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# Inhibition of apoptosis by the actin-regulatory protein gelsolin

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**Gelsolin is an actin-regulatory protein that modulates actin assembly and disassembly, and is believed to regulate cell motility *in vivo* through modulation of the actin network. In addition to its actin-regulatory function, gelsolin has also been proposed to affect cell growth. Our present experiments have tested the possible involvement of gelsolin in the regulation of apoptosis, which is significantly affected by growth. When overexpressed in Jurkat cells, gelsolin strongly inhibited apoptosis induced by anti-Fas antibody, $C_2$-ceramide or dexamethasone, without changing the F-actin morphology or the levels of Fas or Bcl-2 family proteins. Upon the induction of apoptosis, an increase in CPP32(-like) protease activity was observed in the control vector transfectants, while it was strongly suppressed in the *gelsolin* transfectants. Pro-CPP32 protein, an inactive form of CPP32 protease, remained uncleaved by anti-Fas treatment in the *gelsolin* transfectants, indicating that gelsolin blocks upstream of this protease. The tetrapeptide inhibitor of CPP32(-like) proteases strongly inhibited Fas-mediated apoptosis, but only partially suppressed both $C_2$-ceramide- and dexamethasone-induced apoptosis. These data suggest that the critical target responsible for the execution of apoptosis may exist upstream of CPP32(-like) proteases in Jurkat cells and that gelsolin acts on this target to inhibit the apoptotic cell death program.**

*Keywords*: apoptosis/CPP32/Fas/gelsolin/Jurkat cells

## Introduction

Apoptosis, or genetically regulated cell death, is crucial for normal development and tissue remodeling in multicellular organisms (Wyllie *et al.*, 1980). In recent years, much effort has been made to identify the components which regulate apoptosis, and it is now recognized that members of the interleukin-1β-converting enzyme (ICE) family of cysteine proteases act as 'executioners' in the cell death process (Martin and Green, 1995). CPP32 (also called Yama or apopain) is a major protease in this growing family, known to be involved and probably essential in various types of apoptosis (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). On the other hand, the Bcl-2 proto-oncogene product (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Tsujimoto *et al.*, 1985) and the related protein Bcl-$x_L$ (Boise *et al.*, 1993) have been shown to exert an apoptosis-inhibitory function (Vaux *et al.*, 1988; Tsujimoto *et al.*, 1989; Nunez *et al.*, 1990). Two viral anti-apoptotic proteins, CrmA and p35, have also been shown to suppress diverse forms of apoptosis (Clem *et al.*, 1991; Ray *et al.*, 1992). Recent studies in which these apoptosis-inhibitory proteins were overexpressed, or where tetrapeptide inhibitors specific for each ICE family protease were used, have generated data helpful for the interpretation of the molecular mechanisms of apoptosis. Thus, the finding of any new inhibitor able to suppress various forms of apoptosis will benefit further study that seeks to understand the machinery of apoptosis.

Gelsolin is an actin-regulatory protein, first isolated from rabbit lung macrophages as a modulator of the cytoplasmic actin gel–sol transformation (Yin and Stossel, 1979). Gelsolin can sever actin filaments and cap the fast-growing ends of the filaments *in vitro*; this promotes actin disassembly. Gelsolin also has the ability to nucleate actin polymerization. These functions are activated by $Ca^{2+}$ and inhibited by polyphosphoinositides (PPIs) (Yin and Stossel, 1980; Kurth and Bryan, 1984; Janmey and Stossel, 1987). Such observations have led to the view that gelsolin regulates actin reorganization in response to changes in the concentrations of $Ca^{2+}$ and PPIs in living cells (Yin, 1988).

The overexpression of gelsolin in NIH 3T3 fibroblasts results in their enhanced motility, indicating that the regulation of cell motility is one of the roles of gelsolin *in vivo* (Cunningham *et al.*, 1991). A recent *in vivo* study has supported this by demonstrating blunted leukocyte motility in transgenic gelsolin-null mice (Witke *et al.*, 1995). Further evidence supports the idea that gelsolin is also able to regulate cell growth: a mouse *gelsolin* gene with a point mutation revealed a tumor suppressive potential against H-*ras* oncogene-transformed NIH 3T3 cells (Mullauer *et al.*, 1993; Fujita *et al.*, 1995). In addition, authentic *gelsolin* transfectants of a human bladder cancer cell line, UMUC-2, greatly reduced colony-forming ability and tumorigenicity *in vivo* (Tanaka *et al.*, 1995).

Several reports have provided evidence that proteins which fulfill apoptosis-regulatory functions may simultaneously possess the ability to regulate cell proliferation, as is the case with Bcl-2 (Borner, 1996; Mazel *et al.*, 1996), Ras (Trent *et al.*, 1996) or p53 (Lassus *et al.*, 1996). We therefore tested the possibility that gelsolin

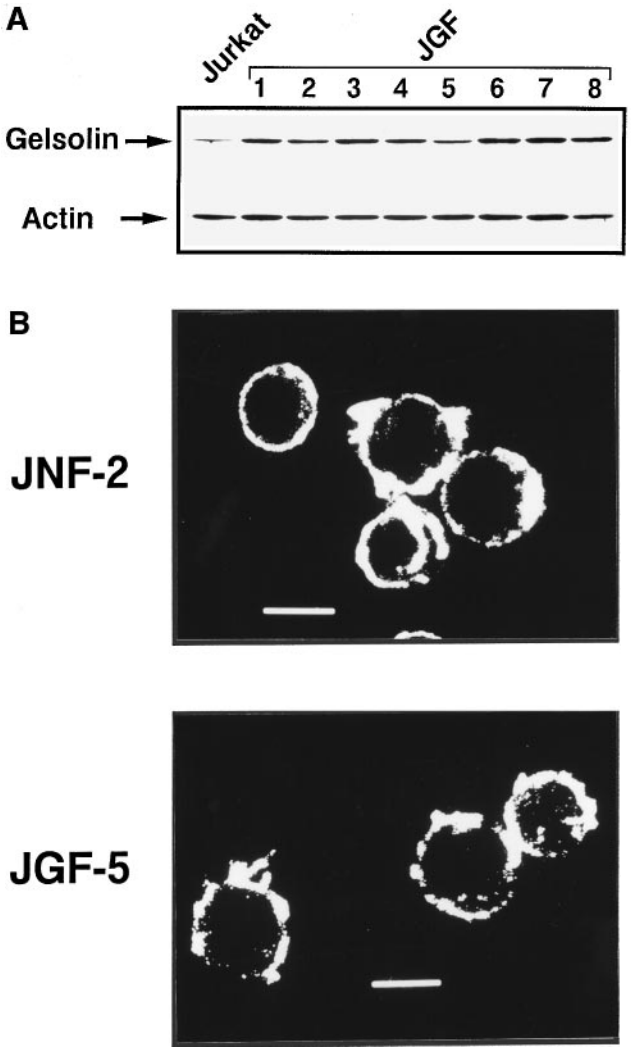


**Fig. 1.** Western blot analysis and F-actin morphology of the parental Jurkat cell line and its transfectants. (**A**) Amounts of gelsolin and actin in parental Jurkat and JGF-1–8 cells examined by Western blot analysis. Proteins (20 μg) were separated on 10% SDS–PAGE and detected as described in Materials and methods. (**B**) F-actin staining of JNF-2 and JGF-5 cells. Shown are representative cells of each clone. Scale bars represent 10 μm.

may have an apoptosis-regulatory function in the Jurkat cell model system. By using three different apoptotic stimuli, all of which activate CPP32(-like) proteases, we found strong evidence that gelsolin acts as an apoptosis inhibitor upstream of CPP32(-like) proteases.

## Results

### *Establishment of Jurkat cell lines that overexpress gelsolin*

To investigate the effects of overexpressed gelsolin on apoptosis, we established stable transfectants of Jurkat cells overexpressing gelsolin, and designated them JGF-1–8. Western blot analysis showed two to three times higher amounts of gelsolin in JGF clones than in either the parental cells (Figure 1A) or the control vector transfectants (designated JNF-1–5, data not shown), with no difference in the amounts of actin (Figure 1A). Phase-contrast microscopy showed that overexpressed gelsolin had induced no significant difference in cellular morphology (data not shown), nor were the distribution or amounts of F-actin affected by gelsolin, as assessed respectively by confocal laser microscopy (Figure 1B) and fluorescence-activated cell sorting (FACS) analyses (data not shown) of cells stained with rhodamine–phalloidin. Two representative clones of each genotype (JNF-2, JNF-5, JGF-5 and JGF-7) were used for further studies.

### *Inhibition of apoptosis by overexpressed gelsolin*

To induce apoptosis, cells were treated with anti-Fas antibody (Itoh *et al.*, 1993), $C_2$-ceramide ($C_2$-Cer) (Obeid *et al.*, 1993) or dexamethasone (Dex) (Cohen and Duke, 1984), and cell viability was assessed by trypan blue exclusion. JGF-5 and JGF-7 cells showed remarkable resistance to all apoptosis-inducing treatments compared with both parental Jurkat and JNF-2 cells (Figure 2A). The extent of apoptotic cell death depended upon the concentrations of anti-Fas antibody; while JNF-2 cells were almost completely killed by anti-Fas antibody at 100 ng/ml for 24 h, 65% of JGF-5 cells were still viable at a 10-fold higher concentration (Figure 2B). The absence of internucleosomal DNA fragmentation in JGF-5 cells treated with anti-Fas antibody (Figure 2C) or with either $C_2$-Cer or Dex (Figure 2D) confirmed gelsolin's anti-apoptotic activity.

Although these stimuli did not induce significant cell death in JGF cells, growth inhibition was observed commonly with each stimulus (data not shown). We therefore investigated the cell cycle profiles of cells treated with the apoptotic stimuli. With no treatment, JNF-2 and JGF-5 cells showed similar cell cycle profiles [Figure 3, (–)]. When cell death was induced using any one of three apoptotic stimuli, sub-$G_0/G_1$ apoptotic populations became apparent in JNF-2 cells, but not in JGF-5 cells (Figure 3). Interestingly, JGF-5 cells showed quite different cell cycle profiles with each stimulus: $G_2$/M arrest with $C_2$-Cer, $G_1$ accumulation with Dex and a slight decrease in S phase with anti-Fas treatment. This result suggests that the three stimuli exert different biological effects on Jurkat cells, although cell death is a common consequence. Gelsolin therefore appears to impinge upon a mechanism central to apoptosis induced by clearly distinct stimuli.

### *Gelsolin alters neither Fas expression nor the amounts of Bcl-2 family proteins*

FACS analysis revealed that gelsolin overexpression did not alter Fas expression on the surface of Jurkat cells (Figure 4A), and Western blot analysis showed no differences in the amounts of Bcl-2 and Bcl-$x_L$ between parental Jurkat cells and the transfected lines (Figure 4B). Bax protein, another member of the Bcl-2 family, has been shown to dimerize with either Bcl-2 or Bcl-$x_L$ and promotes apoptosis (Oltvai *et al.*, 1993; Sedlak *et al.*, 1995), and the level of this protein was also unaltered (Figure 4B). These results indicate that gelsolin exerts its anti-apoptotic activity neither through the reduction of Fas antigen nor by changing the levels of Bcl-2 family proteins.

### *Gelsolin inhibits the activation of CPP32(-like) proteases*

Recent studies have shown that members of the ICE family of proteases play a central role in apoptosis (Miura

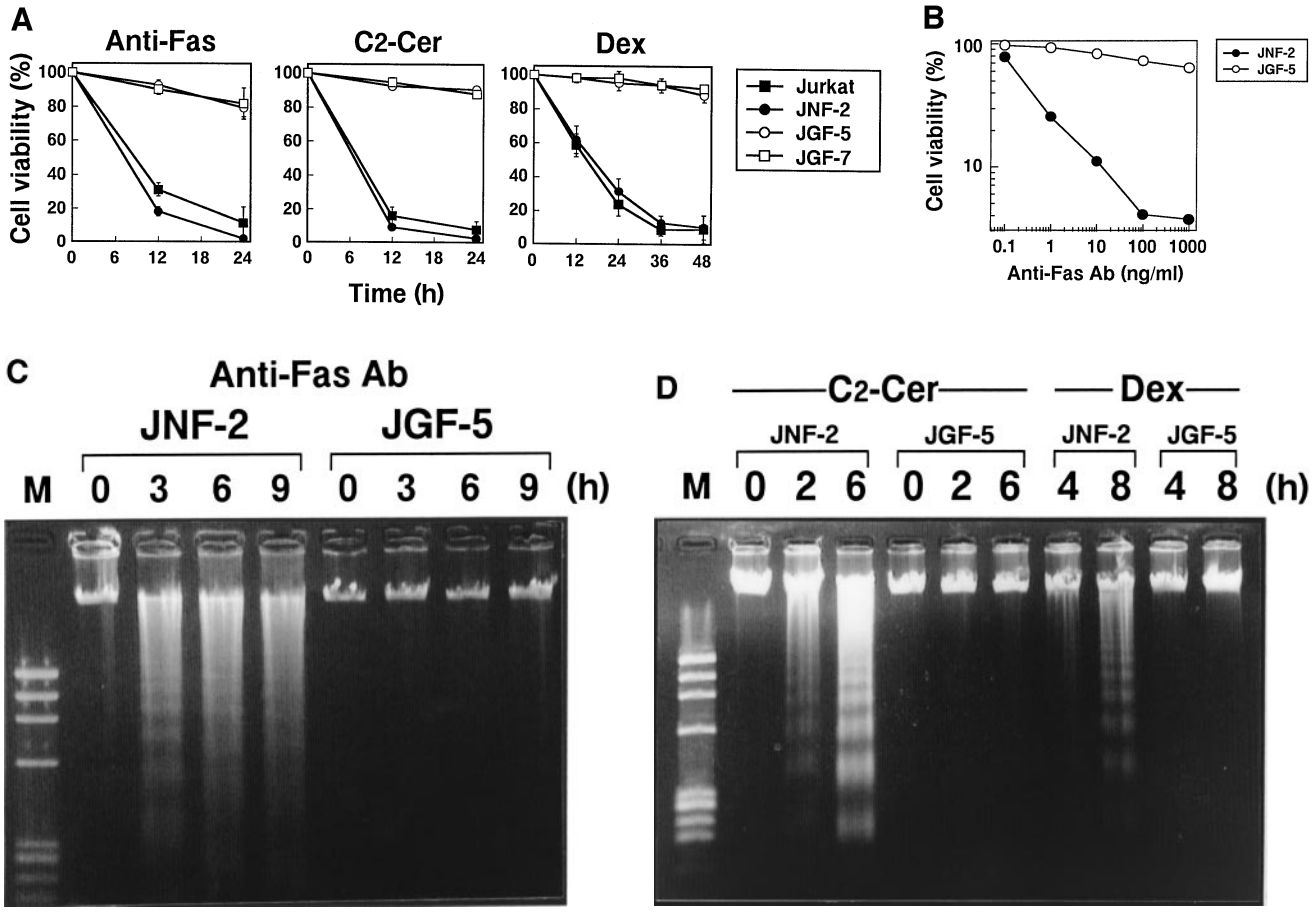


**Fig. 2.** Inhibition of apoptosis by overexpressed gelsolin. (**A**) Time course analysis of the viability of parental Jurkat, JNF-2, JGF-5 and JGF-7 cells treated with anti-Fas antibody, $C_2$-Cer or Dex. Results are shown as means ± SD of values obtained from three independent experiments. (**B**) Dose dependence of viability (24 h) of JNF-2 and JGF-5 cells on the concentration of anti-Fas antibody. Results are shown as mean values from two independent experiments. (**C** and **D**) DNA fragmentation assay. DNA was extracted from JNF-2 or JGF-5 cells treated with each stimulus for the indicated periods and subjected to a 2% agarose gel electrophoresis. M, DNA size marker (ϕX174-*Hae*III digest).

*et al.*, 1993; Wang *et al.*, 1994; Faucheu *et al.*, 1995; Fernandes-Alnemri *et al.*, 1995; Munday *et al.*, 1995). To examine the possible involvement of gelsolin in the regulation of the activity of ICE family proteases, we measured the activity of ICE(-like) proteases and CPP32(-like) proteases using peptide substrates in cells treated with anti-Fas antibody, $C_2$-Cer or Dex, with or without overexpressed gelsolin. We were unable to find any ICE(-like) protease activity in apoptotic Jurkat cells, nor could we detect either *ice* mRNA or ICE protein in these cells (data not shown), consistent with previous observations (Schlegel *et al.*, 1996). In contrast, CPP32(-like) protease activity in control JNF-2 cells was clearly elevated with all inducers of apoptosis (Figure 5A), and the increase preceded the appearance of apoptotic cells (Figure 2A). In JGF-5 cells, however, the induction of CPP32(-like) protease activity was strongly suppressed (Figure 5A), suggesting that gelsolin blocks apoptosis, in part, by inhibiting the usual increase in CPP32(-like) protease activity seen in response to inducers.

CPP32 protease is synthesized as a 32 kDa inactive precursor (pro-CPP32) which is proteolytically cleaved to produce a mature enzyme composed of 17 and 12 kDa subunits (Fernandes-Alnemri *et al.*, 1994; Nicholson *et al.*, 1995; Tewari *et al.*, 1995). To determine whether gelsolin inhibits CPP32(-like) proteases which have already been activated, or prevents the initial activation of these proteases, we examined the cleavage of the pro-CPP32 protein in response to apoptosis-inducing treatments. The results in Figure 5B indicate that the pro-CPP32 protein disappears upon the induction of apoptosis by anti-Fas antibody in parental Jurkat and JNF-2 cells, reflecting the proteolytic cleavage of pro-CPP32 generating active CPP32 protease. In contrast, the pro-CPP32 protein remains uncleaved in identically treated JGF-5 cells. Similar results were obtained with $C_2$-Cer or Dex treatment (data not shown). Taken together, these results strongly suggest that gelsolin suppresses a common upstream step in the pathway leading to the activation of CPP32(-like) proteases in response to diverse stimuli, rather than inhibiting these proteases directly.

To determine if ICE family proteases are required for the apoptosis of Jurkat cells, we next treated cells with anti-Fas antibody, with $C_2$-Cer or with Dex in the presence of Ac-DEVD-CHO, a CPP32/apopain-specific inhibitor (Nicholson *et al.*, 1995), or of Ac-YVAD-CHO, an ICE-specific inhibitor (Thornberry *et al.*, 1992). As shown in Figure 6, Ac-DEVD-CHO effectively inhibited Fas-mediated apoptosis in Jurkat cells at 0.1 mM while Ac-YVAD-CHO had no inhibitory effect at concentrations as high as 1 mM, suggesting that CPP32(-like) proteases, but not ICE(-like) proteases, are essential for Fas-mediated

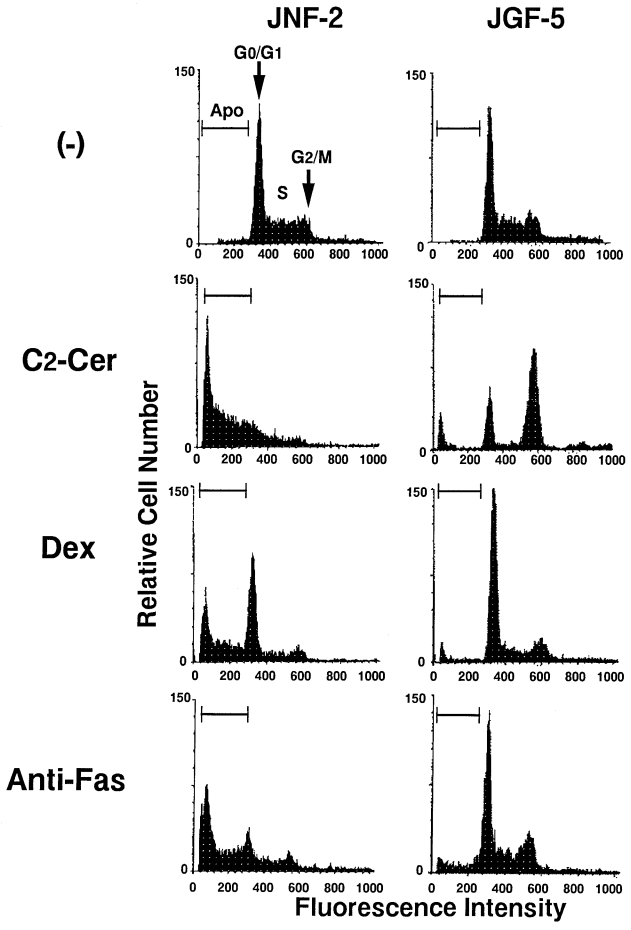


**Fig. 3.** Cell cycle profiles of control or gelsolin transfectants treated with apoptotic stimuli. JNF-2 or JGF-5 cells were treated as in Figure 2A, and harvested at 24 h ($C_2$-Cer and anti-Fas) or 36 h (Dex) for cell cycle analysis. A sub-$G_0/G_1$ fraction representing the apoptotic cell population is shown as 'Apo'. JGF-7 cells exhibited almost identical profiles to JGF-5 cells.

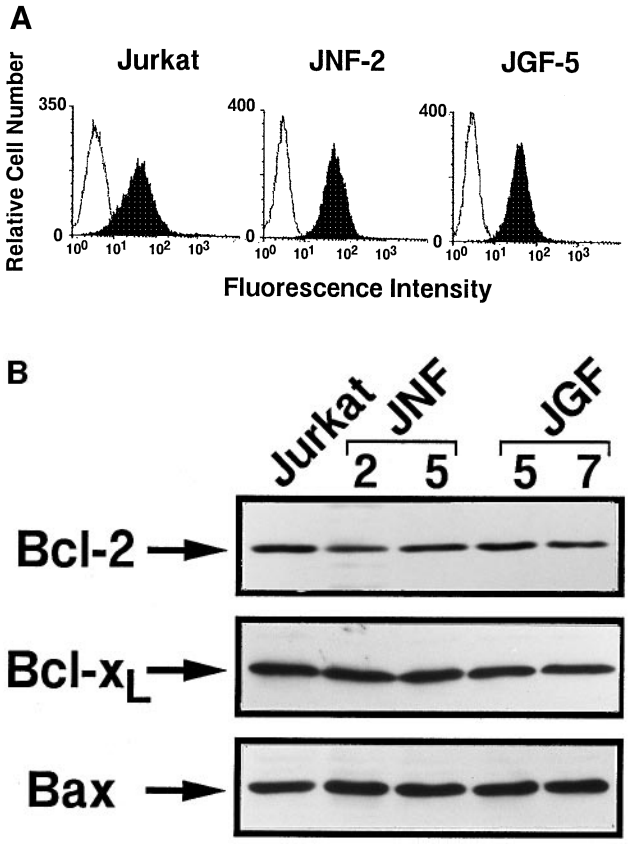


**Fig. 4.** Expression of Fas antigen and Bcl-2 family proteins in the parental Jurkat cell line and its transfectants. (**A**) Cell surface expression of Fas antigen in parental Jurkat, JNF-2 and JGF-5 cells. JNF-5 and two other JGF clones showed almost identical results. (**B**) Amounts of Bcl-2, Bcl-$x_L$ and Bax proteins in parental Jurkat, JNF-2, JNF-5, JGF-5 and JGF-7 cells. Proteins (50 μg) from exponentially growing cells were separated on 12% SDS–PAGE and analyzed as in Materials and methods.

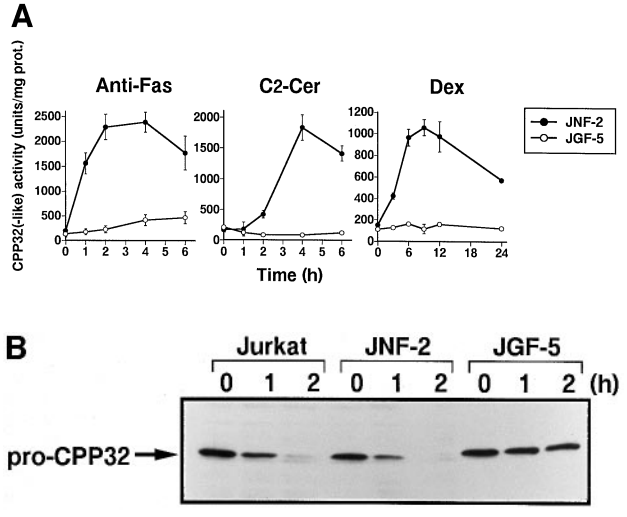


**Fig. 5.** Blocked activation of CPP32(-like) proteases by gelsolin. (**A**) Inhibition of the increase in CPP32(-like) protease activity in JGF-5 cells. Results are shown as means ± SD of values obtained from three independent experiments. (**B**) Western blot analysis of pro-CPP32 proteins in parental Jurkat, JNF-2 and JGF-5 cells with anti-Fas treatment. Cells were treated with (1 or 2 h) or without (0 h) anti-Fas antibody (500 ng/ml), and proteins (40 μg) were separated on 12% SDS–PAGE. Pro-CPP32 proteins were detected as described in Materials and methods.

apoptosis in Jurkat cells. This is consistent with previous observations (Schlegel *et al.*, 1996) and with our observation that ICE protease is not expressed in this model system. However, Ac-DEVD-CHO had only a partially inhibitory effect on $C_2$-Cer- or Dex-induced apoptosis in these cells (Figure 6). This suggests that CPP32(-like) proteases are also involved in $C_2$-Cer- or Dex-induced apoptosis of Jurkat cells, but that they are only partially requisite in response to these agents. Thus, other ICE family proteases or other key steps, which are insensitive to both of the inhibitors above, but can be inhibited efficiently by gelsolin overexpression, may act as an essential mediator of these forms of apoptosis. These experiments are consistent with gelsolin inhibiting apoptosis by blocking a target upstream of the CPP32(-like) proteases which is essential for, and common among, the pathways of apoptosis induced by markedly different stimuli.

## Discussion

These studies have shown that when overexpressed in Jurkat cells, gelsolin inhibits apoptosis induced by three different stimuli. A similar anti-apoptotic activity of

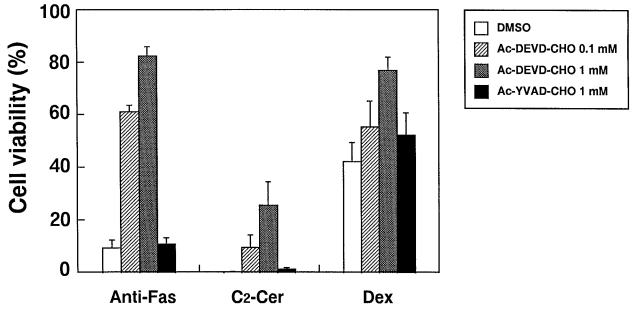


**Fig. 6.** Effects of tetrapeptide inhibitors for ICE(-like) or CPP32(-like) proteases on apoptosis in untransfected Jurkat cells. Results are shown as means ± SD of values obtained from four independent experiments.

gelsolin has been observed in mouse fibroblast NIH 3T3 cells and other human tumor cell lines with several different apoptotic stimuli (N.Sakai, M.Ohtsu, H.Fujita and N.Kuzumaki, in preparation), indicating that this activity is not restricted to Jurkat cells. Several possible mechanisms may explain this inhibitory activity, including modulation of the actin network and inhibition of certain ICE family proteases.

Since gelsolin has actin-regulatory functions, modulation of the actin network might be responsible for the inhibition of apoptosis. However, Western blot analysis showed that the total amount of cellular actin was not altered in response to gelsolin overexpression. Moreover, F-actin analysis with rhodamine–phalloidin staining revealed no apparent change in the distribution or amounts of F-actin in the *gelsolin* transfectants. These results suggest that obvious alterations in the actin network have not occurred in response to gelsolin overexpression, and therefore did not contribute to the apoptosis-inhibitory effect of gelsolin. Since monomeric (G-) actin has been shown to inhibit DNase I activity (Peitsch *et al.*, 1993), an increase of G-actin, although undetectable in our study, might inhibit DNase I and consequently suppress DNA fragmentation. However, since DNA fragmentation is probably a late event in the apoptotic pathway, inhibition of DNase I is unlikely to explain the blockage of CPP32(-like) protease activation and apoptosis observed in the *gelsolin* transfectants.

One study has reported that anti-CD3-induced growth arrest in Jurkat cells accompanies rapid and major actin reorganization, and that treatment with cytochalasin D, an inhibitor of actin polymerization, not only prevents the change in cell shape but also blocks anti-CD3-mediated signal transduction leading to growth arrest (Parsey and Lewis, 1993). Although this suggests that modulation of actin reorganization may also block signal transduction from the Fas antigen, we were unable to find any early changes in F-actin morphology in Jurkat cells treated with anti-Fas antibody (data not shown). In addition, inhibition of actin polymerization by treatment with cytochalasin D did not prevent Fas-induced apoptosis (data not shown), suggesting that signaling along this pathway does not require actin reorganization. Additional experiments with gelsolin mutants which lack various actin-regulatory functions are now underway to address this possibility more completely.

The anti-apoptotic proteins CrmA and p35 recently have been shown to inhibit apoptosis by directly inhibiting specific ICE family protease(s) (Rabizadeh *et al.*, 1993; Bump *et al.*, 1995; Tewari and Dixit, 1995), most likely by functioning as substrates for, and as competitive inhibitors of, these enzymes (Xue and Horvitz, 1995). Gelsolin itself might also be a substrate, and thus an inhibitor of certain ICE family proteases. We can find two potential cleavage sites for CPP32 in the amino acid sequence of human cytoplasmic gelsolin (Kwiatkowski *et al.*, 1986). These sequences are $DQTD^{352}G$ and $SEPD^{588}G$, which respectively resemble the cleavage sites DQMDG in p35 (Bertin *et al.*, 1996) and SEPDS in the sterol-regulatory element-binding protein (SREBP)-1 (Wang *et al.*, 1996). Recently, FLICE/MACH1/Mch-5 has been identified as a new member of the ICE family of proteases which possesses dual functions. One function is to interact with Fas antigen intracellularly, and another is to operate as an ICE family protease at the apex of the apoptotic protease cascade (Boldin *et al.*, 1996; Muzio *et al.*, 1996). It thus seems likely that the cross-linking of Fas antigen leads to the activation of this protease which in turn activates the intermediate ICE family proteases, and these proteases activate CPP32(-like) proteases which are probably the final effectors of the protease cascade (Fraser and Evan, 1996). More recently, Mch-4, a novel protease structurally related to FLICE/MACH1/Mch-5, has been cloned from a Jurkat cDNA library and shown to have the ability to cleave pro-CPP32 protease (Fernandes-Alnemri *et al.*, 1996). In this relatively simple pathway of Fas-mediated apoptosis, it is tempting to speculate, due to the very potent inhibition of apoptosis by gelsolin, that it may inhibit additional ICE family proteases (including FLICE/MACH1/Mch-5, Mch-4) upstream of the CPP32(-like) proteases.

Ceramide has also been proposed to be a regulator of apoptosis by virtue of serving as a novel lipid second messenger in several signal transduction pathways, including one culminating in apoptosis (Hannun and Obeid, 1995), and recent reports suggest that ceramide is an endogenous mediator of Fas-mediated apoptosis (Cifone *et al.*, 1993; Tepper *et al.*, 1995). In this study, however, we present evidence that $C_2$-Cer acts as an alternative apoptotic stimulator rather than as a mediator of Fas-induced apoptosis. $C_2$-Cer treatment induced apoptosis and the activation of CPP32(-like) proteases in Jurkat cells, both of which were inhibited by overexpressed gelsolin. On the other hand, the inhibitor of CPP32(-like) proteases, Ac-DEVD-CHO, was revealed to be partially inhibitory to $C_2$-Cer-induced apoptosis, suggesting that $C_2$-Cer stimulates an additional cell death pathway distinct from CPP32(-like) protease activation. Additionally, the biological effects of $C_2$-Cer are probably not the same as those of increased endogenous ceramide (Pronk *et al.*, 1996), consistent with our observations. Since $C_2$-Cer has been shown to activate a stress-activated protein kinase (SAPK/JNK), which results in c-Jun activation-dependent apoptosis (Verheij *et al.*, 1996), SAPK/JNK activation may therefore transduce the $C_2$-Cer-generated signal via this distinct signal transduction pathway.

The mechanism of Dex-induced apoptosis remains largely unknown. Since the glucocorticoid receptor (GR) is required for Dex-induced apoptosis, GR-mediated induction of 'lysis genes' or repression of 'survival genes' is

thought to be responsible for apoptosis (Helmberg *et al.*, 1995). However, the identity and nature of these lysis and survival genes remain obscure, and it is unknown how the modification of the expression of such genes leads to CPP32(-like) protease activation. Based on our observations that Dex treatment arrests cells in phases of the cell cycle that are distinct from those seen in response to the binding of Fas antigen or the addition of $C_2$-Cer, and the fact that GR activation results in rapid binding of the receptor to DNA, it also appears that Dex may induce apoptosis by a discrete pathway. If this is the case, gelsolin must therefore block the effects of the Fas-, $C_2$-Cer- and Dex-induced pathways which all result in apoptosis in this model system. Thus, the identification of the target(s) of gelsolin, which appear to be shared by different apoptotic pathways, should make substantial contributions to understanding the global apoptotic machinery.

The data presented here, that at relatively low intracellular levels gelsolin can act as a potent inhibitor of apoptosis induced by different agents, provide new insights into the involvement of actin-regulatory proteins in the modulation of apoptosis. Although we have presented evidence consistent with the idea that gelsolin acts as an inhibitor of apoptotic proteases, further experiments are underway to determine the precise mechanism responsible for gelsolin's anti-apoptotic activity.

## Materials and methods

### *Cell culture and transfection*

Jurkat cells, a lymphoblastoid T-cell line, were maintained in RPMI-1640 medium containing 10% fetal bovine serum. Transfections were carried out with either a human gelsolin expression plasmid LKCG (Cunningham *et al.*, 1991) or an empty vector LK444 (Gunning *et al.*, 1987) using Lipofectin. Stable transfectants were selected in the presence of 1 mg/ml of G418 (Geneticin) to obtain the *gelsolin* transfectants JGF-1–8 and the control transfectants JNF-1–5. The reagents were all purchased from Gibco BRL.

### *Western blot analysis*

Cells were extracted in RIPA buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% deoxycholate, 1 mM EDTA, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). The indicated amounts of protein were separated on 10 or 12% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. The following antibodies were used as primary antibody; anti-human gelsolin monoclonal antibody (GS-2C4, BioMaker), anti-human actin monoclonal antibody (Bohringer Mannheim), anti-Bcl-2 polyclonal rabbit antibody raised against GST–human Bcl-2 fusion protein, anti-human Bcl-$x_L$ polyclonal rabbit antibody (Santa Cruz Biotech.), anti-human Bax polyclonal rabbit antibody (Santa Cruz Biotech.) and anti-human CPP32 monoclonal antibody (Transduction Laboratories). Either peroxidase-conjugated goat F(ab′)$_2$ anti-mouse IgG+M (Jackson ImmunoResearch Lab.) or peroxidase-conjugated goat F(ab′)$_2$ anti-rabbit IgG (TAGO) was used as secondary antibody. Detection of the bound antibodies was performed using the ECL system (Amersham).

### *Fluorescence microscopy and flow cytometry*

F-actin staining of cells was performed as previously described (Phatak *et al.*, 1988), using rhodamine–phalloidin (Molecular Probes, Inc.). Stained cells were examined and photographed with a Bio-Rad MRC1024 Laser Scanning Confocal Imaging System. For cell cycle analysis, ethanol-fixed cells were stained with propidium iodide (100 μg/ml) in the presence of RNase A, then analyzed with a FACScan flow cytometer (Beckton Dickinson). Amounts of surface Fas antigen were determined by FACS analysis using anti-Fas antibody (clone CH-11, Medical and Biological Laboratories) as described (Tepper *et al.*, 1995).

### *Analysis of apoptosis*

Cell viability was determined by trypan blue exclusion, and the existence of apoptotic cells was also confirmed by the appearance of sub-$G_0/G_1$ peak fractions in cell cycle analysis. To induce apoptosis, cells were seeded at $1\times10^6$ /ml into 24-well plates and treated with anti-Fas antibody (CH-11, 100 ng/ml), $C_2$-Cer (100 μM) or Dex (Wako, 200 μg/ml). Cell viability was assessed at the indicated times. For the assessment of dose response with anti-Fas antibody, cells were treated with the antibody at the indicated concentrations, and cell viability was examined after 24 h incubation. DNA fragmentation assay was performed as previously described (Itoh *et al.*, 1993), with the same culture conditions as above. For the experiments with ICE family protease inhibitors, cells were seeded at $1\times10^6$ /ml into 96-well plates and treated as above in the absence or presence of 0.1 or 1 mM tetrapeptide CPP32/apopain inhibitor Ac-DEVD-CHO (Peptide Institute) (Nicholson *et al.*, 1995), or 1 mM ICE inhibitor Ac-YVAD-CHO (Peptide Institute) (Thornberry *et al.*, 1992). The corresponding amounts of vesicles (ethanol or/and dimethylsulfoxide) were added to control wells, with no significant effect on cell viability. After 24 h (anti-Fas or $C_2$-Cer) or 48 h (Dex) incubation, cell viability was assessed. The inhibitors were present from 1 h prior to and throughout the experiments.

### *Measurement of ICE(-like) and CPP32(-like) protease activity*

Cells were treated in 24-well plates as described above and harvested at the indicated times. After washing with phosphate-buffered saline, cells were incubated in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA containing 10 μM digitonin at 37°C for 10 min. After clarification by centrifugation at 15 000 r.p.m. for 10 min, cleared lysate (60 μg of protein) was incubated at 37°C with 50 μM DEVD-MCA (substrate for apopain, Peptide Institute) (Nicholson *et al.*, 1995) for 30 min or YVAD-MCA (substrate for ICE, Peptide Institute) (Thornberry *et al.*, 1992) for 60 min (data not shown for YVAD-MCA). The amounts of released 7-amino-4-methylcoumarin (AMC) were measured with a spectrofluorometer (Perkin-Elmer LS50B) with excitation at 380 nm and emission at 460 nm. One unit was defined as the amount of enzyme required to release 1 pmol of AMC per min at 37°C.

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