), consistent with initiation of the death signal at the receptor. We conclude that almost all FLICE in the cell is activated after induction of apoptosis through CD95.

FLICE binding to the DISC results in autocatalytic cleavage to its active components

CAP4/FLICE does not interact with CD95 directly but binds to the adaptor molecule FADD (Chinnaiyan *et al.*, 1996a). To test whether binding to FADD alone was enough to trigger autoprocessing of FLICE, ^{35}S-labeled *in vitro* translated FLICE was bound to either His-tagged FADD or a GST–FADD fusion protein coupled to the appropriate beads (Figure 5A, lanes 3 and 6). Taking into account that a post-translational modification of FADD might be required for proper FLICE activation, FADD beads were also incubated with lysates from unstimulated (Figure 5A, lanes 4 and 7) or stimulated (Figure 5A, lanes 5 and 8) SKW6.4 cells prior to addition of ^{35}S-labeled FLICE. After incubating the beads at 4°C for 24 h all unbound proteins were removed by washing. A smaller form of FLICE was also generated during the *in vitro* translation, likely representing an N-terminally truncated FLICE due to differential initiation at the second methionine (arrow in Figure 5A, lane 1). This was confirmed by precipitation with the anti-peptide antibodies anti-FLICE-N and anti-FLICE-C (see below; Figure 6A). Furthermore, both molecular weight and pI exactly match the calculated values (Figure 6B). Interestingly, this N-terminal truncated form did not bind to FADD. This demonstrates that the first of the two DEDs in FLICE needs to be intact to allow FLICE/FADD engagement. None the less, binding to FADD alone is not sufficient for activation of FLICE, since no cleavage was observed. When ^{35}S-labeled FLICE was added to immunoprecipitated CD95 from unstimulated (Figure 5B, lanes 1 and 3) or stimulated (Figure 5B, lanes 2 and 4) cells, only the stimulated DISC specifically induced cleavage of FLICE into fragments similar to the ones seen in the *in vivo* analysis (Figure 3). Both a 26 kDa fragment (p26), identical to CAP5 (see below), and full-length FLICE were detected attached to the DISC (bound to beads) (Figure 5B, lane 2). In addition, however, an intermediate FLICE cleavage product was found at 43 kDa (p43). Both p43 and the p26 FLICE prodomain remained bound to the DISC after washing, suggesting that both contained the first DED at the FLICE N-terminus (Figure 5B, lane 2). In the supernatant of this assay FLICE fragments were detected that did not associate with the DISC components anymore, p18, p12 and p10, as well as p26 (Figure 5B, lane 4). p18, p12 and p10 likely represent the active

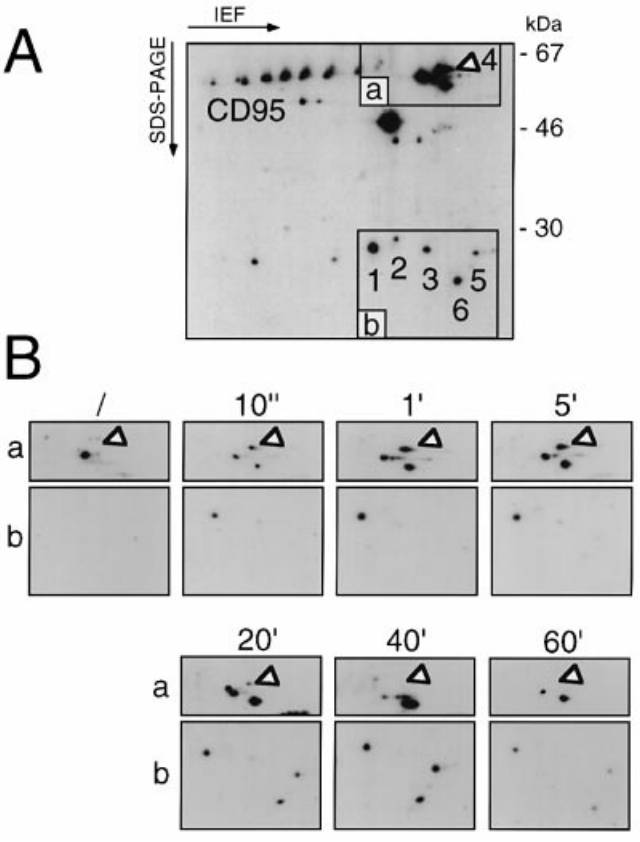

Fig. 3. Two novel DISC components are detected after prolonged stimulation. (**A**) DISC analysis of metabolically labeled Raji cells after treatment with anti-APO-1 for 30 min. The positions of CD95, CAP1-4 and the two novel components CAP5 and CAP6 are indicated. (**B**) Time course of CAP4 cleavage in the DISC. Raji cells were treated with anti-APO-1 for different periods of time, after which they were rapidly cooled to 4°C. Subsequently, the DISC was analyzed on two-dimensional gels. The parts of the gels shown represent the region of the gel containing CAP4/FLICE (a) and the region containing CAP3–6 (b), as indicated in (A).

enzyme subunits. This assumption was confirmed by using this supernatant to cleave PARP, which we have previously reported to be a substrate for FLICE (Muzio *et al.*, 1996). PARP was efficiently cleaved by the supernatant of an *in vitro* FLICE assay, generating a characteristic 85 kDa PARP fragment (Figure 5C, lane 2). To verify that the activity in the supernatant was due to FLICE, we generated a second rabbit polyclonal antibody recognizing the very C-terminus of FLICE (anti-FLICE-C). Depleting the supernatant of the active FLICE subunits with this antibody indeed resulted in a loss of PARP cleaving activity (Figure 5C, lane 3).

Identification of the different FLICE cleavage products in vivo and in vitro

To confirm the identity of all FLICE cleavage fragments, immunoprecipitation of *in vitro* processed ^{35}S-labeled FLICE was done using the antibodies anti-FLICE-N and anti-FLICE2. p26 was identified as the FLICE prodomain, since it was only immunoprecipitated by anti-FLICE-N, which recognizes a peptide at the very C-terminus of the prodomain (Figure 6A, lane 4). In addition, part of p26 remained bound to the DISC, indicating that it contained the first DED. Under denaturing conditions anti-FLICE-C immunoprecipitated only p12 and p10, indicating that these two fragments contained the FLICE C-terminus (Figure 6A, lane 5). These results were confirmed by a two-dimensional gel-based analysis of all FLICE cleavage products. On a two-dimensional gel all FLICE fragments had characteristic molecular weights and pI values (Figure 6B). Comparison with the primary protein sequence allowed us to assign cleavage sites (Figure 6C). Usually, the first cleavage site in ICE-like proteases is the aspartate residue following the active site. Consistent with the migration of these fragments on a two-dimensional gel (Figure 6B), cleavage at Asp374 results in formation of p43 and p12. p43 is then further cleaved, likely at Asp216, resulting in two fragments, p26 and p18. Finally, processing of p12 (pI 4.63) at its N-terminal Asp384 residue results in a pI shift to 6.5, as seen on the two-dimensional gel (Figure 6B).

As mentioned, p43 was not found *in vivo* (see Figure 3A). Similarly, the p12 fragment could not be detected *in vivo* by immunoprecipitation or Western blotting using anti-FLICE-C (Figure 9F; data not shown). Therefore, p43 and p12 likely represent cleavage intermediates that can only be detected in the *in vitro* FLICE assay carried out at 4°C. On the other hand, the additional cleavage of the prodomain found *in vivo* resulting in formation of CAP6 was not detected *in vitro*. Taken together, all our data suggest that FLICE cleavage is a two step mechanism that is too rapid to be detected *in vivo*. In the first step FLICE is cleaved at Asp374, generating p43 and p12, and in the second step cleavage occurs at Asp216 and Asp384, giving rise to the active enzyme p18/p10 and the prodomain (Figure 6C).

Activation of FLICE requires binding to the DISC

The complete turnover of cytosolic FLICE seen in Figure 4A could be achieved in two ways: (i) FLICE in the DISC could be activated and active FLICE subunits might, once released from the DISC, cleave FLICE in the cytoplasm; (ii) FLICE could be turned over at the DISC and would not be able to cleave cytosolic FLICE. To answer this question, an enzymatically inactive FLICE molecule was generated by replacing the essential cysteine residue in the active center with serine (FLICE C360S). To distinguish this mutant protein from wild-type (wt) FLICE during SDS–PAGE, C-terminally truncated FLICE C360S was used (ΔFLICE C360S). When ^{35}S-labeled wt FLICE was subjected to an *in vitro* FLICE assay the characteristic subunits p18, p12 and p10 were formed (Figure 7A, lane 1). Processing of ΔFLICE C360S resulted in generation of the Δp12 and Δp10 subunits and p18, consistent with its C-terminal truncation (Figure 7, lane 2), demonstrating that ΔFLICE C360S was as efficiently processed as the wt protein. To test whether the active wt FLICE subunits would accept uncleaved ΔFLICE C360S as a substrate, both were incubated together (Figure 7A, lane 3). No cleavage of ΔFLICE C360S was observed, indicating that the active FLICE subunits (p18/p10), once released from the DISC, did not accept FLICE as a substrate, while they readily cleaved PARP (Figure 5C, lane 2).

Similarly, incubation of FLICE with the CD95 DISC for different times showed a clear increase in FLICE processing from 2 to 24 h (Figure 7B, lane 1 and 2). This increase required the presence of an active CD95 DISC,

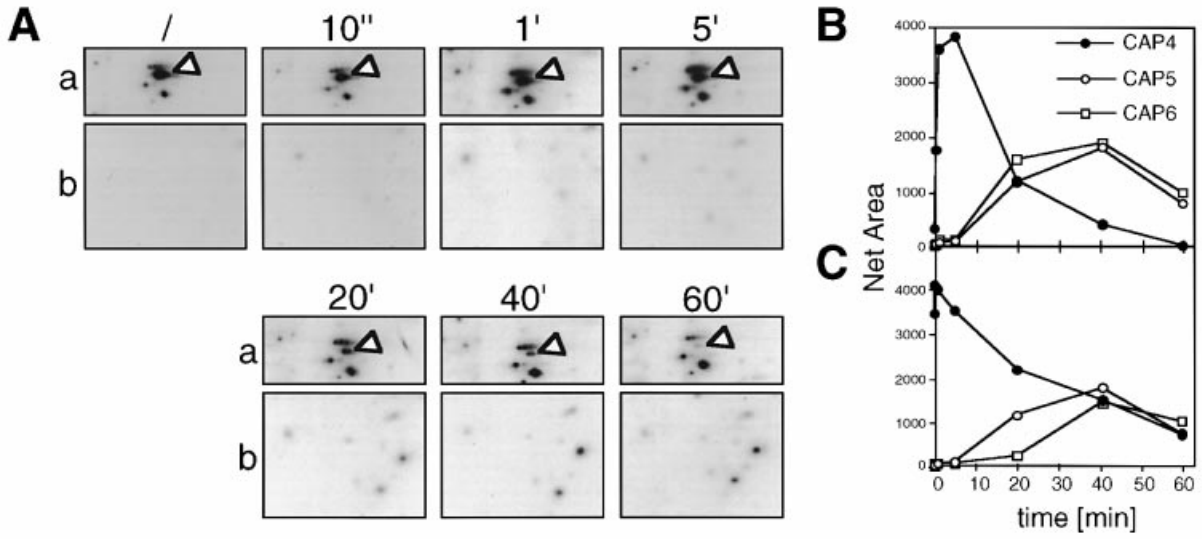

Fig. 4. All cytosolic FLICE is cleaved. (**A**) Kinetics of FLICE cleavage in the cytosol. Metabolically labeled Raji cells were treated with anti-APO-1 for different periods of time as indicated. Cells were lysed after stimulation and cleared from the CD95 DISC present. Subsequently, cytosolic FLICE was immunoprecipitated with anti-FLICE1. Analysis was as in Figure 3. (**B** and **C**) Densitometric analysis of CAP4, CAP5 and CAP6 spots. The intensities of CAP4, CAP5 and CAP6 either from CD95 DISC immunoprecipitations (B) as shown in Figure 3B or from cytosolic FLICE immunoprecipitations (C) as shown in (A) were determined and plotted against time of treatment with anti-APO-1.

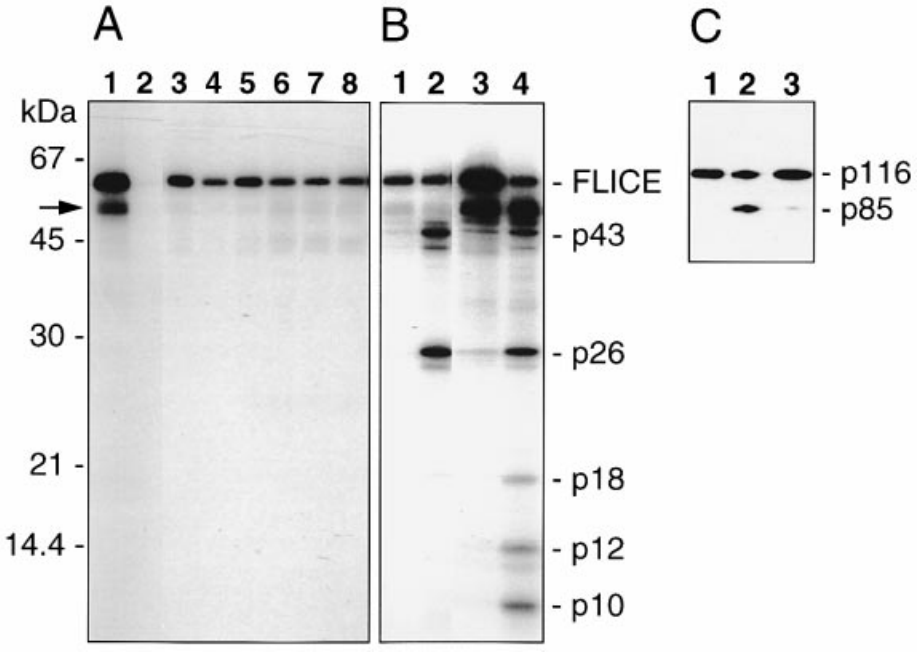

Fig. 5. FLICE is cleaved and activated by the DISC. (**A**) *In vitro* translated ^{35}S-labeled FLICE was either directly analyzed by SDS–PAGE (lane 1) or first incubated for 24 h at 4°C in FLICE reaction buffer with either GST beads alone (lane 2), 10 μg His-tagged FADD coupled to Ni^{2+} beads (lanes 3–5) or 10 μg GST–FADD coupled to GST beads (lanes 6–8). In addition, prior to the incubation with ^{35}S-labeled FLICE both GST–FADD or His-tagged FADD were also incubated for 1 h at 37°C with lysate from 10^6 SKW6.4 cells either unstimulated (lanes 4 and 7) or stimulated for 5 min with anti-APO-1 (lanes 5 and 8). After incubation with FLICE the beads were washed and bound proteins were separated by 15% SDS–PAGE. The position of the additional 50 kDa band, representing the N-terminal truncated FLICE, is marked by an arrow. (**B**) CD95 was immunoprecipitated from either untreated (lanes 1 and 3) or anti-APO-1-treated (5 min) unlabeled SKW6.4. cells (lanes 2 and 4). Immunoprecipitates were washed four times and incubated with *in vitro* translated ^{35}S-labeled FLICE. After 24 h the supernatant (lanes 3 and 4) of this *in vitro* FLICE assay was separated from the beads (lanes 1 and 2). The beads were washed three times and both beads and supernatants were loaded on an SDS–polyacrylamide gel. The positions of FLICE and its fragments p43, p26, p18, p12 and p10 are indicated. (**C**) Analysis of PARP cleavage by addition of rPARP to the supernatant of an *in vitro* FLICE assay with immunoprecipitated CD95 from untreated SKW6.4 cells (lane 1), from anti-APO-1-treated cells (lane 2) or from anti-APO-1-treated cells first precleared twice with the anti-FLICE2 antibody (lane 3). After incubation for 8 h at 37°C, the supernatants were subjected to SDS–PAGE and then immunoblotted with a mAb against the p85 subunit of rPARP.

since it was not observed when the CD95 DISC was removed after 2 h during this 24 h assay (Figure 7B, lane 3). We therefore conclude that in the CD95 pathway FLICE can only be activated at the DISC level.

zVAD-fmk inhibits CD95-mediated apoptosis by blocking FLICE activation at the DISC level

To test which of the peptide ICE inhibitors blocks FLICE activity the inhibitors were titrated into an

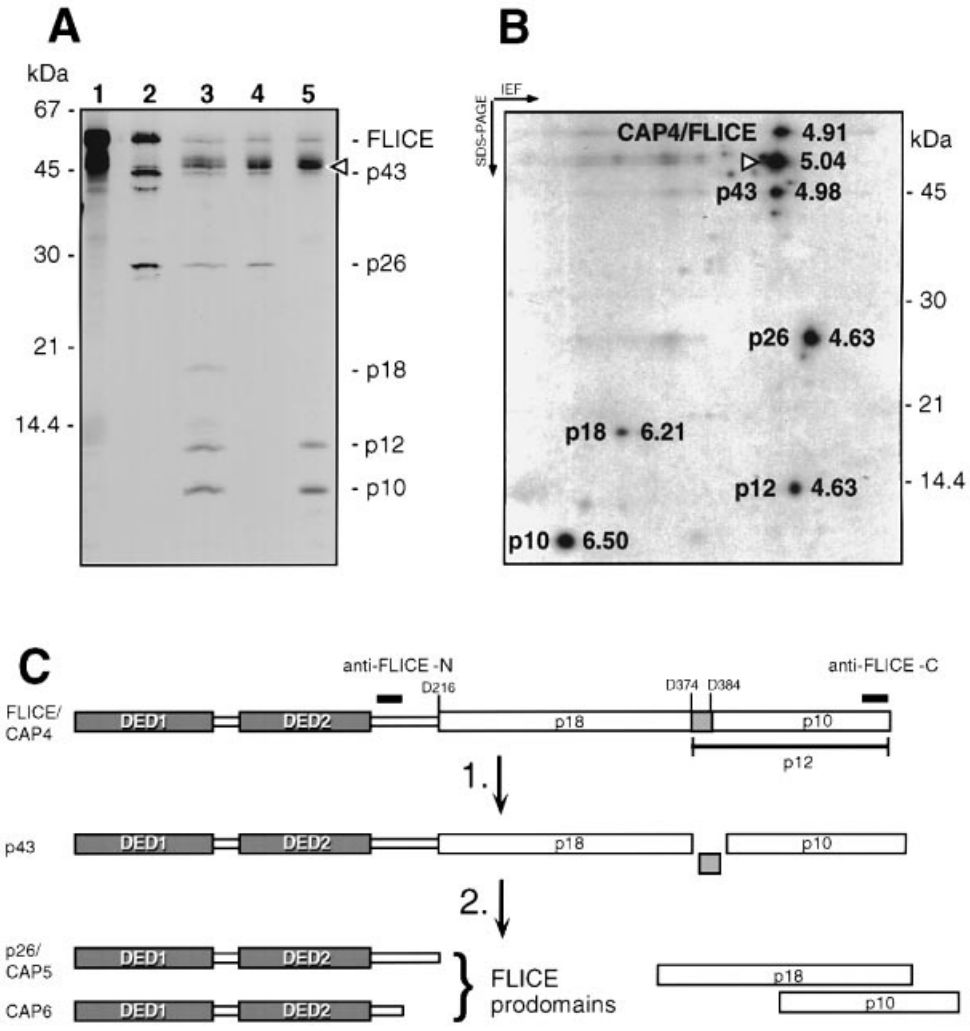

Fig. 6. Identification of the cleavage products of FLICE. (**A**) *In vitro* translated ^{35}S-labeled FLICE (lane 1). ^{35}S-labeled FLICE was cleaved by the CD95 DISC of anti-APO-1-treated (5 min) SKW6.4 cells. Beads (lane 2) and supernatants (lanes 3–5) of this assay were prepared as described in Figure 5B. Supernatants were either loaded directly (lane 3) or immunoprecipitated with anti-FLICE-N, under native conditions (lane 4), or anti-FLICE-C, under denaturing conditions (lane 5). The migration position of an N-terminal truncated FLICE is labeled by an arrowhead. (**B**) Two-dimensional gel analysis of ^{35}S-labeled FLICE after incubation in an *in vitro* FLICE assay. Both molecular weight and calculated pI values of putative cleavage fragments are indicated next to each cleavage product. Only p26 shows a small discrepancy between calculated and apparent pI. In addition, the arrowhead indicates the position of the N-terminal truncated FLICE. (**C**) Proposed mechanism of FLICE cleavage as described in the text. Identified cleavage sites and the binding sites of the two antibodies anti-FLICE-N and anti-FLICE-C are indicated.

in vitro FLICE assay (Figure 8A). zVAD-fmk, zDEVD-fmk (similar results were observed with Ac-DEVD-CHO) and zIETD-fmk, an inhibitor designed to mimic the first cleavage site in Yama, were effective inhibitors of FLICE activation by the DISC *in vitro*, whereas Ac-YVAD-CHO had no effect at the concentrations used. Moreover, DISC analysis of whole cells treated with zVAD-fmk revealed complete inhibition of the conversion of CAP4 to CAP5 and CAP6 (Figure 8C and E), indicating that zVAD-fmk blocks FLICE activation also *in vivo*.

Overexpression of crmA blocks the CD95 pathway without inhibiting FLICE activation

Overexpression of crmA in BJAB cells completely blocks CD95-mediated apoptosis (Figure 9A), while overexpression of an inactive crmA mutant did not alter anti-CD95 sensitivity (Figure 9A). We therefore tested the effects of *in vitro* translated ^{35}S-labeled crmA in a FLICE assay with DISC from unstimulated (Figure 9B, lane 1) or stimulated (Figure 9B, lane 2) cells. Interestingly, crmA served as a substrate for FLICE, as it was efficiently cleaved (Figure 9B). However, addition of crmA did not affect the ability of the DISC to cleave *in vitro* translated FLICE (results not shown). Furthermore, crmA did not inhibit cleavage of recombinant PARP by the immunoprecipitated DISC, whereas the peptide inhibitor Ac-DEVD-CHO readily inhibited this activity (Figure 9C).

Using the two-dimensional gel system we tested whether overexpression of crmA would inhibit FLICE processing *in vivo*. In both transfectants, i.e. BJAB expressing active wt crmA or inactive mutated crmA, FLICE processing was normal in the DISC, since formation of CAP5 and CAP6 was initiated at a comparable rate (Figure 9D). At a later time point FLICE was completely converted and similar amounts of active subunits were detected in the cytosol of both

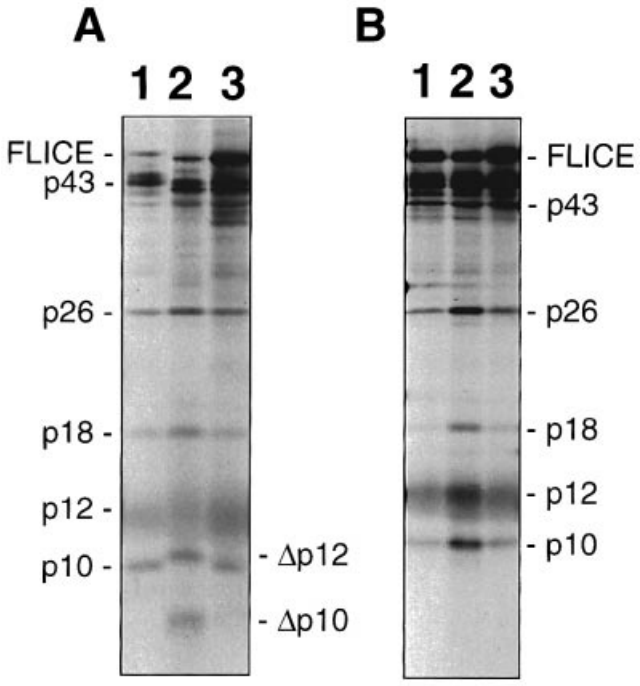

Fig. 7. The CD95 DISC is required for FLICE processing. (**A**) Cleavage of *in vitro* translated ^{35}S-labeled wt FLICE (lane 1) or ΔFLICE C360S (lane 2) in an *in vitro* FLICE assay. Processing of wt FLICE resulted in the characteristic cleavage products p43, p26, p18, p12 and p10 (lane 1), while cleavage of ΔFLICE C360S gave rise to smaller p12 (Δp12) and p10 (Δp10) products (lane 2) due to its C-terminal truncation. Lane 3, unprocessed ΔFLICE C360S added for 24 h to activated wt FLICE, which was obtained by incubating ^{35}S-labeled wt FLICE with a CD95 DISC for 24 h. (**B**) *In vitro* translated ^{35}S-labeled FLICE was incubated for either 2 (lanes 1 and 3) or 24 h (lane 2) with a CD95 DISC in an *in vitro* FLICE assay. The reaction in the supernatants of all samples was either stopped (lanes 1 and 2) by the addition of 5× reducing sample buffer or incubated for another 22 h and then analyzed (lane 3).

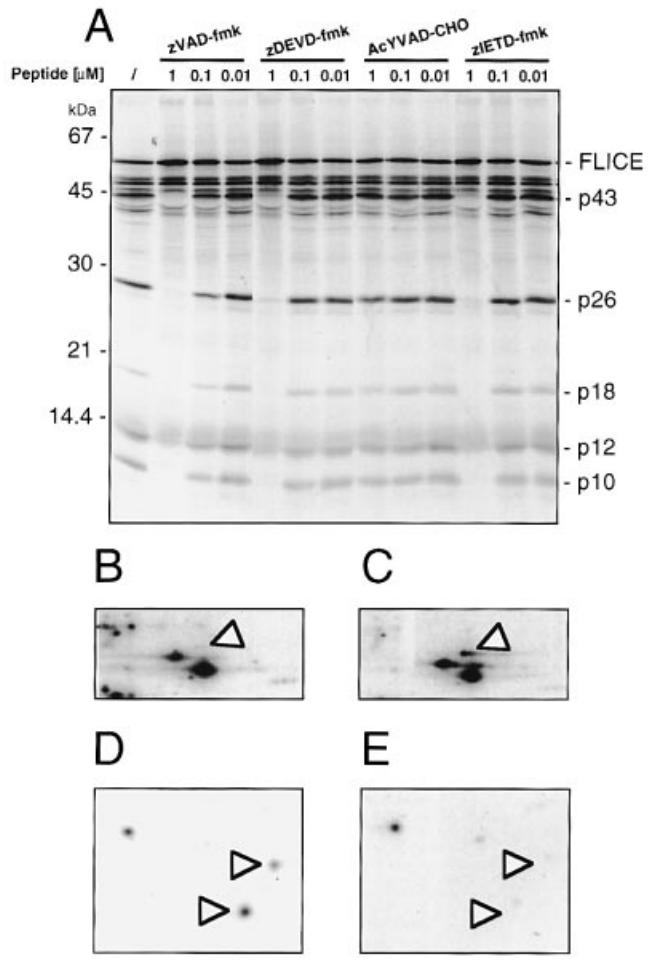

Fig. 8. zVAD-fmk inhibits FLICE activation *in vitro* and *in vivo.* (**A**) *In vitro* FLICE cleavage assay with the CD95 DISC from anti-APO-1-treated SKW6.4 cells. Prior to the addition of ^{35}S-labeled FLICE different peptide inhibitors were added at the concentrations indicated. (**B**–**E**) *In vivo* inhibition of CAP5 and CAP6 formation. Raji B cells were either left untreated (B and D) or pretreated for 30 min with 20 μM zVAD-fmk (C and E), prior to addition of anti-APO-1 for 30 min. The CD95 DISC was analyzed on a two-dimensional gel. The regions of the gel containing CAP4/FLICE (B and C) or CAP5 and CAP6 (D and E) are shown. Migration positions of CAP4 (B and C) and CAP5 and CAP6 (D and E) are indicated by an arrowhead.

transfectants (Figure 9E). Taken together, the data show that FLICE in the DISC can be inhibited by zVAD-fmk, zDEVD-fmk (Ac-DEVD-CHO) and zIETD-fmk, but not by Ac-YVAD-CHO or crmA.

Discussion

It has become increasingly clear that members of the ICE-like protease family are essential for induction of apoptosis. Two of the ICE-like proteases that have been shown to be involved in CD95-mediated apoptosis are ICE and Yama/CPP32/apopain. Thymocytes from ICE-deficient mice were resistant to CD95-induced apoptosis (Kuida *et al.*, 1995). In addition, two inhibitors have been shown to specifically inhibit ICE, the serpin class inhibitor crmA (Komiyama *et al.*, 1994; Schlegel *et al.*, 1996) and the peptide Ac-YVAD-CHO (Walker *et al.*, 1994; Nicholson *et al.*, 1995), which both block CD95-mediated apoptosis (Enari *et al.*, 1996; Hasegawa *et al.*, 1996). It has been shown that a Yama-like activity acts downstream of an ICE-like protease in the CD95 apoptotic pathway (Enari *et al.*, 1996). Recent data, however, suggest that both ICE-like proteases, ICE and Yama, are not crucial in all cell types for the CD95 pathway. A number of cell lines that do not express ICE are highly sensitive for CD95-mediated apoptosis. Jurkat T cells and two human carcinoma cell lines were shown to be ICE negative and CD95 apoptosis could not be blocked by the ICE-specific inhibitor Ac-YVAD-CHO (Hasegawa *et al.*, 1996; Schlegel *et al.*, 1996). The Yama-specific inhibitor Ac-DEVD-CHO, however, readily blocked apoptosis in these cells, indicating that a Yama-like ICE protease is involved in CD95 signaling. A recent report now shows that neither ICE nor Yama are essential for CD95 killing induced by CTLs (Darmon and Bleackley, 1996). These data indicate that an additional ICE-like protease activity upstream of both ICE and Yama must exist. We have recently identified a new member of the ICE protease family, FLICE (Muzio *et al.*, 1996). Since FLICE is part of the CD95 DISC, it must be upstream of all cytosolic ICE proteases. We now show that FLICE is generally used in a number of different cell types. Activation of FLICE requires the CD95 DISC. *In vitro* FLICE activity is inhibited by Ac-DEVD-CHO but not by Ac-YVAD-CHO, confirming that FLICE has a Yama-like substrate specificity. FLICE is also effectively

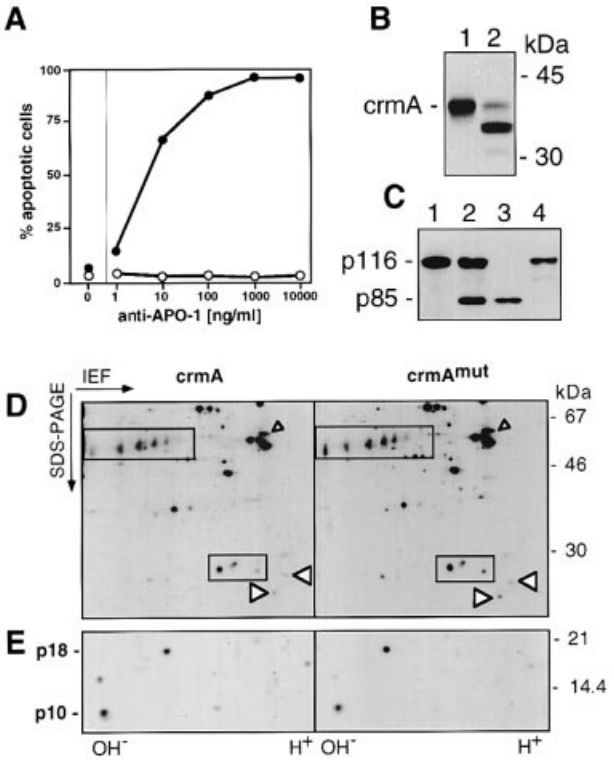

Fig. 9. CrmA does not inhibit FLICE processing. (**A**) BJAB cells overexpressing either wt crmA or mutant crmA were incubated for 16 h with different concentrations of anti-APO-1. After this incubation cells were harvested and the number of apoptotic cells was determined. (**B**) CD95 DISC immunoprecipitated from either untreated (lane 1) or anti-APO-1-treated (lane 2) SKW6.4 cells and incubated for 24 h at 4°C with *in vitro* translated ^{35}S-labeled crmA. (**C**) Analysis of PARP cleavage by addition of rPARP to immunoprecipitated CD95 from untreated SKW6.4 cells (lane 1) or from anti-APO-1-treated cells (lanes 2–4). PARP cleavage was without additions (lane 2) or in the presence of *in vitro* translated crmA (lane 3) or 10 μM zDEVD-fmk (lane 4). PARP detection was as described in Figure 5C. (**D** and **E**) Metabolically labeled BJAB cells, overexpressing either wt (left) or mutant crmA (right), were treated for 15 (D) or 60 min (E) with anti-APO-1. Cells were then lysed and either the CD95 DISC (D) or the p10 and p18 FLICE subunits, using anti-FLICE-C (E), were immunoprecipitated and analyzed using two-dimensional gel electrophoresis. Arrowheads in (D) point to CAP4, CAP5 and CAP6, while the positions of p18 and p10 are indicated in (E).

inhibited by zVAD-fmk both *in vivo* and *in vitro*. Overexpression of crmA completely blocks the CD95 pathway. However, it did not inhibit FLICE, indicating that FLICE activation alone may not be sufficient for CD95 signaling. At least one crmA-sensitive ICE-like protease must be located downstream of FLICE in the pathway. Future studies should identify the crmA sensitive ICE-like protease located downstream of DISC-bound FLICE.

We now show that FLICE can only be cleaved when bound to the DISC. Binding of FLICE to the DISC likely forces FLICE to change its conformation and to become proteolytically active. Initial cleavage at Asp374 generates N-terminal p43 and C-terminal p12 intermediates. Further processing likely occurs at Asp374 and Asp384, generating the active p18 and p10 subunits and the FLICE prodomain p26. Autoproteolytic activation is common among ICE-like proteases under certain conditions. Both ICE and Yama have been demonstrated to cleave themselves (Ramage *et al.*, 1995; Wang,X. *et al.*, 1996; Yamin *et al.*, 1996). Pro-ICE needs to oligomerize to be activated (Gu *et al.*, 1995) similar to (pro-)FLICE, which needs to hetero-oligomerize with FADD bound to the DISC. The initial ICE cleavage occurs at Asp297, the first of the two distal cleavage sites (Ramage *et al.*, 1995; Yamin *et al.*, 1996), similar to what we have found for FLICE. Further processing results in splicing out of a 19 or 10 amino acid spacer in ICE and FLICE respectively. It has been shown that pro-ICE performs intermolecular proteolysis during which it undergoes a conformational change (Yamin *et al.*, 1996). Interestingly, we clearly observed cleavage of enzymatically inactive ΔFLICE C360S. This also points to an intermolecular cleavage in which the wt FLICE molecules precipitated with the DISC cleaved ΔFLICE C360S. Although the analogy with activation of ICE is striking, at this point we cannot conclude whether FLICE is activated due to oligomerization or due to another factor present in the DISC, such as CAP3.

In vivo FLICE activation can now be described as follows. Aggregation of CD95, induced, for example, by addition of anti-APO-1, results in an instant recruitment of FADD (CAP1/2), FLICE (CAP4) and CAP3 to the receptor (Figure 10 I). Once at the receptor, FLICE is converted to its active subunits (p18/p10), which are released from the receptor and can now cleave various substrates (Figure 10 II). During the activation all cytosolic FLICE becomes quantitatively cleaved. Functional processing of FLICE generates two fragments which were identified on two-dimensional gels as CAP5 and CAP6 and were found in the DISC (Figure 10 III) but also in the cytosol. However, active FLICE does not accept cytosolic FLICE as a substrate. Therefore, the appearance of CAP5 and CAP6 in the cytosol must occur by a turnover mechanism of FLICE at the level of the DISC.

Using the anti-FLICE-C Ab, which recognizes the FLICE p10 subunit, in a Western blot experiment, cleaved p10 subunits were detected as early as 1 min after anti-CD95 stimulation of SKW6.4 cells (data not shown). FLICE cleavage therefore represents the fastest proteolytic event in any known form of apoptosis.

We have recently used granzyme B to activate FLICE *in vitro* (Muzio *et al.*, 1996). We now show that physiological activation of FLICE during CD95-mediated apoptosis occurs through its binding to the CD95 DISC. Attempts to reconstitute a DISC *in vitro* using *in vitro* translated [^{35}S]FLICE and the recombinant cytoplasmic tail of CD95 together with either GST–FADD or after incubation with SKW6.4 cell lysate, which results in binding of endogenous FADD (Kischkel *et al.*, 1995), have been unsuccessful (our unpublished data). In addition, an anti-FADD immunoprecipitate from either unstimulated or stimulated cells did not contain any FLICE cleavage activity. A reason for this could be the absence of CAP3 in these cleavage assays. The role of CAP3 in the DISC remains as yet elusive. Although initial data indicate that CAP3 represents the prodomain of FLICE, it does not seem to be a FLICE cleavage product for two reasons: First, its presence in the DISC is not changed during conversion of FLICE to CAP5 and CAP6. Second, no product which resembles CAP3 is formed in the *in vitro* FLICE assay. None the less, together with FLICE we recently also obtained sequence information on CAP3 (Muzio *et al.*, 1996). We have now repeated the sequencing of CAP3

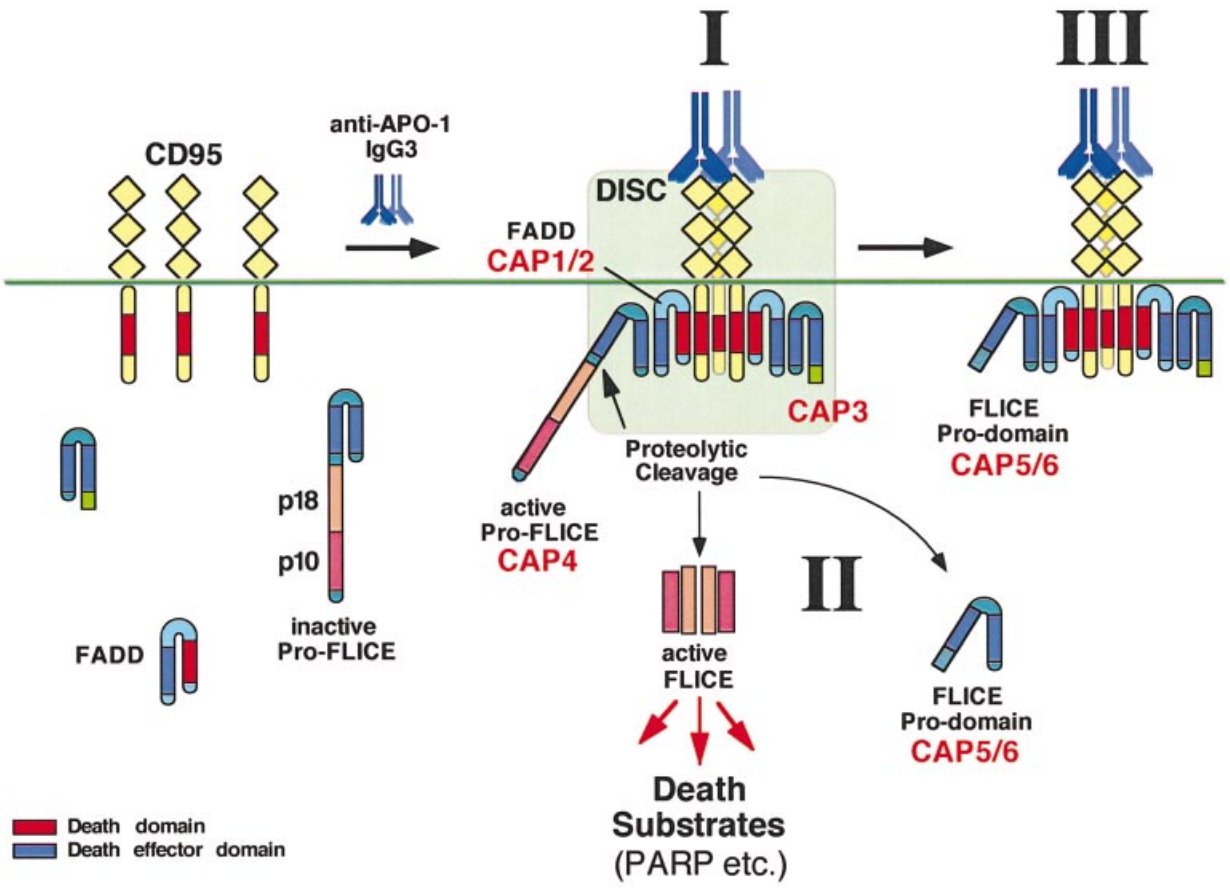

Fig. 10. Model for the activation of FLICE. (I) Crosslinking of CD95 with anti-APO-1 rapidly recruits CAP1,2/FADD, CAP4/FLICE and CAP3 to the receptor, due to specific DD–DD and DED–DED interactions. (II) Cleavage of FLICE by a two step, autoproteolytic mechanism results in the release of active FLICE into the cytosol, where it can cleave its substrates. (III) After activation part of the prodomain (CAP5 and CAP6) remains bound to the DISC, while most of it is found in the cytosol, likely as a result of a cleavage process that involves turnover of FLICE at the receptor level.

twice (data not shown), each time with the same result: the two sequenced peptides found in CAP3 were identical to two of the sequenced peptides located in the two DEDs of FLICE. The complete identity of CAP3 is unknown at present, but since CAP3 has been observed in the DISCs of all cell types tested (see Figure 1; data not shown) it might have an important regulatory function.

Alternatively, CAP3 could represent one of the reported isoforms of FLICE lacking a functional ICE protease domain (Boldin *et al.*, 1996). However, so far none of the reported N-terminal MACHβ isoforms match CAP3 in molecular weight or pI. In addition, so far we did not find any other DISC-associated putative FLICE splice forms, suggesting that FLICE is the physiological form that is recruited by the CD95 receptor.

Materials and methods

Cells

The leukemic T cell lines HUT78 and H9, the B lymphoblastoid cell line SKW6.4, the group I Burkitt lymphoma BL-60 cell line transfected with CD95 (K50) (Oehm *et al.*, 1992), the colon carcinoma cell line HT29, the hepatoma cell line Hep G2 and the mouse fibroblastoid cell line L929-huCD95, which was transfected with human CD95 (Ratter *et al.*, 1996), were all maintained in RPMI 1640 (Gibco Biocult, Eggenheim, Germany), 10 mM HEPES, pH 7.3, 10% fetal calf serum (Conco, Wiesbaden, Germany), 2 mg/ml gentamycin (Gibco) in 5% CO_2. BJAB cells transfected with wt crmA or a mutated inactive crmA were kindly provided by Dr Dixit (University of Michigan, Ann Arbor, MI) and were cultured as described elsewhere (Chinnaiyan *et al.*, 1996b).

Antibodies and reagents

Rabbit anti-peptide antibodies against FLICE were generated and affinity purified as previously described (Peter *et al.*, 1995). Peptides used for immunization were CKERSSSLEGSPDEFSNGE (anti-FLICE-N) and CVEVSNKDDKKNMGKQMPQ (anti-FLICE-C), encompassing positions 183–201 and 466–479 respectively of the FLICE primary structure (Muzio *et al.*, 1996). Anti-APO-1 is an agonistic mAb (IgG3, κ) recognizing an epitope on the extracellular part of APO-1 (CD95/Fas) (Trauth *et al.*, 1989). The mouse mAb against PARP (C-II-10) was a kind gift of Dr A.Burkle (German Cancer Research Center, Heidelberg, Germany). The horseradish peroxidase-conjugated antibody goat anti-mouse IgG was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). His-tagged FADD (Muzio *et al.*, 1996) and GST–FADD (Chinnaiyan *et al.*, 1995) constructs were a kind gift of Dr Dixit. Glutathione beads were purchased from Promega (Heidelberg, Germany). The Ni^{2+}–NTA beads were from Qiagen (Hilden, Germany). The peptide inhibitors benzoxycarbonyl-Val-Ala-Asp (*O*-methyl)fluoromethyl ketone (zVAD-fmk), zDEVD-fmk and zIETD-fmk were obtained from Enzyme System Products (Dublin, CA). Acetyl-Tyr-Val-Ala-Asp aldehyde (Ac-YVAD-CHO) and Ac-DEVD-CHO were purchased from Bachem (King of Prussia, PA). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma (St Louis, MO).

Plasmids

To generate FLICE C360S, a point mutation (G→C at position 1081) was introduced by the use of PCR. Four primers were designed, two of which overlapped with each other on separate strands and contained a C→G mutation at the position to be changed (1, CCCCCTGA**G**AA-GCCTG-AATAAAAAACAC; 2, GTGTTTTTTATTCAGGCTT**C**TCA-

GGGGG) while the other two overlapped with a sequence in FLICE either upstream (3, CCTGGGAGAAGGAAAGTTGG) or downstream (4, GGCTGAGGCATCTGTT-TCCC) of the site to be mutated. PCR was then performed using primer pairs 1 plus 4 and 2 plus 3. Resulting products were separated from the primers on a 1.2% agarose gel and the PCR products (with the mutation and the 28 bp overlap) were pooled and subjected to a second PCR using the outside primers 3 and 4. The resulting product was then cleaved with *Eco*RI and *Xho*I and ligated into the *Eco*RI and *Xho*I cut original pcDNA3-FLICE vector, generating pcDNA3-FLICE-C360S. Due to a mismatch in the PCR an additional clone was obtained with a premature stop codon at the far C-terminus, creating pcDNA3-ΔFLICE-C360S.

Analysis of CAP4 isoforms by mass spectrometry

The strategy used for the MS analysis was described previously (Shevchenko *et al.*, 1996a). Briefly, CAP4 spots were cut out from the gel and in-gel digested with trypsin as described (Shevchenko *et al.*, 1996b). After overnight digestion an aliquot of the digest solution (representing 1–2% of total volume of a digest) was taken and analyzed by MALDI MS. MALDI mass spectra were obtained on a modified Bruker REFLEX time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with the SCOUT multiprobe inlet and a gridless delayed extraction ion source as described elsewhere (Jensen *et al.*, 1996). The rest of the tryptic peptides were then extracted from the gel particles, pooled extracts dried down and subjected to nanoelectrospray tandem mass spectrometric sequencing as previously described (Wilm *et al.*, 1996). Nanoelectrospray tandem MS was performed on a triple quadrupole mass spectrometer API III (Sciex, Ontario, Canada) equipped with a nanoelectrospray ion source (Wilm and Mann, 1996).

Metabolic labeling, DISC analysis and immunoprecipitation

Metabolic labeling and DISC analysis were performed essentially as described before (Kischkel *et al.*, 1995). In short, 3×10^7 cells were washed twice with phosphate-buffered saline (PBS) and labeled in 15 ml RPMI without cysteine and methionine (Gibco) for 20 h with 0.5 mCi ^{35}S ProMix (Amersham, Braunschweig, Germany). Untreated or anti-APO-1 (2 μg/ml)-treated cells were washed with PBS and lysed in lysis buffer [30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl flouride and small peptide inhibitors (Kischkel *et al.*, 1995), 1% Triton X-100 (Serva) and 10% glycerol]. The CD95 DISC was then precipitated for 1–3 h at 4°C with protein A–Sepharose (Pharmacia, Freiburg, Germany). Immunoprecipitation of FLICE or the FLICE subunits was performed at 4°C for 2 h with 20 μg anti-FLICE-N or anti-FLICE-C Ab precoupled to 30 μl protein A–Sepharose. All beads were washed five times with lysis buffer. To immunoprecipitate p10 and p12 under denaturing conditions, samples were boiled for 2 min in the presence of 0.5% SDS. After addition of 9 vol. lysis buffer without SDS immunoprecipitation was with 20 μg anti-FLICE-C Ab coupled to 30 μl protein A–Sepharose. All immune complexes were subjected to IEF/SDS–PAGE analysis as described (Huber and Peter, 1994). For densitometric quantification of ^{35}S-labeled CAP proteins two-dimensional autoradiographs were scanned with a Macintosh Apple One scanner and analyzed on a computer using the Scan Analysis Software from Biosoft (Ferguson, MO). Protein sequence analysis to determine the molecular weight and pI of FLICE fragments was with the protean module of Lasergene software (DNAstar, Madison, WI).

In vitro translation and in vitro cleavage assay

FLICE, mutant FLICE and crmA were *in vitro* translated using a T7 polymerase-directed reticulate lysate system (TNT; Promega). *In vitro* cleavage assays were performed as follows. CD95 DISC was immunoprecipitated from 5×10^7 unlabeled cells as described above. Subsequently, the beads (containing the DISC) were incubated in 50 μl reaction buffer [50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% 3-(cyclohexylamino)-1-propanesulfonic acid, 10 mM dithiothreitol and 20% sucrose] for 24 h at 4°C, unless otherwise indicated in the text, with either *in vitro* translated FLICE, *in vitro* translated crmA or 300 ng recombinant PARP (rPARP), which was a kind gift from Dr G.De Murcia (University of Strasbourg, France). Resulting products were either analyzed by two-dimensional IEF/SDS–PAGE as described before (Huber and Peter, 1994) or, after boiling for 3 min in a standard reducing sample buffer, analyzed by 15% SDS–PAGE with subsequent amplification (Amplify, Amersham), drying of the gels and autoradiography (FLICE and crmA) or by 10% SDS–PAGE with subsequent immunoblotting.

Western blotting

Hybond nitrocellulose membrane (Amersham) was blocked with 2% bovine serum albumin in PBS containing 0.05% Tween-20 (PBS/Tween) for at least 1 h, washed with PBS/Tween and incubated with the mAb C-II-10 (1:5 dilution of hybridoma supernatant). The blots were washed with PBS/Tween and developed with goat anti-mouse IgG (1:5000). After washing with PBS/Tween the blots were developed by the enhanced chemiluminescence method following the manufacturer's protocol (Amersham).

Cell stimulation and cytotoxicity assay

Samples of 10^6 cells were incubated in 24-well plates (Costar, Cambridge, MA) with 1 μg/ml anti-APO-1 in medium (5×10^5 cells/ml) for 16 h at 37°C unless otherwise stated in the figure legends. Quantification of DNA fragmentation as a measure of apoptosis was carried out essentially as described elsewhere (Nicoletti *et al.*, 1991). Briefly, cells were centrifuged in a minifuge (Heraeus, Hanau, Germany) at 4000 r.p.m. for 5 min and washed once with 1 ml PBS. Cells were then carefully resuspended in 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X-100 containing 50 μg/ml propidium iodide (Sigma). After incubation at 4°C in the dark for at least 24 h the percentage of apoptotic nuclei was determined using a FACScan© (Beckton and Dickinson, Heidelberg, Germany).

Acknowledgements

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