FLICE Is Predominantly Expressed as Two Functionally Active Isoforms, Caspase-8/a and Caspase-8/b*

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Induction of apoptosis by the cell surface receptor CD95 (APO-1/Fas) has been shown to involve activation of a family of cysteine proteases (caspases). Recently, a new member of this family has been identified, designated FLICE (caspase-8/MACH/Mch5). FLICE is part of the CD95 death-inducing signaling complex and is therefore the most upstream caspase in the CD95 apoptotic pathway. A total of eight different isoforms of FLICE (caspase-8/a–h) have been described. To determine which isoforms are expressed in different cells we have generated a panel of monoclonal antibodies directed against all functional domains of FLICE. Using these antibodies we could show that only two of the FLICE isoforms (caspase-8/a and caspase-8/b) were predominantly expressed in cells of different origin. Both isoforms were recruited to the CD95 death-inducing signaling complex and were activated upon CD95 stimulation with similar kinetics. Taken together, only two of the eight published caspase-8 isoforms could be detected in significant amounts at the protein level.

Apoptosis, or programmed cell death, plays an essential role in development, homeostasis, and defense in multicellular organisms (1, 2). Several cell surface receptors, such as CD95 (APO-1/Fas), TNF receptor 1, DR3 (APO-3/TRAMP/Wsl-1), DR4 (TRAILR) (3) belonging to the TNF receptor/nerve growth factor receptor superfamily, have been shown to trigger apoptosis upon binding of their cognate ligands or specific agonistic antibodies. Stimulation of CD95 has been shown to result in aggregation of its intracellular death domains, leading to the recruitment of a set of signaling proteins (CAP1–4) and the formation of the death-inducing signaling complex (DISC) (4, 5). In the DISC, CAP1 and CAP2 were identified as the adapter molecule FADD (MORT-1) (4, 6, 7) that couples through its C-terminal death domain to the cross-linked CD95 receptor. The N-terminal death effector domain of FADD enables recruitment of CAP4, which was identified as FLICE (MACH2/1/MCH5/caspase-8) (8–10).

FLICE belongs to a family of cysteine proteases (caspases, related to the Caenorhabditis elegans cell death gene ced-3 that have been shown to play a key role in the induction of most forms of apoptosis (11). Caspases are synthesized as inactive proenzymes that have to be activated by proteolytic cleavage after specific aspartate residues (12). Recently, we have shown that FLICE is activated by association with the CD95 DISC, leading to the release of the active subunits p18 and p10 into the cytosol (13). There, they can activate other caspases, in turn resulting in the specific cleavage of a number of “death substrates.” During CD95 triggering all cytosolic FLICE is activated at the DISC (13). After activation at the DISC a part of the FLICE prodomain remains bound to the DISC.

A large number of caspases have been identified including caspase-1 (ICE) (14, 15), caspase-2 (ICH-1/Nedd-2) (16, 17), caspase-3 (CPP32/Yama/apopain) (18–20), caspase-4 (ICP2/TX/ICE-rel-I) (21–23), caspase-5 (ICE-rel-III/TY) (23, 24), caspase-6 (Mch2) (25), caspase-7 (Mch3/ICE-LAP3/CMH-1) (26–28), caspase-8 (FLICE/MACH/Mch5) (8–10), caspase-9 (Mch6/ICE-LAP6) (29, 30), caspase-10 (Mch4/FLICE2) (10, 31), and caspase-11 (ICH-3) (32). However, the role of these caspases in different cell death pathways in various tissues remains elusive. Caspase-3, for example, has been shown to be proteolytically activated upon CD95-induced cell death. In mice deficient of caspase-3, however, the CD95 apoptosis pathway was not affected in most tissues (33).

In addition to the large number of different caspases, various isoforms of these molecules have been described at the mRNA level. Some of these isoforms have been found to be inactive splice variants such as ICE6 (34), MCH2β (25), or MCH3β (26). Others function as dominant inhibitors of apoptosis such as ICEe (34) or ICH-1s (17). For caspase-8, eight different isoforms (designated as caspase-8/a–h), including FLICE (CAP4/MACH4) (8), MACH2 and MACH3, MACHβ1–4 (9), and Mch5 (10) have been described at the mRNA level.

In this study, expression of the different FLICE isoforms on the protein level in various cell lines was determined by monoclonal antibodies covering the three functional domains of caspase-8. Only two caspase-8 isoforms were detected on the protein level of all cell lines tested. Both isoforms were recruited and activated by the CD95 DISC with identical kinetics.

EXPERIMENTAL PROCEDURES

Cell Lines—The monocytic cell line MonoMac, the T-cell lymphoma HUT78, the B-lymphoblastoid cell line SKW6.4, the Burkitt lymphoma Raji, the Burkitt-like lymphoma BJJAB, the colon carcinoma cell line HT-29, the breast carcinoma cell line MCF-7, the cervix carcinoma HeLa, the myroryhadosarcoma cell line KYM-1 (kind gift from M. Grell, Stuttgart, Germany), the small lung cell carcinoma line SCLC22H (kind gift from J. Fischer, Heidelberg, Germany), and the neuroblastoma SHEP (kind gift from M. Schwab, Heidelberg, Germany) were cultured in RPMI + 10% fetal calf serum, 0.05 mg/ml gentamycin, and 0.05 mg/ml HEPES. The hepatoma cell line HepG2, the gastric cancer line Hs746T (both were a kind gift from M. Muller-Schilling, Heidelberg, Germany), and the embryonic kidney line 293T were cultured in...
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RESULTS

Generation of Anti-FLICE Monoclonal Antibodies—Using either GST fusion proteins with recombinant FLICE prodomain (GST-N-FLICE, amino acids 1–180) or the prodomain (GST-C-FLICE, amino acids 181–479) as an immunogen, different mouse anti-FLICE monoclonal antibodies were generated. Specificity of the antibodies was first established by Western blotting with recombinant GST-N-FLICE or GST-C-FLICE. Fourteen different fusion proteins with recombinant FLICE prodomain were used as immunogens, the FLICE cleavage products, and the domains recognized by the anti-FLICE mAbs N2, C15, and C5 as described in A and B and described in the text. DED, death effector domain.

FLICE was used in immunoprecipitation experiments (Fig. 1B). In addition to full-length FLICE, the N2 antibody immunoprecipitated the p43 and the p26 cleavage products, both containing the prodomain of FLICE. Interestingly, the band below full-length FLICE was not immunoprecipitated by N2 (Fig. 1B). This band represents N-terminal truncated FLICE due to the usage of an internal start site in the in vitro translation (13). Therefore, the N2 mAb recognizes an epitope located within the first death effector domain of FLICE. The C5 mAb directed against the C terminus of FLICE immunoprecipitated all FLICE cleavage products containing the p18 domain. The antibody C5 precipitated p12 and p10, both representing the very C terminus of FLICE. Therefore, C5 is directed against the p10 subunit of FLICE.

Two FLICE Isoforms Are Predominantly Expressed in Various Cell Lines—A number of isoforms of caspase-8 have been described at the mRNA level (9, 10). Our mAbs against the three major domains of FLICE (the prodomain and the active subunits p18 and p10) enabled us to test which of the reported caspase-8 isoforms were actually expressed in vivo. To this end several cell lines representing different tissues were tested for

Fig. 1. Characterization of anti-FLICE mAbs. A, 50 ng of either purified GST-N-FLICE (N) or GST-C-FLICE (C) were subjected to SDS-PAGE and immunoblotted with anti-FLICE mAbs N2, C15, or C5 as indicated. B, in vitro translated [35S]labeled FLICE was processed by incubation with immunoprecipitated CD95 DISC as described previously (13). After boiling the samples in 1% SDS to avoid association, the different cleavage products were either directly analyzed by SDS-PAGE (input) or subjected to immunoprecipitation with anti-FLICE mAbs C5, C15, or N2. The immunoprecipitates were also analyzed by SDS-PAGE and autoradiography of the dried gel. C, scheme of the GST-FLICE fusion proteins, the FLICE cleavage products, and the domains recognized by the anti-FLICE mAbs N2, C15, and C5 as analyzed in A and B and described in the text. DED, death effector domain.

Antibodies and Reagents—The affinity-purified rabbit anti-peptide antibodies anti-FLICE-N and anti-FLICE-C against the FLICE peptides 183–201 and 466–479, respectively, were generated as described previously (13). For the anti-FLICE mAbs, BALB/c mice were immunized four times by injection of 300 μg of either purified GST-N-FLICE or GST-C-FLICE. Spleen cells from immunized animals were fused with the Ag8 myeloma. 2 weeks after fusion culture supernatants from wells positive for growth were tested in an enzyme-linked immunosorbent assay with HIS-FLICE as coated antigen. Hybridomas that produced anti-FLICE mAbs were cloned several times by limited dilution yielding subclones positive for the desired antibody. The anti-FLICE mAbs used in this study were C5 (IgG2a), C15 (IgG2b), and N2 (IgG1). The mouse mAb anti-APO-1 (IgG3, k) recognizes an epitope on the extracellular part of human APO-1 (CD95/Fas) (35). The horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b were purchased from Dianova (Hamburg, Germany). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma.

Fusion Proteins and Immunoprecipitation—Using standard polymerase chain reaction and cloning techniques the following fusion proteins were generated: His-FLICE, GST-N-FLICE (amino acids 1–180), and GST-C-FLICE (amino acids 181–478). Fusion proteins were purified as described previously (4). For immunoprecipitation mAbs (10 μg) were coupled to anti-IgG Agarose beads (Sigma) (N2), to protein A Sepharose beads (Sigma) (C15), or to protein A/G-plus Agarose (Santa Cruz Biotechnology) (C5). After addition of in vitro activated [35S]FLICE and incubation for more than 1 h at 4 °C, beads were washed three times with lysis buffer. The amount of DISC-associated FLICE was determined as follows: 5 × 10⁵ SKW6.4 cells were either first treated with 5 μg/ml anti-APO-1 for 5 min at 37 °C and then lysed (stimulated condition) or first lysed and then supplemented with 2 μg/ml anti-APO-1 (unstimulated condition). [35S] labeling, cell lysis, and immunoprecipitation of CD95 were done as described elsewhere (4).

Western Blotting—For Western blot detection of cytosolic proteins postnuclear supernatants equivalent to 1 × 10⁶ cells or 50 μg of total protein as determined by the BCA method (Pierce) were separated by 12% SDS-PAGE. After electrophoresis all samples were transferred to Hybond nitrocellulose membrane (Amersham Corp.), blocked with 2% bovine serum albumin in PBS/Tween (PBS + 0.05% Tween 20) for at least 1 h, washed with PBS/Tween, and incubated with supernatant of anti-FLICE hybridomas diluted 1:5 in PBS/Tween for 16 h at 4 °C. Blots were washed with PBS/Tween and developed with goat anti-mouse IgG1 (N2, IgG2a (C5), or IgG2b (C15) (1:20000)). After washing with PBS/Tween, the blots were developed with the chemiluminescence method (ECL) following the manufacturer’s protocol (Amersham Corp.).

In Vitro FLICE Activation Assay—The CD95 DISC was immunoprecipitated from 5 × 10⁶ anti-APO-1-treated SKW6.4 cells (5 min) as described above, and immunoprecipitates were incubated with in vitro translated [35S]labeled FLICE (TNT, T7 coupled reticulocyte lysate system, Promega) in FLICE cleavage buffer (50 m M HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, and 10% sucrose) for 24 h at 4 °C. The cleavage reactions were stopped by addition of 1% SDS. After boiling for 3 min, samples were diluted 1:10 in lysis buffer and subjected to immunoprecipitation as described above. The immunoprecipitates were separated on 15% SDS-PAGE, and the amplified dried gel slices were subjected to autoradiography.

Dulbecco’s modified Eagle’s medium + 10% fetal calf serum, 0.05 mg/ml gentamycin, and 0.05 mg/ml HEPES. All cells were of human origin.
FLICE expression by Western blotting using the N2, C15, and C5 anti-FLICE mAbs (Fig. 2). Surprisingly, all three antibodies detected only two bands of 55 and 53 kDa of equal intensity in almost all cells. Other caspase-8 isoforms were undetectable. The only reported caspase-8 isoform that was not expected to be detected with the antibodies used was caspase-8/e (Fig. 3 and Table I).

Expression levels of the two detected caspase-8 isoforms were very different, spanning a range from high expression in the B-cell line SKW6.4 or the myeloblastic leukemia cell line KMY-1 to low expression in the embryonic kidney cell line 293T. Interestingly, the small lung cell carcinoma line SCLC22H was negative for FLICE expression. The third band detected only by the C5 mAb in the hepatoma line HepG2 likely represents a nonspecific background band because a FLICE product as detected by the C terminus-specific mAbs C15 and C5, respectively indicating that both isoforms did not differ in their C terminus ICE-like domains (Fig. 5A). Recognition of caspase-8 isoforms by different antibodies Anti-FLICE-N and anti-FLICE-C are rabbit anti-peptide antibodies generated against the FLICE peptides 183–201 and 466–479, respectively. N2, C15, and C5 are mouse monoclonal antibodies recognizing the prodomain, the p18 subunit, and the p10 subunit of FLICE, respectively.

Both FLICE Isoforms Are Recruited to the DISC—We have recently shown that FLICE is recruited to the CD95 receptor in a stimulation-dependent manner forming the DISC (4, 8). To test whether the second FLICE isoform is also recruited to the CD95 receptor, we analyzed the DISC by one-dimensional as well as by two-dimensional Western blotting, using the C15 anti-FLICE mAb. As shown in Fig. 4A, both FLICE isoforms were recruited to the CD95 receptor in a stimulation-dependent manner. The comparison between the two-dimensional Western blot and the DISC precipitation from 35S-labeled cells (Fig. 4B) confirmed that the upper FLICE isoform was identical with CAP4, whereas the lower isoform was hidden underneath a background spot in the 35S-DISC precipitation that was also recruiting to the CD95 receptor in a stimulation-dependent manner.

Both FLICE Isoforms Are Activated upon CD95 Triggering—As both FLICE isoforms were recruited to the DISC we tested next whether both are cleaved upon triggering of CD95. Therefore, we analyzed the lysate of either untreated cells or cells stimulated with the anti-APO-1 antibody for 1 h in a Western blot experiment using the three different anti-FLICE mAbs. Prolonged stimulation of CD95 resulted in almost complete cleavage of both FLICE isoforms (Fig. 5A, lanes 2, 4, and 6). Cleavage of both full-length FLICE bands during stimulation resulted in the formation of only one p18 and p10 cleavage product as detected by the C terminus-specific mAbs C15 and C5, respectively indicating that both isoforms did not differ in their C terminus ICE-like domains (Fig. 5A, lanes 1–4). To confirm this we made use of the rabbit antibody anti-FLICE-C, which was directed against the very C terminus. This antibody was able to precipitate both FLICE isoforms, confirming that...
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they did not differ in their C terminus (Fig. 5A, lanes 9 and 10). However, the N terminus-specific anti-FLICE mAb N2 detected two different FLICE cleavage products, p26 and p24, after CD95 stimulation (Fig. 5A, lanes 5 and 6). In addition, two bands, p43 and p41, were also detected, representing intermediates after cleavage between the p18 and the p10 subunit of FLICE, because they were also weakly detected by the C15 antibody (Fig. 5A, lanes 1, 2, 5, and 6). Therefore, the two isoforms of FLICE differed in the size of their prodomains. Given the molecular weight and pI of 53 kDa and 4.91, respectively, and the difference in the prodomain, the described isoform of caspase-8 that most likely represents the second band is caspase-8/b (MACHo2) (Fig. 3). This isoform differs from FLICE in a box of 15 amino acids that is not present in caspase-8/b (9). To further test this assumption we made use of the rabbit antibody anti-FLICE-N, which was directed against these 15 amino acids. Using SDS-boiled lysates from unstimulated cells the anti-FLICE-N antibody only precipitated CAP6 representing the prodomain of FLICE after proteolytic activation (13). To test whether these new DISC components were recruited to and activated by the CD95 DISC, we tested next whether they showed any differences in cleavage kinetics. Therefore, we analyzed the cleavage of caspase-8/a and 8/b at the DISC level and in the cytosol at various time points after CD95 stimulation. Consistent with the fast kinetics of CD95-mediated apoptosis both isoforms were recruited to the DISC within 10 s (Fig. 6A). Also after 10 s the two cleavage intermediates p43 and p41 as well as the prodomain cleavage products p26 and p24 were detectable in the DISC. In the cytosol all the FLICE cleavage products p26, p24, p18, and p10 were detectable as early as 10 s after activation (Fig. 6B). The cleavage products p26 and p24 as a readout for the activation of caspase-8/a and 8/b, respectively, increased in the cytosol during stimulation with identical kinetics, demonstrating that both isoforms were activated simultaneously. Interestingly, at the DISC level there was only a slight increase in the p26 and p24 cleavage products starting after 10 min when the amount of full-length FLICE began to decline (Fig. 6A). This suggests that there is only a limited capacity of the DISC to bind death effector domain-containing proteins. Notably, FLICE cleavage at the DISC level preceded cleavage in the cytosol, confirming that FLICE turnover takes place at the DISC level where all cytosolic FLICE is processed.

![Image](76x555 to 279x729)

**Fig. 4.** Both FLICE isoforms are recruited to the CD95 DISC.

CD95 was immunoprecipitated from either untreated (unstim.) or anti-APO-1-treated (5 min) stimulated SKW6.4 cells. Immunoprecipitates were analyzed by immunoblotting lysate (lys.) of 10⁶ untreated (−) or 1 h anti-APO-1-treated (+) SKW6.4 cells using the anti-FLICE mAbs C15, C5, or N2 as indicated. Similarly, the SDS-boiled lysate of 10⁷ untreated (−) or 1 h anti-APO-1-treated (+) SKW6.4 cells was immunoprecipitated using the rabbit antibody anti-FLICE-N (N-ip) or anti-FLICE-C (C-ip). The immunoprecipitates were immunoblotted with the anti-FLICE mAb N2. The two bands recognized by the C5 antibody in both untreated and treated cells are likely to be the result of unspecific binding. B, the CD95 DISC was immunoprecipitated from 10⁷ anti-APO-1-treated (10 min) SKW6.4 cells as described in Fig. 3 (left). FLICE was immunoprecipitated from 10⁷ anti-APO-1-treated (30 min) SKW6.4 cells using the anti-FLICE-N antibody (right). Both immunoprecipitates were subjected to two-dimensional gel electrophoresis and immunoblotted with the anti-FLICE mAb N2. Migration positions of the full-length proteins (caspase-8/a and 8/b), the cleavage intermediates (p43 and p41), and the cleaved prodomains, which are identical to the recently described new DISC components CAP5 and CAP6 (13), are indicated by arrowheads.

![Image](323x401 to 549x729)

**Fig. 5.** The two expressed FLICE isoforms represent caspase-8/a and 8/b.

A, in vitro cleavage products of caspase-8/a and 8/b were analyzed by immunoblotting lysate (lys.) of 10⁶ untreated (−) or 1 h anti-APO-1-treated (+) SKW6.4 cells using the anti-FLICE mAbs C15, C5, or N2 as indicated. Similarly, the SDS-boiled lysate of 10⁷ untreated (−) or 1 h anti-APO-1-treated (+) SKW6.4 cells was immunoprecipitated using either the rabbit antibody anti-FLICE-N (N-ip) or anti-FLICE-C (C-ip). The immunoprecipitates were immunoblotted with the anti-FLICE mAb N2. The two bands recognized by the C5 antibody in both untreated and treated cells are likely to be the result of unspecific binding. B, the CD95 DISC was immunoprecipitated from 10⁷ anti-APO-1-treated (10 min) SKW6.4 cells as described in Fig. 3 (left). FLICE was immunoprecipitated from 10⁷ anti-APO-1-treated (30 min) SKW6.4 cells using the anti-FLICE-N antibody (right). Both immunoprecipitates were subjected to two-dimensional gel electrophoresis and immunoblotted with the anti-FLICE mAb N2. Migration positions of the full-length proteins (caspase-8/a and 8/b), the cleavage intermediates (p43 and p41), and the cleaved prodomains, which are identical to the recently described new DISC components CAP5 and CAP6 (13), are indicated by arrowheads.
now demonstrate that only two caspase-8 isoforms are expressed at detectable levels in a number of different cell lines. Interestingly, the Burkitt lymphoma line Raji also expressed only these two isoforms as protein, although five different caspase-8 mRNA species were cloned from this cell line (9).

Both expressed caspase-8 isoforms were recruited to the CD95 receptor in an activation-dependent manner. By comparison of the two-dimensional Western blot with immunoprecipitated DISC from 35S-labeled cells, we could confirm that the expressed caspase-8 isoform of 55 kDa corresponds to FLICE (caspase-8/a, MACHo1), originally described as CAP4 (4). We identified the second expressed caspase-8 isoform as caspase8/b (MACHo2), because this protein could not be immunoprecipitated by the rabbit anti-peptide antibody anti-FLICE-N directed against a box of 15 amino acids not present in caspase-8/b (MACHo2).

Different caspase-8 isoforms have been suggested to function as modulators of the activation of caspase-8 in CD95- or TNF-induced apoptosis (9). Caspase-8c (MACHo3) has been demonstrated to protect against CD95- and TNF-induced apoptosis, whereas caspase-8/d (MACHo1) was suggested to enhance the cytotoxic activity of the active caspase-8 isoforms (caspase-8/a and 8/b) (9). However, none of these isoforms were detected by the anti-FLICE mAbs in significant amounts in any of the cell lines tested. Whether both expressed active isoforms caspase-8/a and 8/b have different functions remains to be determined. The fact that both were expressed in a one to one ratio in all cell lines tested as well as the identical CD95-induced activation kinetics suggests the possibility that both isoforms are necessary in equal amounts for the signal transduction of the CD95 receptor.

Recently, the number of apoptosis-inducing receptors has increased. The fact that FLICE was expressed in almost every cell line, some of which do not have the CD95 receptor or do not respond to CD95 triggering, raises the possibility that FLICE is also utilized by the signaling pathways of the other “death receptors” such as TNF receptor 1, DR3 (APO-3/TRAMP/Wsl/LARD), or DR4 (TRAILR) (3). Future studies should clarify the involvement of the different caspase-8 isoforms in other cell death signaling pathways.

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