the *egl-1* single mutant or the wild type (Fig. 4*c*). Moreover, whereas the *egl-1* single mutant still adapted to serotonin, the *egl-1*; *unc-2* double mutant was strongly adaptation defective (Fig. 2*d*). Thus *unc-2* mutants appear to lay eggs constitutively at least in part because their egg-laying muscles are hypersensitive and fail to adapt to endogenous serotonin. Where might the Unc-2 protein act to regulate egg laying? Mosaic analysis suggested that Unc-2 functions in neurons, yet the *egl-1* experiment demonstrated that Unc-2 does not require the HSNs to promote serotonin adaptation. An attractive hypothesis to explain these data is that Unc-2 modulates the release of FMRFamide, which can potentiate serotonin response from the VCs, and perhaps from the HSNs as well. Serotonin adaptation could occur if activation of the Unc-2 calcium channel causes an inhibition of FMRFamide release and thus a decrease in serotonin response (Fig. 4*d*).

In summary, we have shown that a voltage-gated calcium channel appears to be required for adaptation to dopamine and serotonin, and for determining the postsynaptic threshold for serotonin response. The *unc*-2 gene, which encodes the α-1 subunit of this channel, is expressed in neurons that control egg-laying behaviour. We propose that, in these neurons, *unc*-2 may participate in serotonin adaptation by modulating the serotonin response, perhaps by controlling the release of a neuropeptide. The *unc-2* message is also expressed in several other neuronal and body muscle cells that may control response to dopamine; determination of the behavioural functions of *unc-2* in these cells may help to elucidate the mechanisms underlying dopamine adaptation. □

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Involvement of an ICE-like protease in Fas-mediated apoptosis

Masato Enari*, Hubert Hug† & Shigekazu Nagata

Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565, Japan

FAS is a type-I membrane protein that transduces an apoptotic signal[1,2]. Binding of Fas ligand or agonistic anti-Fas antibody to Fas kills the cells by apoptosis[3]. Studies in the nematode *Caenorhabditis elegans* have suggested that proteases such as interleukin-1β-converting enzyme (ICE) or the product of the *C. elegans* cell-death gene *ced*-3 are involved in apoptotic signal transduction[4]. The activity of ICE can be inhibited by the product of *crmA*, a cytokine-response modifier gene encoded by cowpox virus[5–7]. We report here that expression of *crmA* inhibits cytotoxicity induced by anti-Fas antibody or tumour necrosis factor (TNF). We have found a specific ICE inhibitor tetrapeptide (acetyl-Tyr-Val-Ala-Asp-chloromethylketone)[8,9] that also prevents apoptosis induced by anti-Fas antibody. These results suggest an involvement of an ICE-like protease in Fas-mediated apoptosis and TNF-induced cytotoxicity.

Expression plasmids encoding *crmA*[10] and human *fas*[1] were introduced into Rat-2 (rat fibroblast) cells together with the neomycin-resistance gene. Among G418-resistant transformants, the Fas-expressing transformants were selected by flow cytometry analysis using mouse anti-human Fas antibody. Three clones (33, 45 and 48) were found to express human Fas on the cell surface (data not shown). Immunoprecipitation using anti-human Fas antibody confirmed the expression of Fas in these transformants (Fig. 1*a*). As found previously[1], immunoprecipitation gave two bands (M_r 43K and 50K) for human Fas, which may reflect different degrees of glycosylation. The expression of *crmA* was then analysed by RNase protection assay using a ^{32}P-labelled RNA fragment carrying the *crmA* gene (nucleotides 1,174 to 1,468). The control *crmA* RNA produced by *in vitro* transcription gave protected bands of 280, 250 and 150 bases (Fig. 1*b*). The upper band was of the size expected, but the other two bands may have been products of digestion at AU-rich regions of the *crmA* RNA. The same protected bands were observed with messenger RNAs from clones 33 and 48, but no such bands were detected with mRNA from the parental Rat-2 cells and clone 45.

The parental Rat-2 cell line and its transformants were then treated with the agonistic anti-human Fas antibody. As shown in Fig. 2*a*, the anti-Fas antibody had no effect on Rat-2 cells. However, clone 45, which expressed human Fas but not CrmA, was killed within 14 hours by the anti-Fas antibody in a dose-dependent manner in the presence of 50 ng ml^{-1} actinomycin D. Almost all cells died within 10 hours of treatment with 1 μg ml^{-1} of the anti-Fas antibody (Fig. 2*b*). However, the transformant clones 33 and 48 that expressed CrmA were resistant to the cytotoxic activity of the anti-human Fas antibody, and even survived incubation for 10 hours with 1 μg ml^{-1} of anti-Fas antibody. The inhibition of Fas-mediated apoptosis by CrmA was dose dependent, that is, the transformed clones expressing small amounts of *crmA* mRNA were weakly protected against Fas-mediated apoptosis (data not shown).

TNF has cytotoxic activity in various cell lines. To examine whether an ICE-like protease is also involved in TNF-induced cytotoxicity, Rat-2 cells and its transformants were treated with TNF. The Rat-2 cells were killed by 250 ng ml^{-1} of mouse TNF in the presence of 12.5 ng ml^{-1} actinomycin D (Fig. 2*c*), although this process was much slower than Fas-mediated cytotoxicity (all cells were killed in ~60 hours). Clone 45, which expresses Fas but not CrmA, was killed by TNF treatment as efficiently as were the parental Rat-2 cells, but clones 33 and 48,

* Permanent address: Graduate School of Integrated Science, Yokohama City University, Yokohama 236, Japan.
† Present address: Zentrum für Molekulare Biologie, Universität Heidelberg, D-69120 Heidelberg, Germany

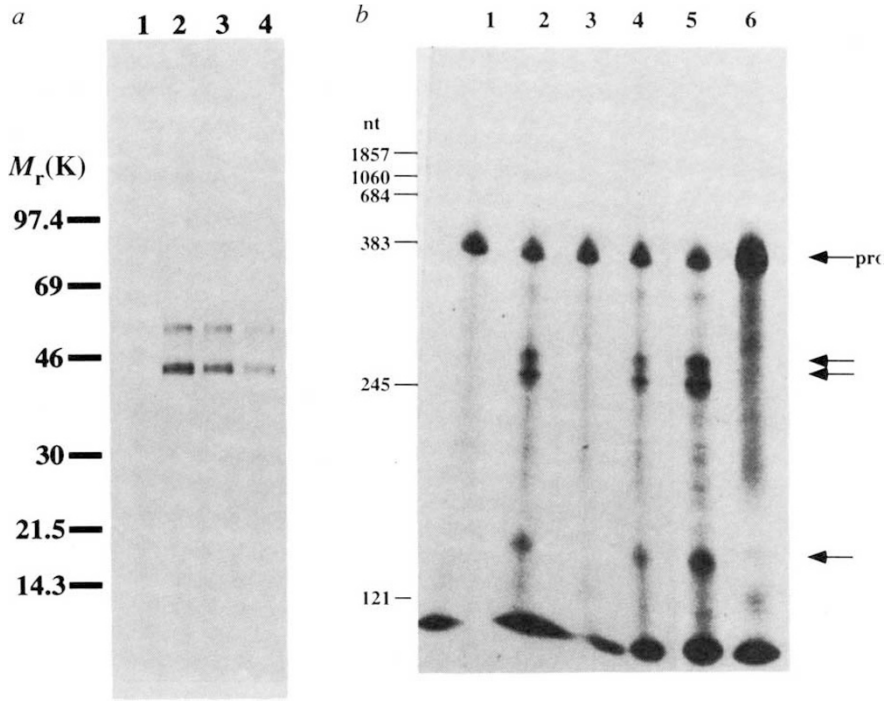

FIG. 1 *a*, Immunoprecipitation of human Fas. The parental Rat-2 (lane 1), and its transformant clones 33 (lane 2), 45 (lane 3) and 48 (lane 4) were biotinylated, lysed in an NP-40-containing buffer, preabsorbed with normal mouse IgG and immunoprecipitated with mouse anti-human Fas antibody as described[13]. The immunoprecipitates were electrophoresed through a polyacrylamide gel, blotted onto a nitrocellulose filter and detected using the ECL system (Amersham). The sizes of marker proteins are shown on the left. *b*, RNase protection assay. ^{32}P-labelled antisense *crmA* RNA (2×10^5 c.p.m.) was hybridized with 1.0 μg poly(A)$^+$ RNA from parental Rat-2 (lane 1) and its transformant clones 33 (lane 2), 45 (lane 3), 48 (lane 4), or with control sense *crmA* RNA (lane 5), digested with a mixture of RNase A and RNase T1, and electrophoresed on a 6% polyacrylamide gel containing 8.3 M urea as described[28]. Undigested probe RNA (lane 6) and size-marker DNAs were electrophoresed in parallel. Marker DNA sizes are shown on the left, and the protected bands are indicted by arrows on the right.

METHODS. The 1.5-kilobase (kb) cowpox virus DNA fragment encoding CrmA[10], provided by D. J. Pickup, was inserted into mammalian expression vector pEF-BOS[29] to generate pEFcrmA. Rat-2 cells (JCRB IFO 50282) were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum. The cells were transfected with 10 μg pEFcrmA, 10 μg pEFF58 (ref. 1) and 0.5 μg pSTneoB by electroporation as described[1], and G418-resistant transformants were selected by culturing cells in medium containing 900 μg ml^{-1} G418 for 3 weeks. For the RNase protection assay, the 1.5-kb *CrmA* fragment was inserted at the *Eco*RI site of pBluescript (pBScrmA), digested with *Pvu*II, and the ^{32}P-labelled antisense RNA corresponding to the C-terminal part of CrmA was synthesized in an *in vitro* transcription system using T7 RNA polymerase (Promega) and [α-^{32}P]CTP (Amersham). To prepare the control sense *CrmA* RNA, pBScrmA was digested with *Hin*dIII, and transcribed *in vitro* using T3 RNA polymerase (Promega). Poly(A)$^+$ RNA was prepared from Rat-2 and its transformants using a mRNA purification kit (Pharmacia).

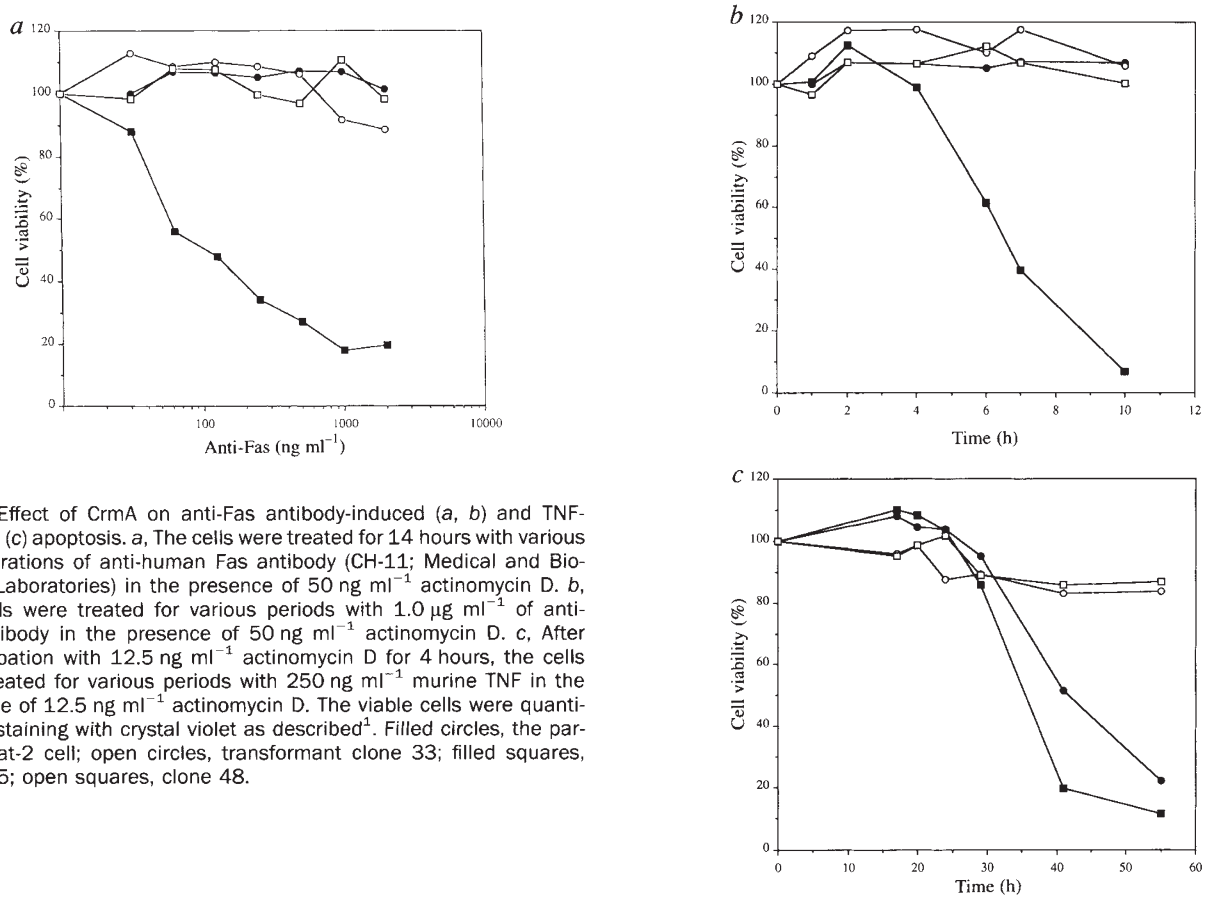

FIG. 2 Effect of CrmA on anti-Fas antibody-induced (*a*, *b*) and TNF-induced (*c*) apoptosis. *a*, The cells were treated for 14 hours with various concentrations of anti-human Fas antibody (CH-11; Medical and Biological Laboratories) in the presence of 50 ng ml^{-1} actinomycin D. *b*, The cells were treated for various periods with 1.0 μg ml^{-1} of anti-Fas antibody in the presence of 50 ng ml^{-1} actinomycin D. *c*, After preincubation with 12.5 ng ml^{-1} actinomycin D for 4 hours, the cells were treated for various periods with 250 ng ml^{-1} murine TNF in the presence of 12.5 ng ml^{-1} actinomycin D. The viable cells were quantified by staining with crystal violet as described[1]. Filled circles, the parental Rat-2 cell; open circles, transformant clone 33; filled squares, clone 45; open squares, clone 48.

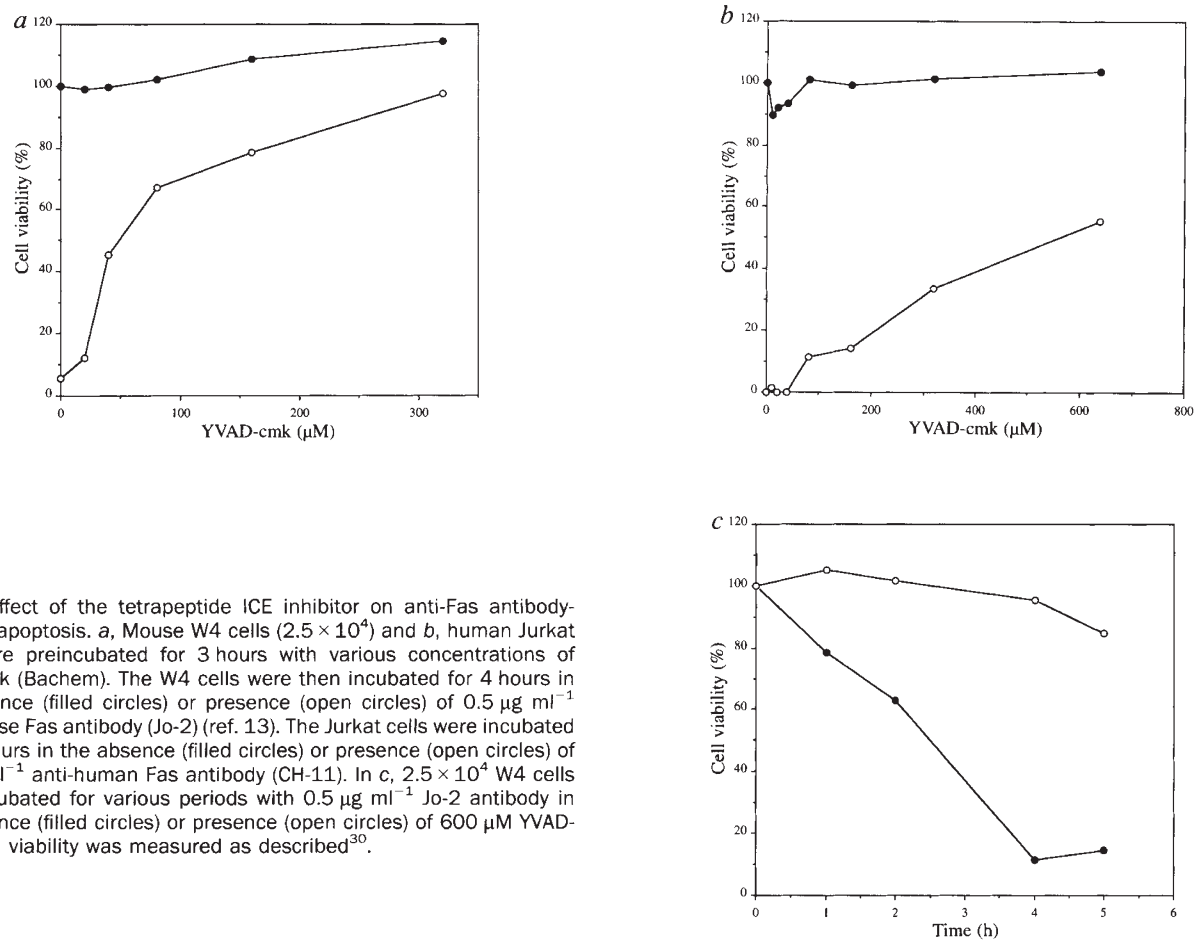

FIG. 3 Effect of the tetrapeptide ICE inhibitor on anti-Fas antibody-induced apoptosis. *a*, Mouse W4 cells (2.5×10^4) and *b*, human Jurkat cells were preincubated for 3 hours with various concentrations of YVAD-cmk (Bachem). The W4 cells were then incubated for 4 hours in the absence (filled circles) or presence (open circles) of 0.5 μg ml^{-1} anti-mouse Fas antibody (Jo-2) (ref. 13). The Jurkat cells were incubated for 23 hours in the absence (filled circles) or presence (open circles) of 1.0 μg ml^{-1} anti-human Fas antibody (CH-11). In *c*, 2.5×10^4 W4 cells were incubated for various periods with 0.5 μg ml^{-1} Jo-2 antibody in the absence (filled circles) or presence (open circles) of 600 μM YVAD-cmk. Cell viability was measured as described[30].

which expressed CrmA, were resistant to TNF-induced cytotoxicity. These results indicate that an ICE-like protease is involved not only in Fas-mediated apoptosis but also in TNF-induced cytotoxicity. It has previously been shown[11] that TNF-induced cytotoxicity is partly inhibited by a serine-protease inhibitor, plasminogen-activator inhibitor type 2. Because plasminogen-activator inhibitor type 2 is a member of the serpin superfamily to which CrmA belongs[7], it is possible that the plasminogen activator inhibitor also inhibits the ICE-like protease by 'cross-class' interaction[12].

The cytotoxicity of the anti-Fas antibody and TNF in Rat-2 cells was seen only in the presence of actinomycin D. We tried to express *crmA* in other cell lines, such as mouse W4 (ref. 13) or human Jurkat cells[14], which can be killed by anti-Fas antibody without actinomycin D. However, stable transformants constitutively expressing CrmA were not obtained because of the strong growth-inhibitory effect of CrmA in these cell lines. To overcome this problem, and to confirm the involvement of an ICE-like protease in Fas-mediated apoptosis in other cells, we used a tetrapeptide of acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk), which works as a specific inhibitor for ICE or ICE-like proteases[8,9]. Treatment of W4 cells with 0.5 μg ml^{-1} anti-mouse Fas antibody or Jurkat cells with 1.0 μg ml^{-1} anti-human Fas antibody killed the cells (Fig. 3). Addition of YVAD-cmk inhibited the killing process in a dose-dependent manner. The kinetic analysis of the death process of W4 in the presence of 600 μM YVAD-cmk confirmed its inhibitory activity against the anti-Fas antibody-induced apoptosis (Fig. 3*c*). However, as found with purified ICE[9], other protease inhibitors, such as TPCK, PMSF and E-64, had no effect on Fas-mediated apoptosis (data not shown).

TNF type-I receptor (TNFRI) and Fas carry a similar killing domain in the cytoplasmic region[15,16] and we suggest here that both receptors use an ICE-like molecule to kill the cells. This indicates that the apoptotic mechanism is well conserved during evolution from nematodes to mammals. Programmed cell death in nematodes is inhibited by CED-9 (ref. 17). Significant inhibition of Fas-mediated apoptosis by Bcl-2 (ref. 18), a mammalian homologue of *ced-9*, is in agreement with this. It is possible that signalling molecules immediately downstream of the receptors are distinct between Fas and TNFRI[19,20] but these pathways would eventually lead to the activation of an ICE-like protease. Because at least three ICE or ICE-like proteases exist in mammalian cells[9,21–23], it would be necessary to determine which ICE-like protease is involved in Fas- and TNFRI-mediated apoptosis. ICE is a tetrameric protein, consisting of $(p20)_2/(p10)_2$ in the cytoplasm[24]. Because Fas-mediated apoptosis occurs in the presence of inhibitors of RNA or protein synthesis, the activation of an ICE-like protease during Fas-mediated apoptosis is probably a post-transcriptional event. A signal(s) from Fas or TNFRI may activate the processing of the precursor of an ICE-like protease[9], or inactivate the CrmA-like cellular protein. The Fas ligand works as an effector of cytotoxic T-lymphocytes (CTL)[25,26] and it is possible that the Fas system is involved in CTL-mediated disease, such as fulminant hepatitis or AIDS[27]. If so then inhibitors of ICE such as the YVAD-cmk, may be of clinical benefit.

Note added in proof: Tewari and Dixit have recently reported that the *crmA* product can inhibit Fas- and TNF-induced apoptosis (*J. biol. Chem.* **270,** 3255–3260 (1995)). □

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Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis

Marek Los*†, Marc Van de Craen‡, Louis C. Penning‡, Heike Schenk*, Michael Westendorp§, Patrick A. Baeuerle†, Wulf Dröge*, Peter H. Krammer§, Walter Fiers‡ & Klaus Schulze-Osthoff*†‖

Divisions of * Immunochemistry and § Immunogenetics, Tumorimmunology Program, German Cancer Research Center, Heidelberg, Germany
† Institute of Biochemistry, Albert-Ludwigs-University, Hermann-Heder-Strasse 7, D79104 Freiburg im Breisgau, Germany
‡ Laboratory of Molecular Biology, University of Ghent, Ghent, Belgium

THE Fas/APO-1 receptor is one of the major regulators of apoptosis[1–7]. We report here that Fas/APO-1-mediated apoptosis requires the activation of a new class of cysteine proteases, including interleukin-1β-converting enzyme (ICE)[8–10], which are homologous to the product of the *Caenorhabditis elegans* cell-death gene *ced*-3 (refs 11, 12). Triggering of Fas/APO-1 rapidly stimulated the proteolytic activity of ICE. Overexpression of ICE, achieved by electroporation and microinjection, strongly potentiated Fas/APO-1-mediated cell death. In addition, inhibition of ICE activity by protease inhibitors, as well as by transient expression of the pox virus-derived serpin inhibitor CrmA or an antisense ICE construct, substantially suppressed Fas/APO-1-triggered cell death. We conclude that activation of ICE or an ICE-related protease is a critical event in Fas/APO-1-mediated cell death.

The signal transduction pathway elicited by Fas/APO-1 is almost completely unknown. Initiation of apoptosis may involve a new class of cysteine proteases, including the product of the *C. elegans* cell-death gene *ced-3*, mammalian interleukin-1β-converting enzyme (ICE) and the related proteases Nedd-2/Ich-1, prICE and CPP-32 (refs 11–17). Overexpression of CED-3, ICE or Nedd-2/Ich-1 in Rat-1 fibroblasts has been shown to result in apoptotic cell death[12,15]. We therefore investigated whether Fas/APO-1-mediated apoptosis involved an ICE-related proteolytic activity. In L929-APO-1 cells[18] or B-lymphoblastoid SKW 6.4 cells, apoptosis triggered by the agonistic monoclonal antibody anti-APO-1 was strongly inhibited by the ICE inhibitor YVAD-CHO, a tetrapeptide aldehyde ($K_i = 0.76$ nM)[8] (Fig. 1*a*). Inhibition was also observed with the protease inhibitor dichloroisocoumarin, but other serine protease inhibitors, such as PMSF and leupeptin, calpain inhibitors and the cysteine protease inhibitor E-64 were not effective (data not shown). In addition, ICE-like proteolytic activity was readily induced by Fas/APO-1 ligation (Fig. 1*b*). The fluorogenic ICE substrate DABCYL-YVADAP-EDANS, which contains the cleavage site of the interleukin-1β precursor[19], was cleaved after treatment of permeabilized cells with anti-APO-1, but no effects were detected using classical cysteine or serine protease-specific substrates (Fig. 1*b*, *c*).

To explore further the participation of ICE in Fas/APO-1-mediated apoptosis, ICE was overexpressed using several techniques. First, murine ICE complementary DNA was microinjected into nuclei of L929-APO-1 cells. After treatment with anti-APO-1, apoptotic cells could be recognized as round-shaped cells revealing membrane blebbing and cytoplasmic condensation. When cells microinjected with ICE cDNA were treated with a suboptimal dose of anti-APO-1, a nearly threefold increase in the number of apoptotic cells was detected compared with cells microinjected with the empty vector alone (Fig. 2*a*). In contrast, microinjection of vaccinia virus-derived *crmA* cDNA, the product of which inhibits ICE activity by forming a serpin-like pseudosubstrate[20–22], significantly suppressed anti-APO-1-induced cell death. These observations suggested the involvement of ICE or an ICE-related protease in the Fas/APO-1 signalling pathway. Although ICE is the only protease known to be inhibited by CrmA, it is possible that a related protease with similar substrate specificity was inhibited by CrmA. To investigate more specifically the role of ICE, we further included an antisense ICE construct. ICE, *crmA* and antisense ICE cDNAs were overexpressed by electroporation, which resulted in transfection efficiencies of more than 80% as assessed by reporter gene plasmids. Figure 2*b* shows that apoptosis was increased by transient expression of ICE after anti-APO-1 treatment, whereas apoptosis induced in their *crmA*- or antisense-ICE-transfected counterparts was reduced. The effects on apoptosis were further evaluated in L929-APO-1 cells after cotransfection with the *lacZ* gene as a marker of gene expression (Fig. 3). In comparison with cells transfected with the vector control, the percentage of round apoptotic cells out of the total number of blue-stained cells was substantially increased in ICE cDNA-transfected cells after anti-APO-1 treatment. As in the previous experiments, no significant difference in cell viability of L929-APO-1 cells was observed without Fas/APO-1 activation. This is in apparent contrast to other cell types undergoing apoptosis by overexpression of ICE alone[12,15]. In line with the previous data, transient overexpression of CrmA or antisense-ICE resulted in an inhibition of anti-APO-1-induced apoptosis of ~50% (Fig. 3*g*).

These data indicate that ICE plays a role in the induction of apoptosis mediated by Fas/APO-1. Although it cannot be excluded that other ICE-related proteases may also be involved, the antisense experiments suggest that ICE is important. Because ICE is structurally and functionally related to the nematode

‖ To whom correspondence should be addressed at Freiburg.