J. Biochem. **132**, 53–62 (2002)

Monitoring of Caspase-8/FLICE Processing and Activation upon Fas Stimulation with Novel Antibodies Directed against a Cleavage Site for Caspase-8 and Its Substrate, FLICE-Like Inhibitory Protein (FLIP)[1]

Yuichi Niikura, Takashi Nonaka,[2] and Shinobu Imajoh-Ohmi[3]

Division of Cell Biology and Biochemistry, Department of Basic Medical Science, Institute of Medical Science, The University of Tokyo (IMSUT), 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639

Received March 18, 2002; accepted April 24, 2002

We generated and characterized novel antibodies specific for a cleavage site of human caspase-8/FLICE and its substrate, FLICE-like inhibitory protein (FLIP). The synthetic peptides used as immunogens were CQGDNYQKGIPVETD (#791) and VSEGQLEDSS-LLEVD (#1342), which corresponded to cleaved regions of N-terminal fragments of caspase-8 and FLIP generated by active caspase-8, respectively. Each antibody purified from rabbit antiserum reacted specifically with the immunogen but not with the peptide corresponding to the unproteolyzed form, as assessed by ELISA. *In vitro* cleavage of GST-FLIP by active caspase-8 generated an N-terminal fragment (GST-p43) and a C-terminal one (p12). Consistent with other *in vivo* data, the FLIP cleavage site follows the Asp residue, LEVD$_{376}$GPAMKNVEF, identified on N-terminal sequencing of the p12 fragment. #1342-antibody (#1342-Ab) recognized the GST-p43 fragment but not the uncleaved protein, thus confirming its specificity. When the antibodies were used for immunoblotting, flow cytometry, and confocal laser microscopy, the proteolysis of caspase-8 and FLIP, and the subcellular localization of their digests could be monitored in apoptotic U937 cells. Interestingly, a significant increase in the percentage of cells exhibiting caspase-8 and FLIP cleavage was observed upon Fas stimulation in interferon-γ–treated U937 cells, in which the susceptibility to Fas is extremely enhanced. In contrast, U937 cells treated with vitamin D_3 or all-*trans* retinoic acid showed Fas-resistance, and caspase-8 processing and FLIP cleavage were strongly inhibited. In conclusion, we established a system based on the cleavage site–directed antibodies to monitor the dynamics of caspase-8 processing and activation during apoptosis. Using this system, we found that Fas-susceptibility changes during U937 differentiation occur upstream of caspase-8 processing/activation.

Key words: caspase-8, cleavage site–directed antibody, differentiation, Fas, FLIP.

Limited proteolysis is one of the most important post-translational modifications of proteins. It is essential for the activation of proenzymes, modulation of protein functions, and initiation of protein degradation (*1, 2*). Apoptosis, which plays an essential role in development, homeostasis, and defense in multicellular organisms (*3*), involves the sequential activation of cysteine proteases known as caspases (*4, 5*). Caspases are expressed as inactive proenzymes in living cells and become activated on proteolytic cleavage at sites following specific aspartate residues. The limited proteolysis results in the generation of an active enzyme lacking an N-terminal prodomain and comprising p18 and p10 polypeptides, which form a dimeric $(p10/p18)_2$ complex (*6*). Of the 14 members of the mammalian caspase family, caspase-8 (FLICE/MACH/Mcl6) (*7, 8*) and caspase-10 (Mch4/FLICE2) (*9, 10*) function as initiators of the apoptotic signaling cascade through death receptors such as Fas (CD95/APO-1). Stimulation of Fas by its cognate ligand or a specific agonistic antibody enables recruiting of adapter molecule Fas-associated death domain protein (FADD) (*11, 12*), and of caspase-8 (*7, 8*) to Fas *via* homophilic death domain (DD) and death effector domain (DED) interactions, respectively. Recruitment of caspase-8 to the death-inducing signaling complex (DISC) accelerates its proteolytic activation, leading to the release of the active complex $(p10/p18)_2$ into

[1] This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

[2] Present address: Department of Molecular Neurobiology, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585

[3] To whom correspondence should be addressed. Tel/Fax: +81-3-5449-5311, E-mail: ohmi@ims.u-tokyo.ac.jp

Abbreviations: Ab, antibody; Ac-IETD-CHO, acetyl-Ile-Glu-Thr-Asp-aldehyde; CBB, Coomassie Brilliant Blue; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; ELISA, enzyme-linked immunosorbent assay; FADD, Fas-associated death domain protein; FITC, fluorescein isothiocyanate; FLICE, Fas-associated death domain protein interleukin-1β–converting enzyme; FLIP, FLICE-like inhibitory protein; GST, glutathione *S*-transferase; IFN, interferon-γ; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood monocyte; PI, propidium iodide; RA, all-*trans* retinoic acid; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; VD_3, $1,25\alpha(OH)_2$ vitamin D_3; z-VAD-FMK, carbobenzoxy-Val-Ala-Asp-fluoromethylketone.

the cytosol. They process and activate downstream effector caspases (caspases-3 and -7), which in turn results in the specific cleavage of a number of death substrates that ultimately leads to apoptosis (*13*).

To date, eight proteins have been identified as targets of caspase-8: caspase-3 ($IETD_{175}$) (*4*), caspase-7 ($IQAD_{198}$) (*4*), caspase-8 ($VETD_{374}$) (*4*), caspase-13 ($LEED_{289}$) (*14*), BID ($LQTD_{59}$) (*15, 16*), RIP ($LQLD_{324}$) (*17*), plectin ($ILRD_{2398}$) (*18*), and FLIP ($LEVD_{376}$) (*19–21*). FLIP was identified by different groups and independently called FLIP (*19*), Casper (*20*), FLAME-1 (*21*), CASH (*22*), CLARP (*23*), I-FLICE (*24*), MRIT (*25*), and Usurpin (*26*). At the mRNA level, FLIP exists as multiple spliced variants, but at the protein level only two endogenous forms, $FLIP_{long}$ ($FLIP_L$) and $FLIP_{short}$ ($FLIP_S$), could be detected (*19*). $FLIP_L$, which is structurally similar to caspase-8, contains two DEDs and a caspase-like domain. This domain lacks residues that are important for its catalysis, most notably a cysteine residue within the active site. $FLIP_S$ also contains two DEDs but lacks a caspase-like domain. In some cases, FLIP induces apoptosis when overexpressed, but typically it acts as a competitive inhibitor by preventing the processing of initiator caspases required for their activation (*27, 28*). Many researchers have reported enhancement of FLIP expression in Fas-resistant cells (*29–35*). A previous study in our laboratory showed that human monoblastic U937 cells differentiate into CD11b-positive monocyte/macrophage-like cells upon treatment with interferon-γ (IFN), 1,25α$(OH)_2$ vitamin D_3 (VD_3), and all-*trans* retinoic acid (RA), and that their susceptibility to Fas- and tumor necrosis factor (TNF) receptor–mediated apoptosis changes: IFN-treated cells become highly susceptible, whereas VD_3- and RA-treated cells become Fas-resistant (*36*). In this pathway, caspase-8 processing/activation is an essential step for the initiation of death signaling. Previously, we could not accurately study caspase processing/activation because there was no system for specifically detecting proteolyzed proteins. This difficulty may be circumvented by the use of antibodies that specifically recognize the cleavage sites of proteolyzed fragments. We have studied proteolysis by using the cleavage site-directed antibodies for protein kinase C_{alpha} and calpain (*37–39*).

In this report, we described a novel system based on cleavage site-directed antibodies for monitoring caspase-8 processing and activation during apoptosis. Using this system we found that changes in Fas-susceptibility occur upstream of caspase-8 processing/activation during U937 differentiation.

EXPERIMENTAL PROCEDURES

Materials—The agonistic anti-Fas/CD95 mAb (clone CH-11, IgM) was purchased from MBL and was used at 50 ng/ml throughout this study. Carbobenzoxy-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK) and acetyl-Ile-Glu-Thr-Asp-aldehyde (Ac-IETD-CHO) were obtained from Bachem and the Peptide Institute, respectively. The differentiation agents, and 1,25α $(OH)_2$ vitamin D_3 (VD_3) and all-*trans* retinoic acid (RA) were purchased from Chugai and Sigma, respectively. Recombinant human IFN-γ (IFN) was kindly provided by Shionogi Pharmaceuticals.

Peptide Synthesis and Antibody Preparation—Peptides were synthesized by the solid phase method with an Applied Biosystems Model 430A peptide synthesizer and purified by reverse-phase high performance liquid chromatography on a C18 column. The following antibodies were generated in rabbits using synthetic peptides, as haptens, conjugated with keyhole lympet hemocyamin (KLH), and purified from antisera by affinity chromatography on the immobilized antigen peptides: anti–human FLIP (α-FLIP) against residues 158–191 (HRIDLKTKIQKYKQSVQGAGTSYRNVLQAAIQK), anti–cleaved FLIP (#1342-Ab) against residues 357–371 (VSEGQLEDSSLLEVD), anti–cleaved caspase-8 (#791-Ab) against residues 360–374 (CQGDNYQKGIPVETD), anti–cleaved PARP (#709-Ab) against residues 215–220 (GVDEVA), and anti-PARP (#634-Ab) against residues 215–224 (GVDEVAKKKS).

ELISA—Peptides were immobilized on 96-well plates (MaxiSorp™, Nunc A/S) at a concentration of 1 nmol/50 μl/well. Each antibody (10 ng/100 μl/well) was added to plates that had been blocked by pre-incubation for 2 h in 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) at room temperature. After a wash, the plates were further incubated with peroxidase-conjugated goat anti–rabbit IgG. Color was developed with *o*-phenylenediamine dihydrochloride (OPD) and measured with a Biomek™1000 (BECKMAN) at an absorbance of 490 nm.

Recombinant Proteins—His-tagged caspase-8 lacking the N-terminal prodomain (referred to as His-p30) and GST-FLIP were expressed and purified from *Escherichia coli* strain JM109. In brief, bacteria carrying the expression plasmid (pQE vector, QIAGEN, and pGEX vector, Amersham Pharmacia Biotech AB) were cultured in Luria-Bertani (LB) media containing 50 μg/ml ampicillin at room temperature to the middle log phase, and then treated with 0.1 mM isopropyl-thio-galactopyranoside (IPTG) for 3 h. The cells were collected by centrifugation, disrupted by sonication in TNG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10% glycerol) containing 1% Triton X-100, and then centrifuged. Recombinant proteins in the supernatant were affinity-purified with the use of Ni-NTA Agarose (QIAGEN) or Glutathion-Sepharose™4B (Amersham Pharmacia Biotech AB). Protein concentrations were determined by the Bradford method with serially diluted BSA samples as controls.

In Vitro Cleavage of FLIP—GST-FLIP (1 nmol) immobilized on glutathione-beads was incubated with a similar ratio of His-p30 for 12 h at 37°C. After centrifugation, the precipitate was washed with TNG buffer, and then bound proteins were solubilized with sodium dodecyl sulfate (SDS)–sample buffer containing 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol (2-ME), and 10 μg/ml Bromophenol Blue in 125 mM Tris-HCl, pH 6.8. Unbound proteins were precipitated with 10% (w/v) trichloroacetic acid (TCA). After centrifugation, the precipitates were solubilized with SDS-sample buffer. Each sample was heat-denatured and then subjected to SDS-PAGE, followed by Coomassie Brilliant Blue (CBB) staining or immunoblotting.

Amino-Terminal Sequencing—After the digests had been subjected to SDS-PAGE, they were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon, Millipore), using a semi-dry blotting apparatus (Model BE-300, Bio-Craft), and then CBB stained. The bands were excised and analyzed with a Beckman LF3000 protein sequencer.

Immunoblotting—Cells (1×10^6 per sample) were washed

with PBS and then treated with 10% (w/v) TCA. After centrifugation, the precipitates were sonicated in 100 μl of SDS-sample buffer and then heat-denatured. Samples (corresponding to 1×10^5 cells) were subjected to SDS-PAGE and then transferred onto a PVDF membrane. The membrane was soaked in tris-buffered saline (TBS) containing 20 mg/ml BSA for 1 h at room temperature and then incubated with antibodies overnight at 4°C. After being washed to remove excess antibody, the membrane was incubated with anti–rabbit or anti–mouse IgG conjugated to alkaline

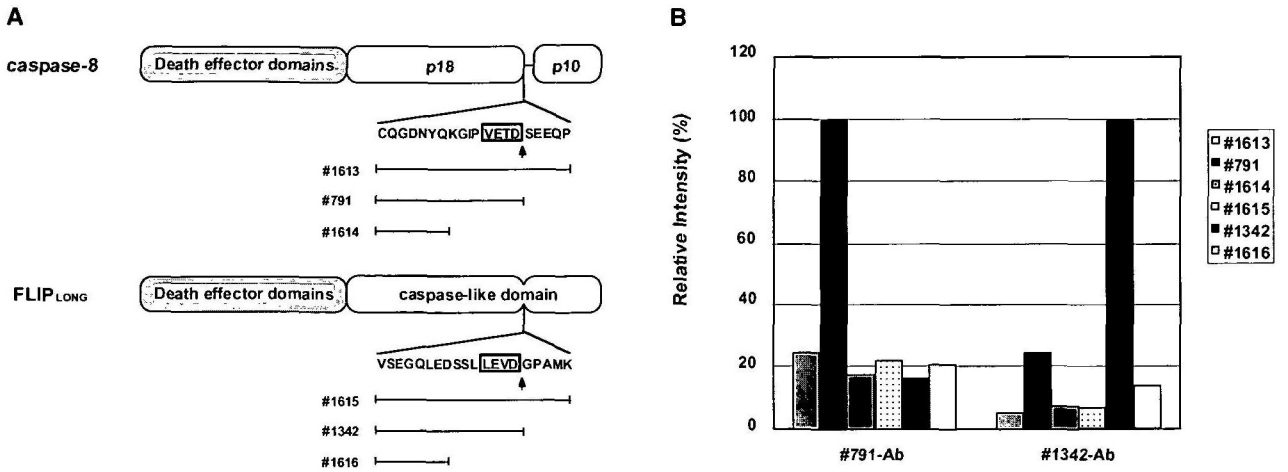

Fig. 1. **Antibodies specific for the cleavage site of caspase-8 (#791-Ab) and FLIP (#1342-Ab).** (A) Schematic representation of human caspase-8/FLICE and FLICE-like inhibitory protein (FLIP). Below the structure of each molecule, the three species of synthetic peptides used in this study are shown. The amino acid sequence of each peptide is indicated in one-letter abbreviations. The arrow following the Asp (D) residue within the box indicates the cleavage site generated by active caspase-8. (B) Specificity of the antibodies for a series of synthetic peptides. The affinity for each peptide is expressed as relative intensity in comparison with the immunogen peptide intensity (100%).

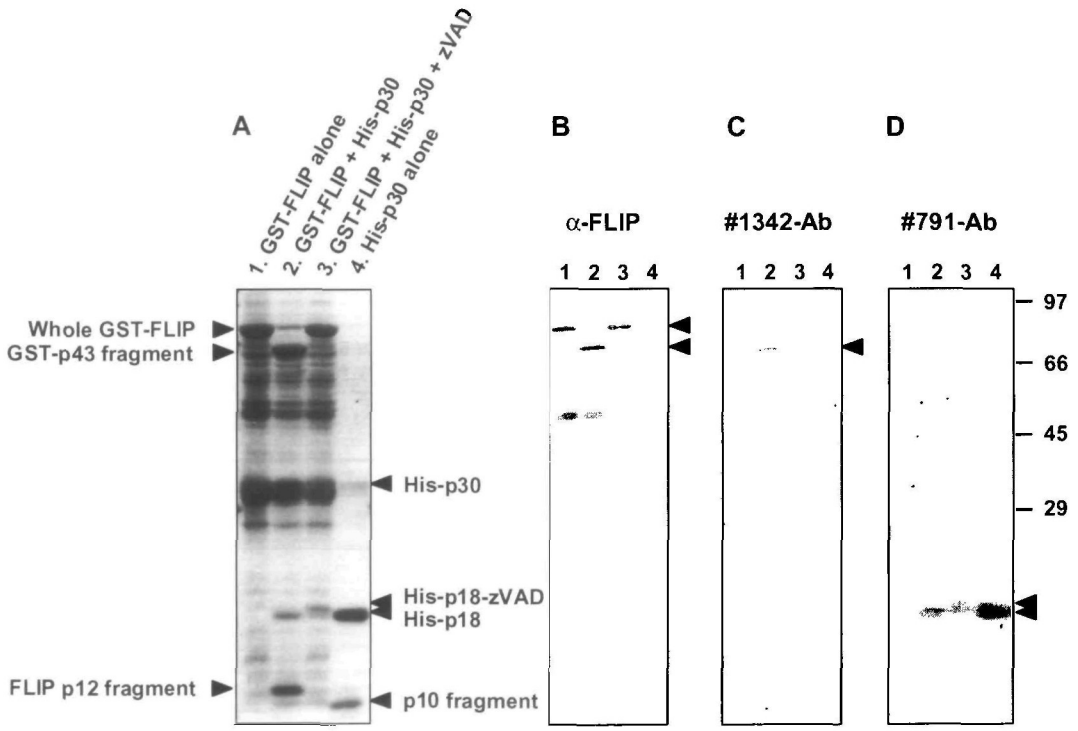

Fig. 2. ***In vitro* cleavage of recombinant FLIP by active caspase-8.** (A) GST-FLIP immobilized on glutathione-beads was incubated with purified caspase-8 (His-p30) expressed in *E. coli* or inactive caspase-8 obtained by prior incubation in the presence of a pan-caspase inhibitor, z-VAD-FMK, as described under "EXPERIMENTAL PROCEDURES." The digests were subjected to SDS-PAGE and visualized by CBB staining. (B–D) Specificity of the antibodies against FLIP and caspase-8 fragments. Immunoblot staining was performed with (B) α-FLIP, (C) #1342-Ab, and (D) #791-Ab. Lane 1, GST-FLIP alone; lane 2, GST-FLIP treated with His-p30; lane 3, GST-FLIP treated with His-p30 in the presence of caspase inhibitor zVAD; and lane 4, His-p30 alone. The positions of molecular mass standards (in kDa) are shown.

phosphatase (Promega). Antigens were visualized by means of the enzymatic reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT).

Cell Culture and Conditions for Differentiation—Human monoblastic U937 cells and human T lymphoblastoid Jurkat cells (provided by the Japanese Cancer Research Resources Bank) were maintained in RPMI-1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 200 μg/ml streptomycin at 37°C under a humidified atmosphere of 5% (v/v) CO_2. For U937 differentiation, cells (less than 2×10^5 cells/ml) were treated with IFN-γ (100 U/ml), VD_3 (10 nM), or RA (1 μM) for 3 days, except where indicated. Differentiation was assessed as CD11b expression by flow cytometric analysis with anti-CD11b mAb (DAKO A/S).

Flow Cytometry and Confocal Laser Microscopy—Cells (5×10^5) were washed with PBS and then fixed with 2% (w/v)

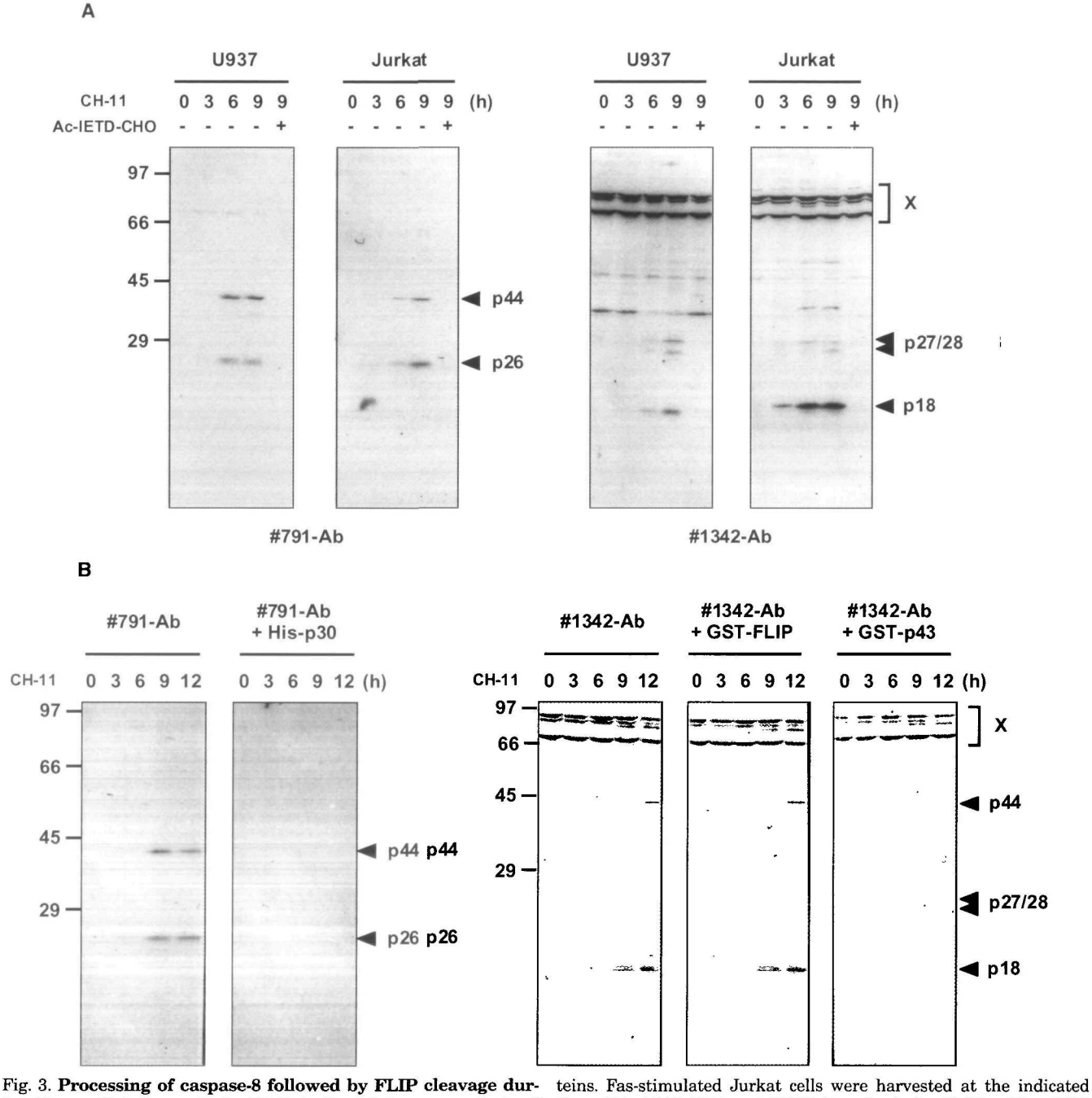

Fig. 3. **Processing of caspase-8 followed by FLIP cleavage during Fas-mediated apoptosis.** (A) Detection of cleavage products of caspase-8 and FLIP. Fas-stimulated U937 and Jurkat cells were harvested at the indicated times (0 to 9 h). Cleavage fragments of caspase-8 and FLIP were visualized with #791- and #1342-Abs, respectively. (B) Adsorption of the antibodies with recombinant proteins. Fas-stimulated Jurkat cells were harvested at the indicated times (0 to 12 h). #791- and #1342-Abs adsorbed with His-p30 or GST-FLIP/GST-p43 were used. X denotes proteins cross-reacting with #1342-Ab. The positions of molecular mass standards (in kDa) are shown.

paraformaldehyde for 30 min at room temperature. They were then washed twice with PBS and permeabilized with PBS containing 0.2% (w/v) Triton X-100. Samples were centrifuged, and the precipitates were resuspended in 100 μl of PBS containing 0.1% (w/v) Triton X-100 and 3% (w/v) human γ-globulin (Jackson ImmunoResearch Laboratories), and then incubated for 10 min on ice. After centrifugation, the precipitates were incubated with rabbit antibodies for 2 h on ice and then labeled with FITC-conjugated goat anti–rabbit IgG (TAGO, Inc.) for 1 h on ice. After being washed with PBS, samples were suspended in PBS containing 30 μg/ml propidium iodide (PI) and then analyzed with a FACScan flow cytometer (Becton Dickinson). The results were calculated with CELLQuest software (Becton Dickinson). The percentage of stained cells was calculated as follows: 100× [fluorescent-positive cells /(fluorescent-positive cells + negative cells)]. The remaining samples were placed on coverslips, and analyzed by confocal laser microscopy (Bio-Rad).

RESULTS

Characterization of Cleavage Site–Directed Antibodies—To investigate proteolysis in apoptotic cells, we developed a system based on the ability of antibodies to recognize a proteolyzed site on caspases and their substrates. Here, we generated antibodies for human caspase-8/FLICE and its substrate, FLIP. Figure 1A presents their structures. Antibodies were purified from rabbit antiserum, and their specificity was determined by ELISA. The synthetic peptides used were as follows: #1613, #791, and #1614, corresponding to uncleaved caspase-8, the cleaved caspase-8 fragment and the N-terminal half of the cleaved fragment, respectively. Similarly, #1615, #1342, and #1616 were designed for FLIP. The affinity of the antibodies for each peptide was expressed as relative intensity compared with that in the case of the immunogen (100%). As shown in Fig. 1B, #791-antibody (#791-Ab) exhibited high affinity for the immunogen #791 peptide but hardly reacted with the #1613 and #1614 peptides (25 and 18%, respectively). Similar results were obtained with #1342-Ab, which showed strong specificity for the #1342 peptide but not for the #1615 and #1616 peptides (7 and 14%, respectively). Of note was that both antibodies did not cross-react with the other's immunogen peptide. These data indicate that both antibodies exhibit high specificity for their immunogen peptides.

In Vitro Cleavage of FLIP—For further characterization of the antibodies, we examined their reactivity toward caspase-8 and FLIP cleaved *in vitro*. Active caspase-8 was expressed in *E. coli* because the caspase zymogen possesses intrinsic proteolytic activity, which allows for auto-processing and activation *in situ*. Almost all His-p30 caspase-8 constructs, which lack the N-terminal prodomain, were converted into 18- and 10-kDa fragments, corresponding to the active form (Fig. 2A, lane 4). Immunoblot analysis showed that #791-Ab reacted specifically with a 18-kDa fragment, indicating that #791-Ab not only recognizes the immunogen peptide but also the processed intact caspase-8 (Fig. 2D). Alternatively, recombinant FLIP was expressed as a GST fusion protein and migrated as an 80-kDa band on SDS-PAGE (Fig. 2A, lane 1). Treatment with active caspase-8 resulted in the breakdown of more than 90% of the GST-FLIP constructs into a 68-kDa fragment that corresponded to the GST-p43 fragment of FLIP (Fig. 2A, lane 2). Both the 80- and 68-kDa proteins were detected by α-

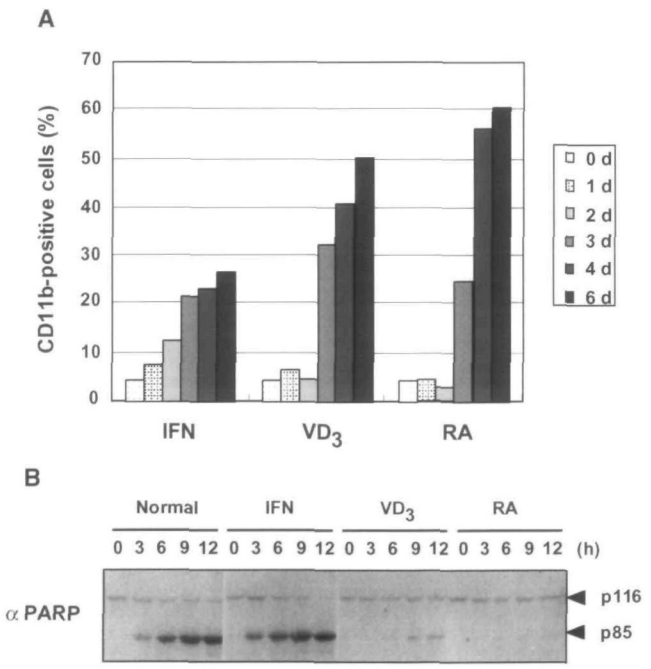

Fig. 4. **U937 cells differentiation caused by interferon-γ (IFN), 1,25α(OH)$_2$ vitamin D$_3$ (VD$_3$), and all-*trans* retinoic acid (RA).** (A) Induction of CD11b on the cell surface during U937 cell differentiation. U937 cells treated with IFN (100 U/ml), VD$_3$ (10 nM), or RA (1 μM) were probed with anti-CD11b mAb followed by FITC-conjugated anti–mouse IgG, and then analyzed with a FACScan as described under "EXPERIMENTAL PROCEDURES." (B) Changes in the Fas death pathway in differentiated U937 cells. Differentiated U937 cells were stimulated with agonistic anti-Fas antibodies (CH-11, 50 ng/ml) and then harvested at different times (0 to 12 h). Cleavage of PARP was used as a biochemical hallmark of apoptosis. Whole PARP (116 kDa) and its cleaved fragment (85 kDa) were stained with anti-PARP Ab, which exhibits higher affinity for the 85-kDa fragment than for the whole molecule.

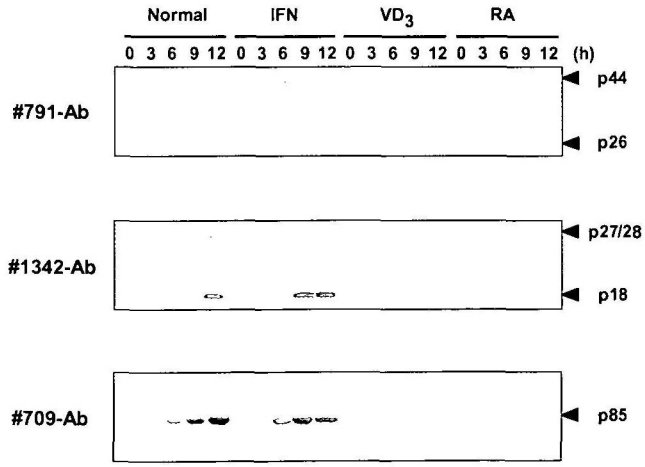

Fig. 5. **Enhanced or suppressed caspase-8 activation upon Fas stimulation in differentiated U937 cells.** Cleavage fragments of caspase-8, FLIP, and PARP were detected with antibodies specific for the cleavage site on each molecule (#791-, #1342-, and #709-Abs, respectively). The samples here are the same as those in Fig. 4B.

FLIP, which recognizes the N-terminal DED that is present in all reported isoforms (Fig. 2B). The GST-p43 fragment, but not GST-FLIP, was recognized specifically by #1342-Ab, thus confirming the antibody's specificity (Fig. 2C). As shown in Fig. 2A, lanes 2 and 3, the appearance of the 12-kDa fragment coincided with down-regulation of GST-FLIP, which was effectively suppressed by z-VAD-FMK, a broad spectrum caspase inhibitor. A FLIP cleavage site has been proposed from the following findings: (i) the molecular masses of the cleavage products, p43 N-terminal and p12 C-terminal fragments; (ii) the existence of (V/L/I)(E/Q)XD_{376}, a preferred sequence for caspase-8, within the equivalent region of the caspase-8 cleavage site; and (iii) prevention of FLIP cleavage on substitution of Asp_{376} with Ala. In our study, the N-terminal sequence of the fragment was determined to be GPAMKNVEF, directly indicating that the cleavage site of FLIP is situated after the Asp residue, $LEVD_{376}$GPAMKNVEF (data not shown). Taken together these data support previous immunoblot results obtained with #1342-Ab. Interestingly, the p12 fragment co-precipitated with the GST-p43 immobilized on glutathione-beads, showing that the p12 fragment binds to the N-terminal fragment of FLIP. Furthermore, 10% of the input caspase-8 co-precipitated with the GST-p43/p12 complex, as determined by CBB staining and immunoblotting. These results are consistent with the previous finding that the caspase-like domain alone is sufficient for interaction with full length caspase-8 (*27, 29, 31*). This raises the possibility that FLIP regulates caspase-8 activity by forming a heterogeneous complex.

Detection of Caspase-8 Processing and Activation in Apoptotic Cells—To monitor caspase-8 activation in apoptotic cells, a time-course study was performed (Fig. 3). Human monoblastic U937 and Jurkat T cells treated with agonistic anti-Fas mAb (CH-11, 50 ng/ml) were harvested at different time points. Cleavage of caspase-8 and FLIP was detected by using #791- and #1342-Ab, respectively. The predominant expression of the long form of FLIP was confirmed with α-FLIP (data not shown). Upon Fas triggering, the release of the 44- and 26-kDa products of caspase-8 was first detectable by 3–6 h of stimulation in both types of cell (Fig. 3A, left). The 44-kDa band represents a caspase-8 missing a C-terminal p10 fragment. The 26-kDa product was presumably derived from the 44-kDa fragment upon further proteolytic cleavage. Unexpectedly, the p18 fragment of caspase-8 was not detected in either type of cell. This suggests that partially proteolyzed p44 and p26 are sufficient to initiate the apoptotic signaling cascade, although rapid degradation of p18 may also be responsible for this effect. Alternatively, the 27-/28- and 18-kDa bands appeared with #1342-Ab, but the 43-kDa band was occasionally detected for Jurkat T cells (Fig. 3A, right). These data do not agree with the results on *in vitro* experiments showing the 43-kDa fragment was a major product. However, the unexpected species detected with #1342-Ab disappeared with the use of polyclonal antibodies adsorbed with recombinant FLIP cleaved by caspase-8 but not the uncleaved form, demonstrating that p18, p27, and p28 were cleavage products of FLIP (Fig. 3B). In the presence of Ac-IETD-CHO, a caspase-8 tropic inhibitor, processing of caspase-8 was inhibited completely, thus preventing FLIP cleavage. These findings show that FLIP is a good substrate for caspase-8 *in vivo* as well as *in vitro*. Therefore, caspase-8 activation may be understood by studying FLIP

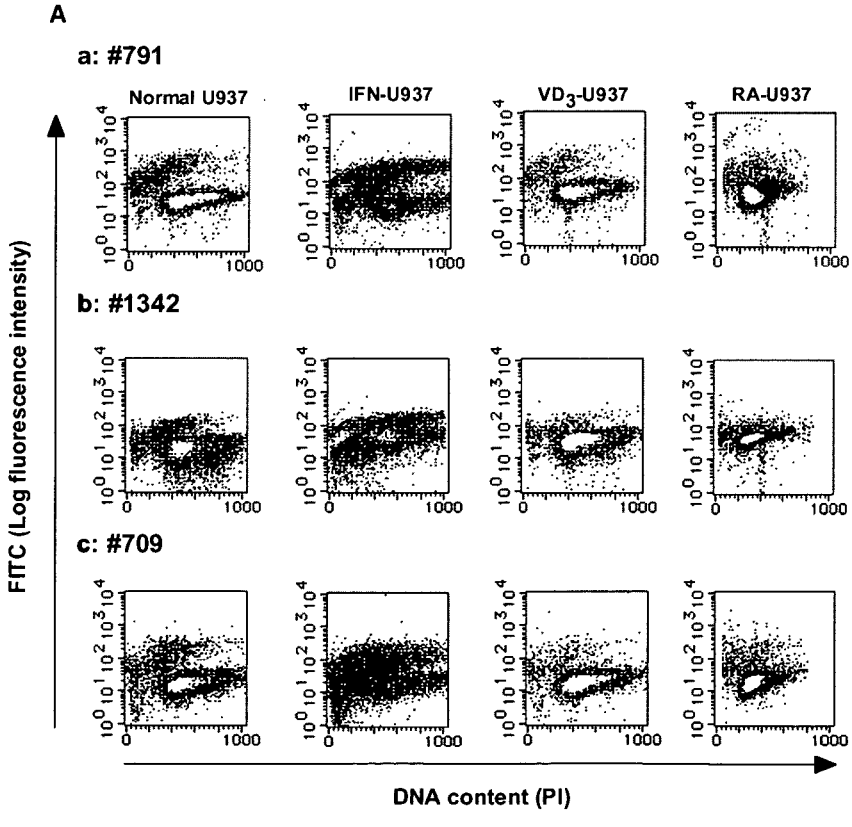

Fig. 6. **Quantitative analysis of the cleavage of caspase-8, FLIP, and PARP in differentiated U937 cells.** (A) Cells were treated with CH-11 (50 ng/ml) for 9 h, followed by staining with (a) #791-Ab, (b) #1342-Ab, or (c) #709-Ab. Ten thousand cells were analyzed with a FACScan flow cytometry instrument with CELLQuest software. (B) The percentage of antibody-reactive cells was calculated as described under "EXPERIMENTAL PROCEDURES." The data represent the averages of three independent experiments.

cleavage with the use of #1342-Ab.

Changes to the Fas Death Pathway in Differentiated U937 Cells—We previously reported that IFN, VD_3, and RA cause the differentiation of U937 cells into CD11b-positive monocyte/macrophage-like cells, and change their susceptibility to Fas-mediated apoptosis (*36*). Figure 4A shows the percentage of CD11b-positive cells detected during U937 differentiation with these reagents. Cells treated with IFN (100 U/ml), VD_3 (10 nM), or RA (1 μM) were probed with anti-CD11b mAb and then analyzed with a FACScan flow cytometer. IFN increased the percentage of CD11b-positive cells after day 1 of treatment, there being 27% CD11b-positive cells on day 6. On the other hand, VD_3 and RA treatment induced CD11b expression after day 3, there being 51 and 61% CD11b-positive cells on day 6, respectively. For further analysis, we studied differentiated cells on day 3 because they showed more typical features of differentiated cells. Moreover, prolonged incubation in the presence of RA induces terminal differentiation followed by apoptotic cell death (data not shown). Figure 4B shows PARP cleavage in response to Fas triggering in differentiated cells. Upon Fas stimulation, the 85-kDa cleavage product of PARP appeared in normal and IFN-U937 cells, but was not detected in VD_3- and RA-U937 cells. Interestingly, down-regulation of PARP was accelerated in IFN-U937 cells. These findings are consistent with our previous data as to apoptotic morphology changes (*36*).

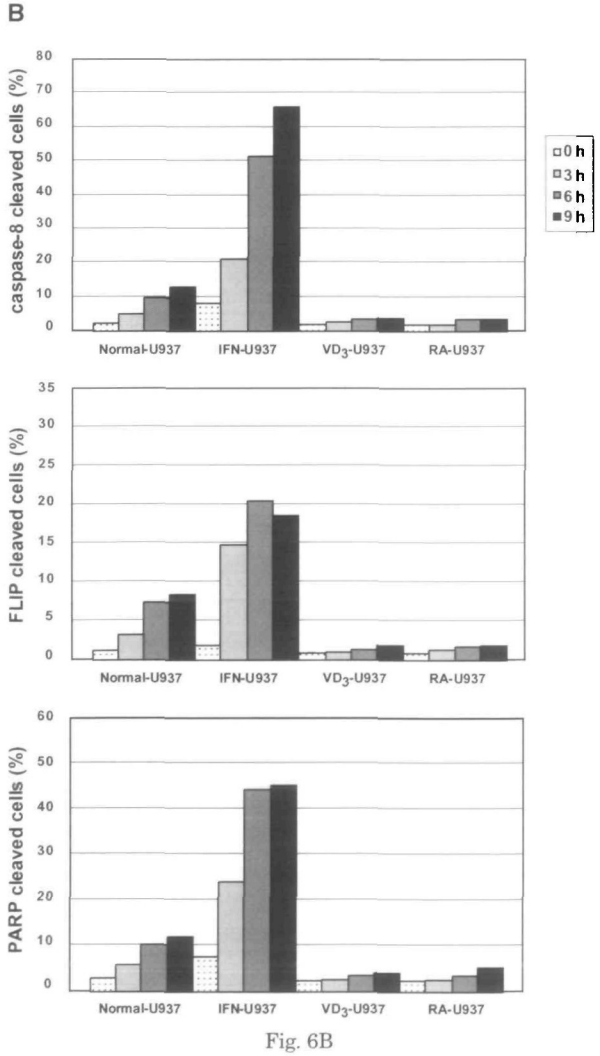

Fig. 6B

Abrogation of Caspase-8 Activation in VD_3- and RA-U937 Cells—To understand the changes in Fas-sensitivity that occur during U937 differentiation, we examined whether or not engagement of Fas was followed by the processing and activation of caspase-8. The zymogen form of caspase-8 was constitutively expressed in each differentiated cell type and the processed form was not detected before Fas stimulation (data not shown). In normal and IFN-U937 cells the processed products, p44 and p26, appeared in response to Fas triggering (Fig. 5, upper). Interestingly, caspase-8 processing was accelerated in IFN-U937 cells compared with in normal cells, thus indicating high susceptibility to Fas-mediated apoptosis in this treatment group. In parallel, a significant increase in FLIP cleavage was observed in IFN-U937 cells (Fig. 5, middle). In contrast, neither caspase-8 processing nor FLIP cleavage was detected in Fas-resistant VD_3- and RA-U937 cells, indicating that the Fas-susceptibility is regulated upstream of caspase-8 processing/activation.

To monitor caspase-8 activation *in situ*, Fas-stimulated cells were analyzed by FACScan flow cytometry (Fig. 6, A and B). Cells showing reactivity to #791-, #1342-, and #709-Abs were present among normal and differentiated U937 cells. Unexpectedly, ΔFITC (the difference in fluorescence intensity between antibody-reactive and non-reactive cells) did not differ between normal and differentiated cells, irrespective of any difference in Fas-susceptibility. Cleavage of caspase-8, FLIP, and PARP after 9 h of stimulation was observed in a large population of IFN-U937 cells (66, 18, and 45%, respectively) in comparison with in normal cells (13, 9, and 12%). However, cleavage of the three was only observed in a small population of VD_3-U937 cells (4, 2, and 4%, respectively) and RA-U937 cells (4, 2, and 5%, respectively). Therefore, the accelerated apoptosis observed in IFN-U937 cells is attributable to an increase in the population of caspase-activating cells but not to enhancement of proteolysis in a cell.

Subcellular Localization of Proteolyzed Caspase-8 and FLIP—The limited proteolysis of caspases and their death substrates may occur anywhere in a cell (*e.g.* membrane, cytosol, cytoskeleton, nucleus, and several organelles). However, changes in the localization of these fragments affect death signaling. It is necessary to monitor digests to understand the functions of the signaling molecules involved in proteolysis. We monitored the fate of the proteolytic fragments of caspase-8 and FLIP in normal and differentiated U937 cells after Fas triggering. Figure 7 shows the subcellular distribution of the cleavage products of caspase-8 and FLIP under a confocal laser microscope. Signals generated with #791- and #1342-Abs were absent in cells prior to Fas triggering (data not shown). After 6 h of stimulation, there was positive #791-Ab reactivity throughout the cytoplasm of apoptotic U937 cells, in which nuclear fragmentation also appeared (Fig. 7a). Thus, the p44 and p26 fragments observed on immunoblot analysis are released from DISC and may activate downstream caspases to transmit extracellular signals to the cytosol along an orchestrated proteolysis pathway. In this process, the digests of FLIP, p27/28 and p18 appeared throughout the cytosol in response to Fas stimulation, but whether the

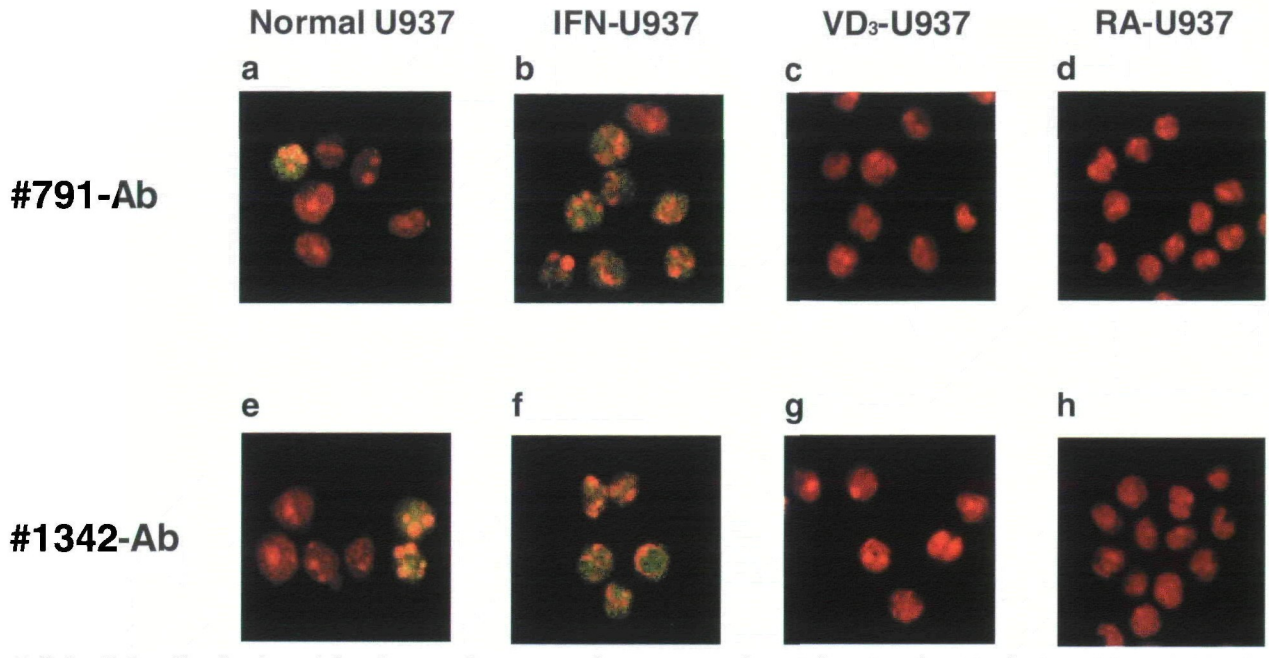

Fig. 7. **Subcellular distribution of the cleavage fragments of caspase-8 and FLIP.** Samples after 6 h of culture (shown in Fig. 6) were placed on coverslips, and then the immunofluorescent signals (green) with #791- and #1342-Abs were examined under a confocal microscope. PI (red) stained the nucleus.

cleavage of FLIP occurs within DISC or the cytosol in apoptotic U937 cells remains unknown (Fig. 7e). The cytoplasmic localization of caspase-8 and FLIP digests was observed in a large population of IFN-U937 cells stimulated with Fas antigen (Fig. 7, b and f), but was not detected in VD_3- and RA-U937 cells (Fig. 7, c and g, and d and h, respectively). These data are consistent with those obtained on immunoblot and FACScan analysis.

DISCUSSION

A cleavage site-directed antibody is defined as an antibody specific to the terminal region of a digest generated from the precursor or native protein by limited proteolysis. Most importantly, the antibody does not recognize the same sequence within the unproteolyzed form. Our study consisted of three parts: (i) production of cleavage site-directed antibodies, (ii) verification of antibody reactivity, and (iii) application of the antibodies to *in situ* analysis.

The quality of a cleavage site-directed antibody depends on the length of the peptide used as the immunogen. Presumably, a difference in the ternary structure between short and long peptides is responsible. When long peptides are used, the antibody recognizes both proteolyzed and unproteolyzed forms because of the existence of two epitopes within the immunogen, the cleavage site and proximal region. Both the #791- and #1342-peptides are 15 amino acids long, with 4 and 5 charged residues (Lys, Asp, or Glu), respectively. Antibodies against these peptides exhibited tight specificity for the cleavage site but not for its proximal sequence. In addition, they recognized intact fragments cleaved by caspase-8 *in vivo* and *in vitro*. Importantly, both #791- and #1342-Ab did not cross-react with the other's immunogen peptide. Although FLIP exhibits strong similarity to caspase-8 in amino acids, the sequences around a cleavage site within caspase-8 and FLIP are quite different, indicating a subsequent difference in the ternary structure around their cleavage sites. Therefore, the ternary structure may dictate the specificity of antibodies for the cleaved regions of their respective immunogens.

Several independent reports have shown that overexpression of FLIP in certain cell lines results in the appearance of p43, a fragment generated on cleavage after Asp_{376}. However, the major cleavage products of FLIP are p27/28 and p18 in U937 and Jurkat cells. This should be true for other cells because ectopic FLIP is usually expressed as a Flag-tag protein and is detected by anti-Flag Ab. Therefore, p26/27 and p18 fragments lacking the Flag-tag would be undetectable. Another report noted that endogenous and ectopically expressed FLIP is recruited into DISC in response to Fas triggering, where p43 is the only fragment of FLIP detected (*27*). This finding is explained by the fact that p43 possesses two functional DEDs to interact with DISC components, whereas p27/28 and p18 lack one or both DEDs. In fact, p27/28 and p18 fragments were observed in the cytoplasm but not on the cell surface of apoptotic U937 cells.

Contrary to *in vivo* results, only the p43 fragment of FLIP was observed *in vitro*, and neither p18/27/28 fragments nor non-specifically cleaved fragments were detected, even upon the addition of a large amount of active caspase-8. However, in a cytosolic extract of untreated U937 cells, endogenous FLIP was completely cleaved into a p18 fragment after the addition of dATP and cytochrome *c* (unpublished data). These results indicate that active caspase-8 recognizes $LEVD_{376}$ within FLIP and that additional factor(s) existing in the cytosol are required for complete cleavage of FLIP. Our present *in vitro* and *in vivo* experiments suggest that FLIP cleavage by caspase-8 with unknown factors results in the removal of an N-terminal

prodomain, and the generation of p18 and p12 fragments, which are topologically equivalent to the p18 and p10 fragments of active caspase-8. They form heterogeneous complexes *in vitro*, as shown in Fig. 2, and perhaps *in vivo*, but whether this process is responsible for caspase-8 inhibition is unclear.

Our previous paper was the first to report the relation between phagocyte differentiation and apoptosis resistance (*36*). In support of our findings, other researchers have since reported Fas-resistance in peripheral blood monocyte (PBMC)-derived macrophages (Mϕ) (*29*), dendritic cells (DC) (*30*), and natural killer cells (NK) (*31*). Moreover, cytotoxic and helper T lymphocytes (*32, 40*), B lymphocytes (*33*), and mast cells (*34*), which play an essential role in cell-mediated and humoral immune responses, have been reported to acquire Fas-resistance through cell activation. In most cases, FLIP is believed to be a key molecule for Fas-resistance because enhanced expression of $FLIP_L$ or $FLIP_S$ in response to differentiation or activation coincides with protection from Fas-induced cell death. In U937 cells, the expression of $FLIP_L$ is strongly up-regulated in response to differentiation stimulation by RA, which is consistent with PBMC-derived Mϕ (unpublished data). However, no FLIP cleavage was observed in RA-U937 cells, indicating that FLIP does not function as a dominant negative for caspase-8 in these cells.

RA and VD_3 are known to induce $p21^{Cip1/WAF1}$, which inhibits cell-cycle progression by binding to G_1 cyclin/CDK complexes and proliferating cell nuclear antigen (PCNA) through its N- and C-terminal domains, respectively. Interestingly, overexpression of the CDK-inhibitory domain but not the PCAN-binding domain of p21 blocks TNF-related apoptosis inducing ligand (TRAIL)-mediated apoptosis, in which the processing of caspase-8 is completely inhibited (*41*). Furthermore, cytoplasmic p21 interacts with caspase-3 to prevent Fas-mediated apoptosis (*42*). The findings mentioned above are consistent with our finding of G_0/G_1 arrest in Fas-resistant VD_3- and RA-U937 cells, but Fas-hypersensitivity in prolific IFN-U937 cells. Therefore, we should address the relation between the cell cycle, differentiation and apoptosis resistance in detail.

Our antibodies proved to be powerful tools for investigating caspase-8 activation *in situ*. The results obtained on flow cytometry and confocal laser microscopy provide us with plenty of quantitative and locative information of high quality. Potential applications of these techniques include analysis of embryos and surgical specimens to reveal novel functions of caspase-8 or FLIP in development and disease. The preparation of a series of characteristic antibodies against other caspases and substrates is under way in our laboratories.

We wish to thank Nozomi Ichikawa for the technical assistance in the peptide synthesis.

REFERENCES

1. Bond, J.S. and Butler, P.E. (1987) Intracellular proteases. *Annu. Rev. Biochem.* **56**, 333–364
2. Neurath, H. (1989) Proteolytic processing and physiological regulation. *Trends Biochem. Sci.* **14**, 268–271
3. Los, M., Wesselborg, S., and Schulze-Osthoff, K. (1999) The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice. *Immunity* **10**, 629–639
4. Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem. J.* **326**, 1–16
5. Nagata, S. (1997) Apoptosis by death factor. *Cell* **88**, 355–365
6. Rotonda, J., Nicholson, D.W., Fazil, K.M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E.P., Rasper, D.M., Ruel, R., Vaillancourt, J.P., Thornberry, N.A., and Becker, J.W. (1996) The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat. Struct. Biol.* **3**, 619–625
7. Boldin, M.P., Goncharov, T.M., Goltsev, Y.V., and Wallach, D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85**, 803–815
8. Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E., and Dixit, V.M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85**, 817–827
9. Fernandes-Alnemri, T., Armstrong, R.C., Krebs, J., Srinivasula, S.M., Wang, L., Bullrich, F., Fritz, L.C., Trapani, J.A., Tomaselli, K.J., Litwack, G., and Alnemri, E.S. (1996) In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA* **93**, 7464–7469
10. Vincenz, C. and Dixit, V.M. (1997) Fas-associated death domain protein interleukin-1β-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling. *J. Biol. Chem.* **272**, 6578–6583
11. Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H., and Wallach, D. (1995) A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.* **270**, 7795–7798
12. Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81**, 505–512
13. Medema, J.P., Scaffidi, C., Kischkel, F.C., Shevchenko, A., Mann, M., Krammer, P.H., and Peter, M.E. (1997) FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J.* **16**, 2794–2804
14. Humke, E.W., Ni, J., and Dixit, V.M. (1998) ERICE, a novel FLICE-activatable caspase. *J. Biol. Chem.* **273**, 15702–15707
15. Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**, 491–501
16. Gross, A., Yin, X.M., Wang, K., Wei, M.C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S.J. (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. *J. Biol. Chem.* **274**, 1156–1163
17. Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z.G. (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev.* **13**, 2514–2526
18. Stegh, A.H., Herrmann, H., Lampel, S., Weisenberger, D., Andra, K., Seper, M., Wiche, G., Krammer, P.H., and Peter, M.E. (2000) Identification of the cytolinker plectin as a major early in vivo substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis. *Mol. Cell. Biol.* **20**, 5665–5679
19. Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E., and Tschopp, J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190–195
20. Shu, H.B., Halpin, D.R., and Goeddel, D.V. (1997) Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* **6**, 751–763
21. Srinivasula, S.M., Ahmad, M., Ottilie, S., Bullrich, F., Banks, S., Wang, Y., Fernandes-Alnemri, T., Croce, C.M., Litwack, G., Tomaselli, K.J., Armstrong, R.C., and Alnemri, E.S. (1997) FLAME-1, a novel FADD-like anti-apoptotic molecule that reg-

ulates Fas/TNFR1-induced apoptosis. *J. Biol. Chem.* **272**, 18542–18545
22. Goltsev, Y.V., Kovalenko, A.V., Arnold, E., Varfolomeev, E.E., Brodianskii, V.M., and Wallach, D. (1997) CASH, a novel caspase homologue with death effector domains. *J. Biol. Chem.* **272**, 19641–19644
23. Inohara, N., Koseki, T., Hu, Y., Chen, S., and Nunez, G. (1997) CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc. Natl. Acad. Sci. USA* **94**, 10717–10722
24. Hu, S., Vincenz, C., Ni, J., Gentz, R., and Dixit, V.M. (1997) I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD- 95-induced apoptosis. *J. Biol. Chem.* **272**, 17255–17257
25. Han, D.K., Chaudhary, P.M., Wright, M.E., Friedman, C., Trask, B.J., Riedel, R.T., Baskin, D.G., Schwartz, S.M., and Hood, L. (1997) MRIT, a novel death-effector domain-containing protein, interacts with caspases and $BclX_L$ and initiates cell death. *Proc. Natl. Acad. Sci. USA* **94**, 11333–11338
26. Rasper, D.M., Vaillancourt, J.P., Hadano, S., Houtzager, V.M., Seiden, I., Keen, S.L., Tawa, P., Xanthoudakis, S., Nasir, J., Martindale, D., Koop, B.F., Peterson, E.P., Thornberry, N.A., Huang, J., MacPherson, D.P., Black, S.C., Hornung, F., Lenardo, M.J., Hayden, M.R., Roy, S., and Nicholson, D.W. (1998) Cell death attenuation by 'Usurpin,' a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell. Death Differ.* **5**, 271–288
27. Scaffidi, C., Schmitz, I., Krammer, P.H., and Peter, M.E. (1999) The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* **274**, 1541–1548
28. Krueger, A., Schmitz, I., Baumann, S., Krammer, P.H., and Kirchhoff, S. (2001) Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. *J. Biol. Chem.* **276**, 20633–20640
29. Perlman, H., Pagliari, L.J., Georganas, C., Mano, T., Walsh, K., and Pope, R.M. (1999) FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. *J. Exp. Med.* **190**, 1679–1688
30. Willems, F., Amraoui, Z., Vanderheyde, N., Verhasselt, V., Aksoy, E., Scaffidi, C., Peter, M.E., Krammer, P.H., and Goldman, M. (2000) Expression of c-FLIP(L) and resistance to CD95-mediated apoptosis of monocyte-derived dendritic cells: inhibition by bisindolylmaleimide. *Blood* **95**, 3478–3482
31. Haux, J., Johnsen, A.C., Steinkjer, B., Egeberg, K., Sundan, A., and Espevik, T. (1999) The role of interleukin-2 in regulating the sensitivity of natural killer cells for Fas-mediated apoptosis. *Cancer Immunol. Immunother.* **48**, 139–146
32. Kirchhoff, S., Muller, W.W., Krueger, A., Schmitz, I., and Krammer, P.H. (2000) TCR-mediated up-regulation of $c\text{-}FLIP_{short}$ correlates with resistance toward CD95-mediated apoptosis by blocking death-inducing signaling complex activity. *J. Immunol.* **165**, 6293–6300
33. Hennino, A., Berard, M., Casamayor-Palleja, M., Krammer, P.H., and Defrance, T. (2000) Regulation of the Fas death pathway by FLICE-inhibitory protein in primary human B cells. *J. Immunol.* **165**, 3023–3030
34. Yoshikawa, H., Nakajima, Y., and Tasaka, K. (2000) Enhanced expression of Fas-associated death domain-like IL-1-converting enzyme (FLICE)-inhibitory protein induces resistance to Fas-mediated apoptosis in activated mast cells. *J. Immunol.* **165**, 6262–6269
35. French, L.E. and Tschopp, J. (1999) Inhibition of death receptor signaling by FLICE-inhibitory protein as a mechanism for immune escape of tumors. *J. Exp. Med.* **190**, 891–894
36. Kikuchi, H., Iizuka, R., Sugiyama, S., Gon, G., Mori, H., Arai, M., Mizumoto, K., and Imajoh-Ohmi, S. (1996) Monocytic differentiation modulates apoptotic response to cytotoxic anti-Fas antibody and tumor necrosis factor α in human monoblast U937 cells. *J. Leukoc. Biol.* **60**, 778–783
37. Kikuchi, H., Imajoh-Ohmi, S., and Kanegasaki, S. (1993) Novel antibodies specific for proteolyzed forms of protein kinase C: production of anti-peptide antibodies available for in situ analysis of intracellular limited proteolysis. *Biochim. Biophys. Acta* **1162**, 171–176
38. Kikuchi, H. and Imajoh-Ohmi, S. (1995) Antibodies specific for proteolyzed forms of protein kinase C α. *Biochim. Biophys. Acta* **1269**, 253–259
39. Kato, M., Nonaka, T., Maki, M., Kikuchi, H., and Imajoh-Ohmi, S. (2000) Caspases cleave the amino-terminal calpain inhibitory unit of calpastatin during apoptosis in human Jurkat T cells. *J. Biochem.* **127**, 297–305
40. Varadhachary, A.S., Peter, M.E., Perdow, S.N., Krammer, P.H., and Salgame, P. (1999) Selective up-regulation of phosphatidylinositol 3′-kinase activity in Th2 cells inhibits caspase-8 cleavage at the death-inducing complex: a mechanism for Th2 resistance from Fas-mediated apoptosis. *J. Immunol.* **163**, 4772–4779
41. Xu, S.Q. and El-Deiry, W.S. (2000) p21(WAF1/CIP1) inhibits initiator caspase cleavage by TRAIL death receptor DR4. *Biochem. Biophys. Res. Commun.* **269**, 179–190
42. Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T., and Miura, M. (1998) Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene* **17**, 931–939