*J. Biochem.* **131**, 319–326 (2002)

# Proteasome Inhibitors Block Ras/ERK Signaling Pathway Resulting in the Downregulation of Fas Ligand Expression during Activation-Induced Cell Death in T Cells[1]

**Yutaka Tanimoto[2] and Harutoshi Kizaki**

*Department of Biochemistry, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502*

Received October 15, 2001; accepted December 3, 2001

**Activation-induced cell death (AICD) plays a critical role in the maintenance of homeostasis and peripheral tolerance in the immune system, and is mediated by Fas ligand (FasL) expression and the interaction between Fas and FasL. In the present study, we examined the role of the ubiquitin-proteasome system in AICD using T cell hybridoma N3-6-71 cells. The peptidyl aldehyde proteasome inhibitor carbobenzoxyl-Ile-Glu(*O-t*-butyl)-Ala-leucinal (PSI) blocked T cell receptor (TCR) stimulation–induced apoptosis in the T cell hybridoma. Fas and FasL gene expression and mouse FasL promoter activity following TCR stimulation were suppressed by PSI pretreatment. Deletion or point mutation of the κB site in the FasL promoter region did not suppress inducible FasL promoter activity effectively. PSI blocked extracellular signal–regulated kinase (ERK) activity induced by TCR stimulation, but had no effect on c-jun N-terminal kinase activation. ERK activation was essential for FasL expression and AICD. The initial tyrosine phosphorylation steps following TCR stimulation, *i.e.*, phosphorylation of CD3ζ and Vav, were not altered by PSI. These data suggest that the ubiquitin-proteasome system has some regulatory function at an intermediate step between the initial tyrosine phosphorylation steps and ERK activation in AICD.**

**Key words: activation-induced cell death, apoptosis, extracellular signal–regulated kinase, Fas ligand, proteasome.**

Engagement of T cell receptor (TCR) on naive T cells triggers the expression of cytokines such as IL-2, clonal expansion, and differentiation to effector cells, while preactivated T cells undergo activation-induced cell death (AICD) as the response to a second antigenic stimulus to terminate an immune response (*1, 2*). AICD is lymphocyte apoptosis that follows lymphocyte activation by any stimulus, including TCR engagement, with the antigen bound to MHC molecules or anti-CD3 antibody, and is crucial for the maintenance of T cell homeostasis and peripheral tolerance (*1, 2*). AICD of peripheral mature T lymphocytes and T cell hybridomas induced by TCR stimulation is dependent on transcriptional activation and expression of Fas ligand (FasL) and ligation to Fas, the receptor for FasL, on the surface of activated T cells (*3–5*). Binding of FasL to Fas triggers rapid apoptosis in the Fas-expressing T cells. However, little is known about transcriptional regulation of the FasL gene in AICD.

The 26S proteasome is an ATP-dependent protease that catalyzes the rapid degradation of abnormal proteins or short-lived regulatory proteins (*6*). Most of these proteins are at first ubiquitinated by a ubiquitin-conjugating system composed of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3), and then degraded by the 26S proteasome. The ubiquitin-proteasome system is involved in a variety of physiological cellular functions including the cell cycle, stress response, antigen processing, and transcriptional regulation (*6*). Recently, this system was shown to be crucial for the regulation of apoptosis. We and others showed that specific inhibitors of the proteasome, carbobenzoxyl-Ile-Glu(*O-t*-butyl)-Ala-leucinal (PSI) or lactacystin, induced apoptosis in mouse RVC lymphoma cells, PC12 pheochromocytoma cells, and U937 monoblast cells (*7–9*). In contrast to the apoptosis-inducing effect of the proteasome inhibitors in these cell types, thymocyte apoptosis induced by dexamethasone, γ-irradiation, or etoposide was prevented by the proteasome inhibitors (*10–13*). Proteasome inhibitors also antagonized apoptosis induced by nerve growth factor withdrawal in rat sympathetic neurons (*12*).

Regarding the relationship between AICD and the ubiquitin-proteasome system, it has been reported that lactacystin inhibited AICD in T cell hybridoma (DO.11.10) by suppressing Fas and FasL expression (*13*). Binding sites for transcription factors including AP-1, nuclear factor of activated T cells (NFAT), and nuclear factor–κB (NF-κB) have been identified on the FasL promoter (*14–16*). NF-κB activation is achieved through the degradation of IκB by the 26S proteasome following TCR stimulation, and protea-

[1] This work was supported by a Grant-in-Aid for Scientific Research (No. 12771110) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

[2] To whom correspondence should be addressed. Tel: +81-43-270-3750, Fax: +81-43-270-3752, E-mail: yutanimo@tdc.ac.jp

Abbreviations: AICD, activation-induced cell death; ERK, extracellular signal–regulated kinase; FasL, Fas ligand; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; PSI, carbobenzoxyl-Ile-Glu(*O-t*-butyl)-Ala-leucinal; TCR, T cell receptor.

some inhibitors inhibit this activation (*17*). The inducible FasL promoter activity is suppressed by IκBα overexpression or mutation of the κB site on the FasL promoter (*14, 16, 18*). An NF-κB overexpression study showed that NF-κB alone is sufficient to activate FasL gene expression (*19*). However, a mutant T cell line lacking expression of the essential NF-κB signaling component IKKγ was reported to have normal FasL gene expression (*20*). The inhibitory mechanisms of proteasome inhibitors on FasL expression in AICD are still unknown.

TCR stimulation triggers activation of multiple protein tyrosine kinases, resulting in phosphorylation of intracellular proteins including CD3ζ, ZAP-70, Vav, Grb2, and LAT (*21*). Recruitment of Grb2/SOS or SLP-76/Vav complexes to the plasma membrane by tyrosine-phosphorylated LAT is required for the activation of Ras, a guanine nucleotide binding protein (*22*). Ras activation by guanine nucleotide exchange factors, SOS and Vav, which convert the GDP-bound form of Ras to the active GTP-bound form, results in the activation of the downstream Raf/MEK/ERK signaling transduction (*22*). Expression studies of dominant negative Ras or MEK mutants in T cells have indicated that the Ras/ERK pathway is required for FasL expression and apoptosis in AICD of T cells (*15, 23*).

To examine the relationship between AICD and the ubiquitin-proteasome system in detail, we studied the effect of proteasome inhibitors on apoptosis, Fas/FasL expression, and signal transduction in T cell hybridoma N3-6-71 cells stimulated by anti-CD3 antibody. We demonstrated that proteasome inhibitors inhibited AICD, activation of Fas/FasL expression, and the promoter activity of FasL. We also showed that ERK activation but not CD3ζ- and Vav-tyrosine phosphorylation were abolished by proteasome inhibitors.

## MATERIALS AND METHODS

*Materials*—Anti-CD3ε antibody was purified from the ascites fluid of SCID mice grafted with 145-2C11 cells by protein A-Sepharose chromatography. The proteasome inhibitors Z-Ile-Glu(Obu$^t$)-Ala-Leu-H (PSI), Z-Leu-Leu-Leu-H (Z-LLL-H, MG-132), and Z-Leu-Leu-Nva-H (MG-115), and the calpain inhibitor Z-Leu-Leu-H (Z-LL-H) were obtained from the Peptide Institute (Osaka). Anti-phospho-specific ERK, anti-ERK, anti-JNK, anti-Vav, and anti-CD3ζ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL protein biotinylation module, anti-phosphotyrosine-HRP conjugate, and ECL Western blotting detection reagents were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). The anti-rabbit IgG-HRP conjugate and protein G-Sepharose were obtained from DAKO (Denmark) and Sigma (St. Louis, MO), respectively. PHAS-I and GST-c-Jun were purchased from Calbiochem (La Jolla, CA).

*Cells and TCR Stimulation*—The murine T cell hybridoma N3-6-71 cells were kindly provided by Dr. T. Tadakuma (National Defense Medical College, Saitama) (*24*). The cells were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (Intergen, Purchase, NY), 50 μM 2-mercaptoethanol, and antibiotics. The cells were activated in tissue culture dishes that had been precoated overnight with 10 μg/ml anti-CD3ε antibody in PBS at 4°C. For the detection of tyrosine-phosphorylated CD3ζ and Vav, cells were suspended in 1 ml of culture medium and stimulated with 1 μg of anti-CD3ε antibody for 5 or 20 min at 37°C.

*Apoptosis Assays*—For the DNA fragmentation assay, cells were harvested and lysed in 0.5% SDS containing 25 mM EDTA and 75 mM NaCl for more than 3 h on ice. Fragmented and intact DNA were separated by centrifugation at 100,000 ×*g* for 30 min at 0°C. The DNA content in the supernatant and in the pellet was measured by the fluorometric method described elsewhere (*25*). The percentage of fragmented DNA was defined as the ratio of DNA in the supernatant (fragmented DNA) to that in the precipitate plus supernatant DNA (total DNA). For the DNA ladder formation assay, cells were harvested and lysed in 50 mM Tris-HCl, pH 7.8, containing 0.5% sodium lauroyl sarcosilate and 10 mM EDTA. Samples were treated with RNase A (0.5 mg/ml) for 15 min at 50°C, then with proteinase K (0.5 mg/ml) for 30 min at 50°C. The samples were loaded onto a 1.8% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) containing 0.5 μg/ml ethidium bromide and resolved using a submerged gel electrophoresis system. Bands were visualized under UV light.

*RT-PCR*—Total RNA was isolated from the cells by the

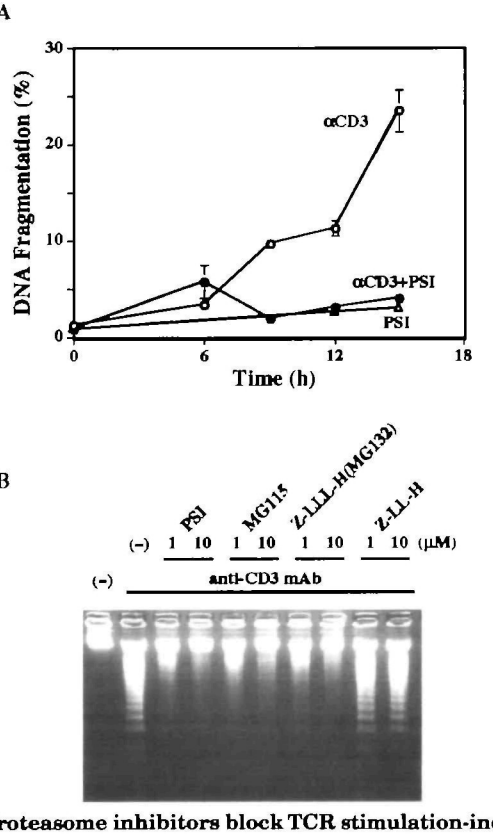


Fig. 1. **Proteasome inhibitors block TCR stimulation-induced apoptosis in N3-6-71 cells.** A: N3-6-71 cells were preincubated with 10 μM PSI for 30 min and stimulated with immobilized anti-CD3 mAb. The graph shows the percentage of DNA fragmentation in N3-6-71 cells treated with anti-CD3 mAb (open circles), anti-CD3 mAb plus PSI (close circles), or PSI (open triangles). B: N3-6-71 cells were stimulated with immobilized anti-CD3 mAb for 15 h in the presence or absence of protease inhibitors. Cells were harvested and analyzed by the apoptotic ladder formation assay.

method of Chomczynski and Sacchi (*26*), and cDNA was synthesized from 10 μg of total RNA in a reaction mixture containing 300 ng of oligo(dT) primer, 50 U of Moloney murine leukemia virus reverse transcriptase, and 40 U of RNase inhibitor. The PCR reaction was performed in a 50-μl reaction mixture containing 2 μl of cDNA, 20 pmol of fluorescein-labeled Fas primers (forward, 5′-CGAGAGTTTAAAGCTGAGGAGG-3′; reverse, 5′-ACTGGAGGTTCTAGATTCA-GGG-3′), Fas ligand primers (forward, 5′-CAACACAAATCTGTGGCTACCG-3′; reverse, 5′-CCCATATCTGTCCAGTAGTGCA-3′), or GAPDH primers (forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′) and 2.5 U of Taq polymerase. The conditions for PCR were 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C for 27 cycles. The PCR products were then resolved by electrophoresis on a 5.0% polyacrylamide gel and quantified by Gene Scan or resolved by electrophoresis on a 1.8% agarose gel containing ethidium bromide in TBE buffer and visualized under UV light. All of the PCR products were confirmed by nucleotide sequencing.

*Plasmid Constructs and Luciferase Assay*—A mouse FasL promoter fragment (approximately 2.4 kb) was amplified by genomic PCR using a Universal GenomeWalker kit (Clontech Laboratories, Palo Alto, CA) (GenBank accession no. AF045739). A FasL reporter plasmid construct was created by insertion of the *Xho*I–*Hin*dIII–flanked FasL promoter fragment into the pGL3-basic luciferase reporter vector (Promega, Madison, WI). Deletions and point mutations in the FasL promoter region were made by the exonuclease/mung bean nuclease method and site-directed mutagenesis according to the manufacturer's instructions, respectively. For transient transfections, $1\times10^6$ cells were incubated for 4 h at 37°C with a mixture of 10 μl of DMRIE-C (Life Technologies) and 5 μg of reporter plasmid DNA in 1 ml of DMEM medium, and then 3 ml of RPMI 1640/15% FCS was added. The cells were stimulated with immobilized anti-CD3ε antibody for 12 h. The cells were harvested and luciferase activity was determined by using a luciferase assay kit (Promega) and a Lumat LB9506 luminometer (Berthold, Germany).

*Immunoprecipitation and Western Blotting*—Cells ($3–5\times10^7$) were lysed by incubation for 30 min on ice with lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 2 mM EGTA, 1 mM $Na_3VO_4$, 1 mM PMSF, and 10 μg/ml leupeptin. The lysate was clarified by centrifugation at 12,000 ×*g* for 15 min. Proteins were immunoprecipitated with 2 μg of anti-CD3ζ or anti-Vav antibody for 1 h and then with 20 μl of protein G-Sepharose (50% w/v slurry) for 1 h at 4°C. The immunoprecipitated proteins were washed, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk in PBS overnight at 4°C, the membranes were incubated with biotinylated anti-CD3ζ or anti-Vav antibody in PBS containing 0.5% skim milk for 1 h at room temperature. The membranes were then probed with streptavidin horseradish peroxidase conjugate and visualized by the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions. For phosphotyrosine immunoblotting, the blots were probed with anti-phosphotyrosine

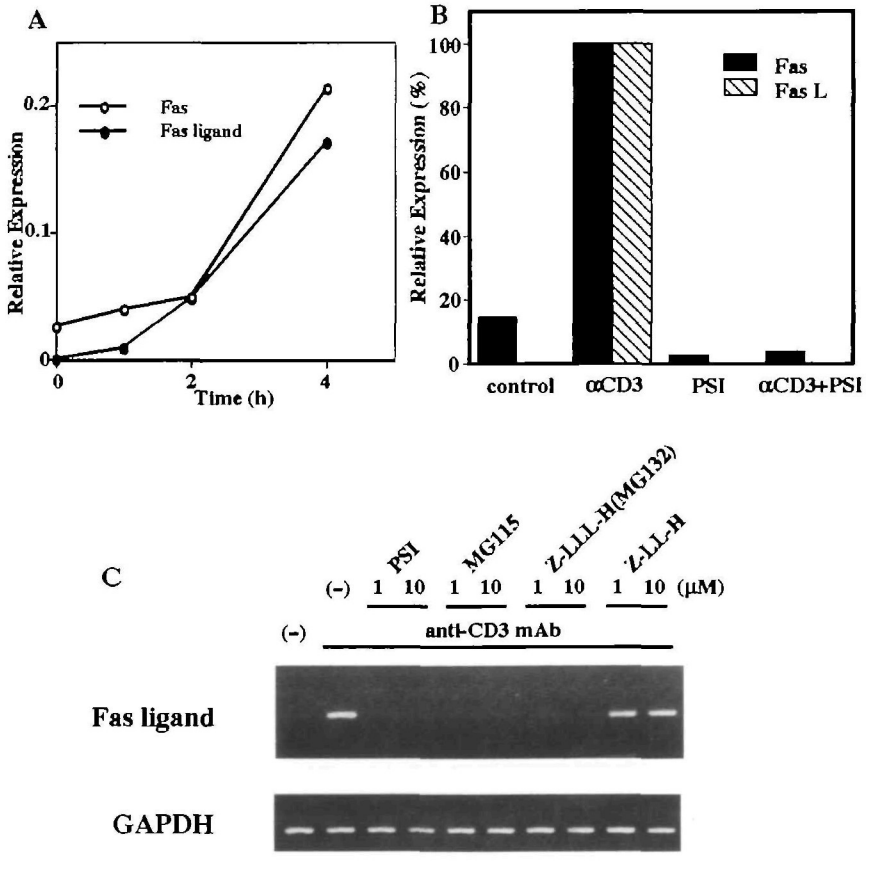


Fig. 2. **Fas and FasL expression in N3-6-71 cells following TCR stimulation.** A: Time course of activation-induced Fas and FasL expression in N3-6-71 cells. The cells were incubated with immobilized anti-CD3 mAb. Fas and FasL expression were assessed by quantitative RT-PCR using the fluorescent-labeled primer method. The results are shown as the relative expression normalized by the GAPDH expression. B: The stimulation of expression of both Fas and Fas ligand expression was inhibited by PSI. The cells were preincubated with 10 μM PSI for 30 min and stimulated with immobilized anti-CD3 mAb for 4 h. The results are shown as the relative expression compared with that in TCR-stimulated N3-6-71 cells. The data are the mean values of three independent experiments. C: Effect of protease inhibitors on Fas and FasL expression induced by TCR stimulation. The cells were stimulated with anti-CD3 mAb in the presence or absence of protease inhibitors. The cells were harvested and analyzed by RT-PCR. The PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining.

horseradish peroxidase conjugate in TBS containing 0.5% bovine serum albumin.

*In Vitro Kinase Assay*—The immunoprecipitates with anti-ERK2 or anti-JNK2 antibody were washed twice with the washing buffer containing 10 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.1 mM $Na_3VO_4$. The precipitates were then incubated for 30 min at 30°C with 2 μg of PHAS-I or GST-c-Jun in kinase buffer containing 10 μCi of [γ-$^{32}$P]ATP, 10 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 15 μM ATP, 1 mM dithiothreitol, and 0.1 mM $Na_3VO_4$. The kinase reaction was stopped with SDS-PAGE sample buffer. The lysates were resolved by SDS-PAGE. The gels were fixed with 10% triacetic acid for 15 min, dried and analyzed using a Molecular Imager (Bio-rad, Herculus, CA).

## RESULTS

*Proteasome Inhibitors Block TCR Stimulation-Induced Apoptosis and Fas/FasL Expression*—To investigate the role of proteasomes in AICD, we tested the effect of proteasome inhibitors on apoptosis induced by anti-CD3 treatment in T cell hybridoma N3-6-71 cells (Fig. 1). N3-6-71 cells were preincubated with 10 μM PSI for 30 min, then incubated with the immobilized anti-CD3 antibody. DNA fragmentation was detected at 9 h, and increased to about 22% at 15 h after TCR stimulation (Fig. 1A). PSI potently inhibited DNA fragmentation to the level in cells treated with PSI alone. Other proteasome inhibitors, MG-132 and MG-115, also inhibited the apoptotic ladder formation effectively at 1 μM and more intensely at 10 μM (Fig. 1B). The DNA fragmentation was not inhibited by Z-LL-H, a calpain inhibitor, at 10 μM, suggesting that the calpain inhibitory activity of MG-132 and MG-115 was not involved in the prevention of apoptosis.

As AICD following TCR stimulation depends on Fas/FasL interaction (*3*), we examined whether the expression of Fas and/or FasL is induced by anti-CD3 treatment in N3-6-71 cells. In unstimulated cells, a low level of Fas mRNA expression was detected, but FasL mRNA expression was not detected by quantitative RT-PCR using fluorescein-isothiocyanate-labeled PCR primers for Fas or FasL (Fig. 2A). Treatment with anti-CD3 antibody potently induced both Fas and FasL expression in a time-dependent manner (Fig. 2A). When the cells were stimulated with anti-CD3 antibody in the presence of neutralizing anti-Fas ligand antibody, DNA fragmentation was completely inhibited (data not shown). These results indicate that anti-CD3 antibody-induced apoptosis in N3-6-71 cells depends on Fas-FasL interaction. Next we examined the effect of PSI on Fas and FasL expression. Pretreatment with PSI strongly blocked the expression of both Fas and FasL mRNAs (Fig. 2B). The level of Fas transcript induced by anti-CD3 antibody in the presence of PSI was lower than that induced in the control (Fig. 2B). However, anti-Fas antibody-induced apoptosis in N3-6-71 cells was not inhibited by PSI (data not shown). The induction of FasL expression by anti-CD3 antibody treatment was also inhibited by the addition of MG-132 or MG-115, whereas Z-LL-H, a calpain inhibitor, failed to block FasL mRNA induction (Fig. 2C).

*Functional Analysis of Mouse FasL Promoter*—Recently, potential binding sites of transcription factors such as NFAT, RE3, and NF-κB on the mouse FasL promoter (Fig. 3A) have been shown to be involved in TCR stimulation-induced apoptosis (*14–16*).

To examine the effect of proteasome inhibitor on FasL promoter activity stimulated by anti-CD3 antibody, the promoter region of the FasL gene was amplified by genomic PCR using the gene-walking method. The promoter fragment was cloned into a luciferase reporter plasmid, and a series of reporter plasmids under the control of a gradually shortened promoter region was constructed. The luciferase activity of the FasL –241 (–241 to +89) reporter construct was stimulated 20-fold by treatment with anti-CD3 antibody as compared with the luciferase activity of unstimulated cells (Fig. 3C). Both deletions of the regions from –155 to –89 and from –89 to –55, which contain the previously identified RE3 and κB sites, respectively, reduced the level of activation by anti-CD3 treatment (Fig. 3C). To investigate the role of the RE3 and κB sequences in the transcriptional activation of the mouse FasL promoter, point mutations of the RE3 and κB sites were introduced in the FasL –241 reporter construct by site-directed mutagenesis (Fig. 3B). The point mutation of the RE3 site resulted in a 75% loss of the inducible promoter activity. In contrast, the point mutation of the κB site was less effective (Fig. 3C).

PSI potently inhibited the inducible promoter activity of the FasL –241 reporter plasmid (Fig. 3C). On the other

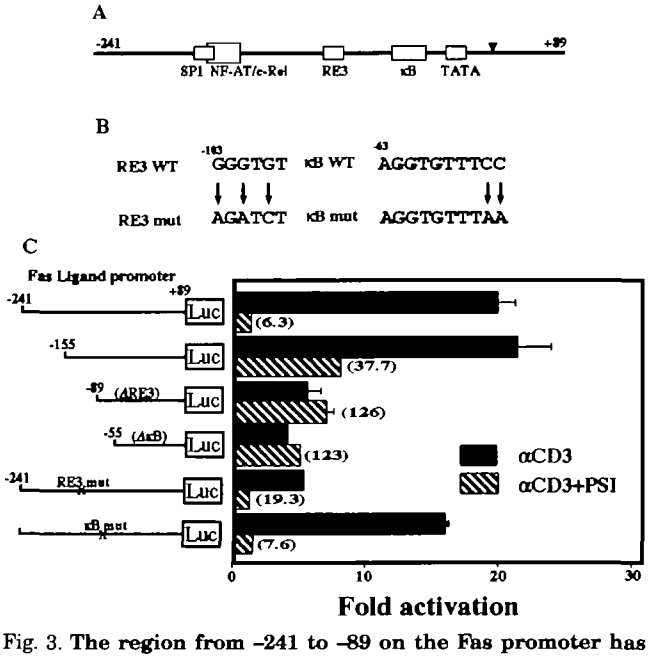


Fig. 3. **The region from –241 to –89 on the Fas promoter has binding sites for transcription factors that are negatively regulated by PSI.** A: Schematic representation of mouse FasL promoter. The arrowhead indicates the transcriptional initiation site (position +1). TATA and other predicted transcription factor binding motifs are indicated by boxes. B: Constructs of reporter plasmids with mutations in RE3- and κB-like binding sites. The base pair changes in the binding site for each transcription factor are indicated by arrows. C: N3-6-71 cells were transfected by lipofection with 10 μg of pGL3 reporter plasmid under the control of the indicated FasL promoter. Transfected cells were stimulated with immobilized anti-CD3 mAb in the presence or absence of PSI for 12 h. Cells were lysed and assayed for luciferase activity. The fold-activation value was calculated by comparison with the activity of the transfectants cultured without treatment. The activity induced by PSI plus TCR stimulation is shown in parentheses as a percentage of that induced by TCR stimulation alone.

hand, progressive deletion of the region from –241 to –155 and from –155 to –89 resulted in increases of the residual promoter activity after PSI treatment from 6.3 to 37.7% and from 37.7 to 126%, respectively (Fig. 3C). These results suggest that the region from –241 to –155 and that from –155 to –89 (presumably RE3) are responsible for the inhibitory effect of PSI on FasL expression.

An NFAT binding site has been identified in the region from –241 to –155 of the FasL promoter (*15, 27*). Moreover, consensus sequences of SP1 and c-Rel were also found to partially overlap the NFAT binding site in this region (*28, 29*). We next examined the effect of point mutations of these sequences (Fig. 4A) on the promoter activity of the Fas ligand –241 reporter plasmid. The point mutations of the SP1-like and the c-Rel–like sites as well as the NFAT binding site markedly reduced the promoter activities regardless of TCR stimulation (Fig. 4B). PSI inhibited the inducible promoter activity of each point mutation less effectively than that of the wild-type promoter. These results suggest that the GGGCGGAAACTTCC sequence (from –168 to –155) is essential for the inducibility in response to TCR stimulation and the inhibitory effect of PSI.

*PSI Inhibits Activation of ERK but Not JNK Following TCR Stimulation*—TCR Stimulation activates the Ras/ERK pathway and Rac/JNK pathway in T cells (*21*), and T cell activation is inhibited by the dominantly interfering Ras and MEK inhibitor PD98059 (*30, 31*). We therefore examined whether PSI affects ERK activation following anti-CD3 antibody stimulation. The activation of ERKs was assessed by assaying the tyrosine phosphorylation (Y204) of ERKs stimulated by anti-CD3 antibody. Phosphorylation of both ERK1 and ERK2 was detected after 5 min of stimulation and up-regulated after 30 min to a maximum level, although the latter was predominant (Fig. 5A). PSI potently inhibited ERKs activation. Proteasome inhibitors MG-115 and MG-132 also blocked the anti-CD3-stimulation–induced ERKs activation (data not shown). To confirm this effect, we measured the kinase activity of ERK2, the dominant form of ERKs, using an *in vitro* kinase assay. PSI reduced the induction of ERK2 kinase activity by TCR stimulation (Fig. 5B). To evaluate the effect of PSI on JNK activation following TCR stimulation, we measured immunoprecipitated JNK1 activity using GST-c-Jun as substrate (Fig. 5C). JNK activation was detectable at 10 min and was further increased at 4 h after anti-CD3 antibody treatment. PSI did not inhibit the activation of JNK activity; rather, pretreatment with PSI accelerated the activation of JNK. To investigate whether the activation of the Ras/ERK signaling pathway is essential for AICD, we analyzed the effect of MEK inhibitor PD98059 on apoptosis and FasL expression following TCR stimulation. PD98059 inhibited the apoptotic DNA ladder formation (Fig. 6A) and FasL mRNA expression (Fig. 6B) in a dose-dependent manner.

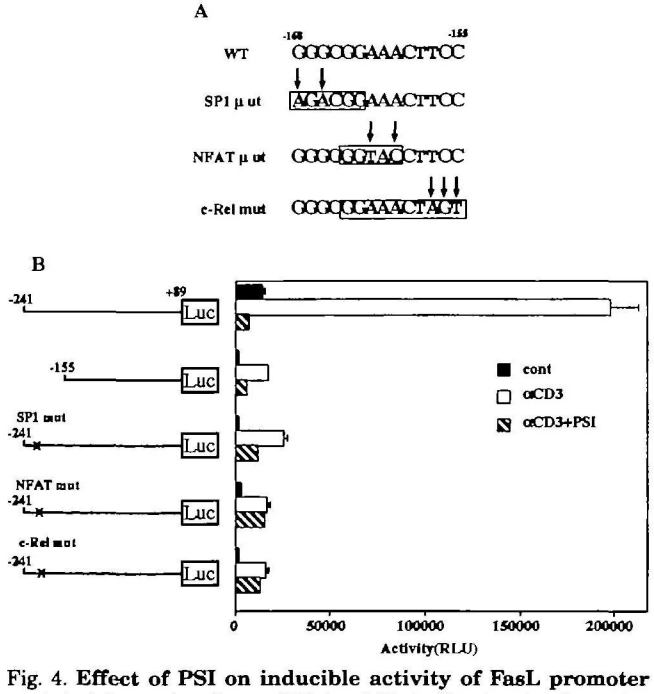


Fig. 4. **Effect of PSI on inducible activity of FasL promoter mutated in region from –241 to –155.** A: Constructs of reporter plasmids with mutations in SP1-, NFAT-, and c-Rel–like binding sites. B: N3-6-71 cells were transfected by lipofection with 10 μg of pGL3 reporter plasmid under the control of the indicated FasL promoter. Transfected cells were stimulated with immobilized anti-CD3 mAb in the presence or the absence of PSI for 12 h. Cells were lysed and assayed for luciferase activity.

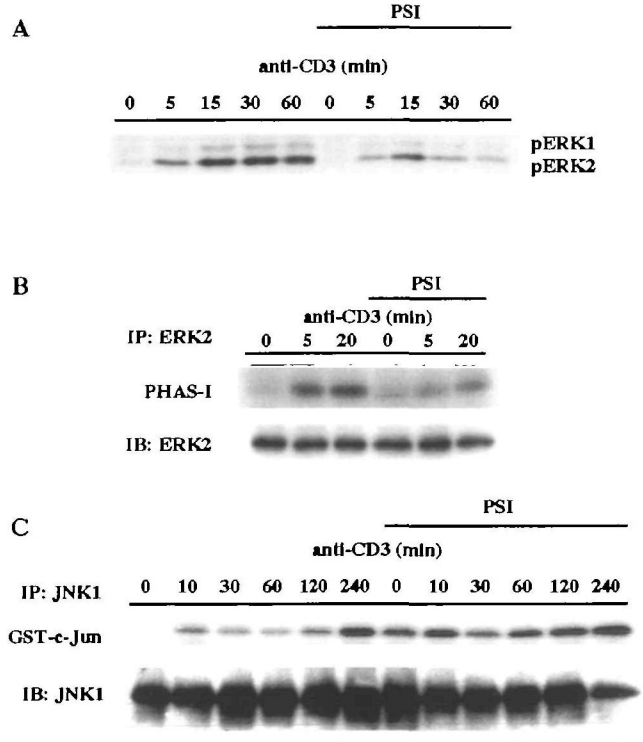


Fig. 5. **PSI inhibits activation of ERK but not JNK following TCR stimulation.** A: N3-6-71 cells were incubated in anti-CD3 antibody–coated dishes in the presence or absence of PSI for various times as indicated. ERK activation was assessed by monitoring the phosphorylation of specific threonine and tyrosine residues. Cell lysates (20 μg/lane) were analyzed by probing the immunoblot with anti-phospho-specific ERK Ab. B and C: N3-6-71 cells were treated as described above. Cells were lysed and the lysates were incubated with 2 μg of anti-ERK (B) or anti-JNK (C) Ab, and then immunocomplexes were precipitated with protein G-Sepharose. ERK and JNK activities in the immunoprecipitates were measured by *in vitro* kinase assays using PHAS-I and GST-c-Jun as the substrate, respectively.

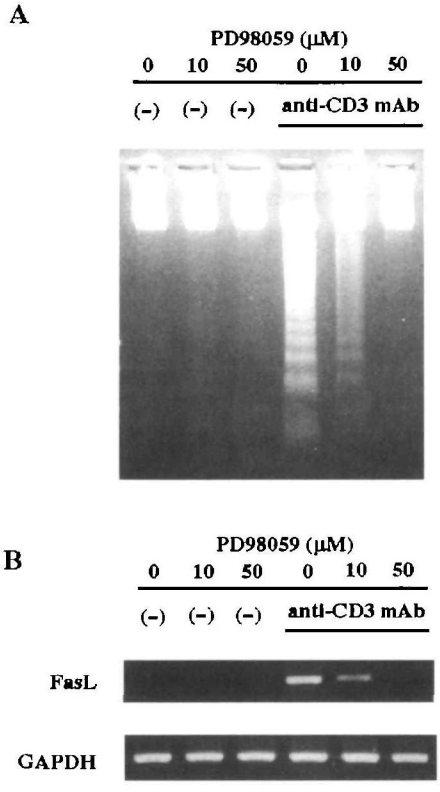


Fig. 6. **MEK inhibitor PD98059 blocks AICD and FasL expression following TCR stimulation.** A: N3-6-71 cells were preincubated with PD98059 for 60 min and stimulated with immobilized-anti-CD3 antibody for 12 h. Cells were harvested and analyzed by apoptotic ladder formation assay. B: N3-6-71 cells were stimulated with anti-CD3 antibody for 4 h in the presence or absence of PD-98059. Cells were harvested and analyzed by RT-PCR.

*Tyrosine Phosphorylation of CD3ζ and Vav Following TCR Stimulation*—TCR stimulation leads to tyrosine phosphorylation of CD3ζ and Vav *via* the activation of protein tyrosine kinases such as Lck and ZAP-70 (*21*). Tyrosine phosphorylation of CD3ζ and Vav transduces TCR signaling to the downstream Ras/ERK pathway or Rac/JNK pathway (*21*). We tested the effect of PSI on tyrosine phosphorylation of CD3ζ (Fig. 7A) and Vav (Fig. 7B) in N3-6-71 cells following anti-CD3 antibody treatment. PSI did not affect the enhancement of CD3ζ or Vav tyrosine phosphorylation by TCR stimulation.

## DISCUSSION

The ubiquitin-proteasome system is important for the regulation of apoptosis as well as the cell cycle, antigen processing, and transcription (*6–9*). Inhibition of the proteasome system prevents dexamethasone- or etoposide-induced thymocyte apoptosis and AICD in T cell hybridomas following TCR stimulation (*10, 11, 13*). Induction of AICD depends on the activation of FasL expression at the transcriptional and protein levels, and on the Fas/FasL interaction-activated apoptosis signaling (*3–5*). It is known that proteasome inhibitors suppress FasL expression during AICD due to the blockage of NF-κB activation by degradation of IκB. However, the other factors affecting the regulation of FasL expression by proteasomes are still unknown.

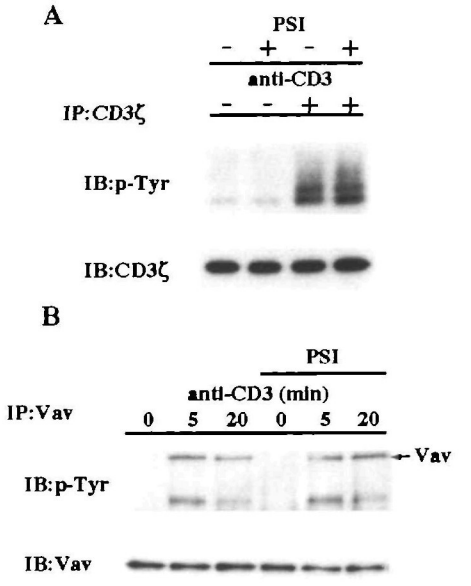


Fig. 7. **Effect of PSI on tyrosine phosphorylation of CD3ζ and Vav following TCR stimulation.** N3-6-71 cells were stimulated with anti-CD3 mAb in the presence or absence of PSI for 20 min (A) or for various times as indicated (B). Cells were lysed and cell lysates were immunoprecipitated with anti-CD3ζ Ab (A) or anti-Vav Ab (B). Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine mAb.

In this study, we showed that the proteasome functions in the activation of the Ras/ERK signaling pathway following TCR stimulation. AICD, the induction of FasL expression, and ERK activation following TCR stimulation were inhibited by PSI and other proteasome inhibitors (MG-132 and MG-115). MG-132 and MG-115 inhibit calpain as well as proteasome activity (*32*). Z-LL-H, which inhibits calpain as potently as the peptidyl aldehyde of tri-leucine MG-132 (Z-LLL-H) but possesses little proteasome inhibitory activity, failed to inhibit AICD (Fig. 1B), the induction of FasL expression (Fig. 2C), and ERK activation (data not shown). Thus, the inhibitory effects of MG-132 and MG-115 are considered likely to be due to the inhibition of the proteasome.

A potential target by which the ubiquitin-proteasome system regulates FasL expression following TCR stimulation is the NF-κB inhibitory protein IκB. The inducible FasL promoter activity is suppressed by IκBα overexpression or mutation of κB sites on the FasL promoter (*14, 16, 18*). NF-κB (RelA) overexpression enhances FasL promoter activity and FasL gene expression (*19*). Indeed, the mutation of the NF-κB binding site in the region from nucleotide –63 to –54 relative to the transcription start site partially suppressed the inducible FasL promoter (Fig. 3C), suggesting that one of the targets for the proteasome inhibitor is the inhibition of NF-κB activation. However, the suppression was not predominant. Although we did not examine the involvement of other NF-κB binding sites in the mouse FasL promoter on FasL gene expression, IKKγ, which is essential for NF-κB activation, has been shown not to be required for FasL expression and AICD (*20*). These findings suggest that proteasome inhibitors suppress FasL expression by inhibiting regulator(s) other than IκB for NF-κB activation.

The GGGCGGAAACTTCC sequence contains an NFAT-responsive element that is important for FasL expression (Fig. 4B). Point mutation of not only the NFAT site but also potential binding sites of SP1 or c-Rel in this segment markedly reduced the inducible FasL promoter activity (Fig. 4B). SP1, which is a general transcription factor, can bind to and act through GC-boxes (*28*). SP1 interacts with many transcription factors including TATA-box binding protein TBP, and TBP associated factors dTAFII110/hTAFII130 and hTAF55. Proteins other than SP1, such as Krüppel-like factors and C2H2 type zinc finger proteins (*28*) also recognize the SP1 site, suggesting that certain factors binding to this site might be PSI responsive. However, we were not able to identify the protein that binds to this site in the FasL promoter. C-Rel is a member of the Rel/NF-κB family, and the GGAAACTTCC sequence is a potential binding site of NF-κB (RelA/p50) as well (*29*). However, we and others have failed to detect inducible NF-κB binding activity using this sequence as a probe in gel shift assays (*16*). Thus the identification of the transcription factor(s) that interacts with the GGGCGGAAACTTCC sequence may be important for clarifying the mechanism of the regulation of FasL expression by the ubiquitin-proteasome system.

TCR engagement leads to the activation of signal transduction pathways such as the Ras/ERK pathway and the Rac/JNK pathway (*21*). MAPKs including ERK1/2, JNK, and p38 MAPK, are involved in the regulation of cell proliferation, differentiation, survival, and apoptosis (*30, 31*). In general, ERK activation promotes cell proliferation and inhibits cell death whereas the activation of JNK and p38 MAPK is associated with the promotion of apoptosis (*31*). However, under some circumstances, the Ras/ERK pathway plays a proapoptotic role (*33*). Apoptosis induction by forced c-Myc expression was enhanced by co-expression of Raf-activating Ras mutant V12 S35 or constitutively active Raf (*34*). In mouse embryo fibroblasts, the expression of v-Ras or v-Raf results in ERK activation and induces apoptosis (*35*). In AICD of T cells, overexpression of dominant negative Ras inhibits FasL expression, and v-Ras expression enhances FasL expression following TCR stimulation (*15*). ERK inhibition by transfection of a dominant interfering mutant of extracellular signal regulated protein kinase kinase (MEK1) or pretreatment with the specific MEK1 inhibitor prevents the induction of FasL expression during AICD (*23, 36*). We also confirmed that MEK1 inhibitor PD98059 effectively blocked AICD and FasL expression as well as PSI (Fig. 6). In this study, we observed that proteasome inhibitors blocked ERK2 activation, FasL expression, and AICD. These data suggest that activation of the Ras/ERK pathway is essential for induction of FasL expression followed by AICD, and that the proteasome inhibitors suppress the proapoptotic function of the Ras/ERK pathway.

TCR stimulation leads to the activation of a non-receptor tyrosine kinase, Lck, which phosphorylates CD3ζ and ZAP-70 (*21*). Subsequent tyrosine phosphorylation of Vav, SLP-76, and LAT through Lck and ZAP-70 is essential for downstream signal transduction (*21*). We observed that tyrosine phosphorylation of CD3ζ and Vav following TCR stimulation was not altered in the presence of PSI (Fig. 7), indicating that the proteasome inhibitor has no effect on the initial tyrosine phosphorylation steps. It may function at some downstream step that is regulated by proteasomes.

In the present study, we showed that RE3 and the GGGCGGAAACTTCC site are responsible for the negative effect of PSI on the FasL expression (Fig. 4) and that the Ras/ERK pathway is regulated by the ubiquitin-proteasome system in AICD. It remains to be elucidated whether transcription factors which bind to the RE3 or the GGGCGGAAACTTCC site are directly regulated by the ubiquitin-proteasome system or indirectly downregulated by the suppression of the Ras/ERK pathway by PSI, and what regulatory molecule(s) in the activation of the Ras/MAPK pathway are regulated by the ubiquitin-proteasome system.

We thank T. Tadakuma and Y. Onishi for helpful discussions and A. Gokan for technical assistance.

REFERENCES

1. Russell, J.H. (1995) Activation-induced death of mature T cells in the regulation of immune responses. *Curr. Opin. Immunol.* **7**, 382–388
2. Janssen, O., Sanzenbacher, R., and Kabelitz, D. (2000) Regulation of activation-induced cell death of mature T-lymphocyte populations. *Cell. Tissue Res.* **301**, 85–99
3. Brunner, T., Mogil, R.J., LaFace, D., Yoo, N.J., Mahboubi, A., Echeverri, F., Martin, S.J., Force, W.R., Lynch, D.H., Ware, C.F., *et al.* (1995) Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* **373**, 441–444
4. Wesselborg, S., Janssen, O., and Kabelitz, D. (1993) Induction of activation-driven death (apoptosis) in activated but not resting peripheral blood T cells. *J. Immunol.* **150**, 4338–4345
5. Nagata, S. (1997) Apoptosis by death factor. *Cell* **88**, 355–365
6. Ciechanover, A. (1994) The ubiquitin-mediated proteolytic pathway: mechanisms of action and cellular physiology. *Biol. Chem. Hoppe Seyler* **375**, 565–581
7. Tanimoto, Y., Onishi, Y., Hashimoto, S., and Kizaki, H. (1997) Peptidyl aldehyde inhibitors of proteasome induce apoptosis rapidly in mouse lymphoma RVC cells. *J. Biochem.* **121**, 542–549
8. Imajoh-Ohmi, S., Kawaguchi, T., Sugiyama, S., Tanaka, K., Omura, S., and Kikuchi H. (1995) Lactacystin, a specific inhibitor of the proteasome, induces apoptosis in human monoblast U937 cells. *Biochem. Biophys. Res. Commun.* **217**, 1070–1077
9. Lopes, U.G., Erhardt, P., Yao, R., and Cooper, G M. (1997) p53-dependent induction of apoptosis by proteasome inhibitors. *J. Biol. Chem.* **272**, 12893–12896
10. Grimm, L.M., Goldberg, A.L., Poirier, G.G., Schwartz, L.M., and Osborne, B.A. (1996) Proteasomes play an essential role in thymocyte apoptosis. *EMBO J.* **15**, 3835–3844
11. Stefanelli, C., Bonavita, F., Stanic, I., Pignatti, C., Farruggia, G., Masotti, L., Guarnieri, C., and Caldarera, C.M. (1998) Inhibition of etoposide-induced apoptosis with peptide aldehyde inhibitors of proteasome. *Biochem. J.* **332**, 661–665
12. Sadoul, R., Fernandez, P.A., Quiquerez, A.L., Martinou, I., Maki, M., Schroter, M., Becherer, J.D., Irmler, M., Tschopp, J., and Martinou, J.C. (1996) Involvement of the proteasome in the programmed cell death of NGF-deprived sympathetic neurons. *EMBO J.* **15**, 3845–3852
13. Cui, H., Matsui, K., Omura, S., Schauer, S.L., Matulka, R.A., Sonenshein, G.E., and Ju, S.T. (1997) Proteasome regulation of activation-induced T cell death. *Proc. Natl. Acad. Sci. USA* **94**, 7515–7520
14. Kasibhatla, S., Genestier, L., and Green, D.R. (1999) Regulation of Fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor κB. *J. Biol. Chem.* **274**, 987–992
15. Latinis, K.M., Carr, L.L., Peterson, E.J., Norian, L.A., Eliason, S.L., and Koretzky, G.A. (1997) Regulation of CD95 (Fas) ligand expression by TCR-mediated signaling events. *J. Immunol.* **158**, 4602–4611

16. Matsui, K., Fine, A., Zhu, B., Marshak-Rothstein, A., and Ju, S.T. (1998) Identification of two NF-κB sites in mouse CD95 ligand (Fas ligand) promoter: functional analysis in T cell hybridoma. *J. Immunol.* **161**, 3469–3473
17. Gerondakis, S., Grumont, R., Rourke, I., and Grossmann, M. (1998) The regulation and roles of Rel/NF-κB transcription factors during lymphocyte activation. *Curr. Opin. Immunol.* **10**, 353–359
18. Teixeiro, E., Garcia-Sahuquillo, A., Alarcon, B., and Bragado, R. (1999) Apoptosis-resistant T cells have a deficiency in NF-κB-mediated induction of Fas ligand transcription. *Eur. J Immunol.* **29**, 745–754
19. Hsu, S.C., Gavrilin, M.A., Lee, H.H., Wu, C.C., Han, S.H., and Lai, M.Z. (1999) NF-κB-dependent Fas ligand expression. *Eur. J. Immunol.* **29**, 2948–2956
20. Rivera-Walsh, I., Cvijic, M.E., Xiao, G., and Sun, S.C. (2000) The NF-κB signaling pathway is not required for Fas ligand gene induction but mediates protection from activation-induced cell death. *J. Biol. Chem.* **275**, 25222–25230
21. Qian, D. and Weiss, A. (1997) T cell antigen receptor signal transduction. *Curr. Opin. Cell Biol.* **9**, 205–212
22. Genot, E. and Cantrell, D.A. (2000) Ras regulation and function in lymphocytes. *Curr. Opin. Immunol.* **12**, 289–294
23. van den Brink, M.R., Kapeller, R., Pratt, J.C., Chang, J.H., and Burakoff, S J. (1999) The extracellular signal-regulated kinase pathway is required for activation-induced cell death of T cells. *J. Biol. Chem.* **274**, 11178–11185
24. Odaka, C., Kizaki, H., and Tadakuma, T. (1990) T cell receptor-mediated DNA fragmentation and cell death in T cell hybridomas. *J. Immunol.* **144**, 2096–2101
25. Onishi, Y., Azuma, Y., Sato, Y., Mizuno, Y., Tadakuma, T., and Kizaki, H. (1993) Topoisomerase inhibitors induce apoptosis in thymocytes. *Biochim. Biophys. Acta* **1175**, 147–154
26. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
27. Latinis, K.M., Norian, L.A., Eliason, S.L., and Koretzky, G.A. (1997) Two NFAT transcription factor binding sites participate in the regulation of CD95 (Fas) ligand expression in activated human T cells. *J. Biol. Chem.* **272**, 31427–31434
28. Suske, G. (1999) The Sp-family of transcription factors. *Gene* **238**, 291–300
29. Grossmann, M., Nakamura, Y., Grumont, R., and Gerondakis, S. (1999) New insights into the roles of ReL/NF-kappa B transcription factors in immune function, hemopoiesis and human disease. *Int. J. Biochem. Cell Biol.* **31**, 1209–1219
30. Cobb, M.H. (1999) MAP kinase pathways. *Prog. Biophys. Mol. Biol.* **71**, 479–500
31. Cross, T.G., Scheel-Toellner, D., Henriquez, N.V., Deacon, E., Salmon, M., and Lord, J.M. (2000) Serine/threonine protein kinases and apoptosis. *Exp. Cell Res.* **256**, 34–41
32. Tsubuki, S., Saito, Y., Tomioka, M., Ito, H., and Kawashima, S. (1996) Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. *J. Biochem.* **119**, 572–576
33. Downward, J. (1998) Ras signalling and apoptosis. *Curr. Opin. Genet. Dev.* **8**, 49–54
34. Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J., and Evan, G. (1997) Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544–548
35. Fukasawa, K. and Vande Woude, G.F. (1997) Synergy between the Mos/mitogen-activated protein kinase pathway and loss of p53 function in transformation and chromosome instability. *Mol. Cell. Biol.* **17**, 506–518
36. Zhu, L., Yu, X., Akatsuka, Y., Cooper, J.A., and Anasetti, C. (1999) Role of mitogen-activated protein kinases in activation-induced apoptosis of T cells. *Immunology* **97**, 26–35