Toso, a Cell Surface, Specific Regulator of Fas-Induced Apoptosis in T Cells

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Summary

Fas is a surface receptor that can transmit signals for apoptosis. Using retroviral cDNA library–based functional cloning we identified a gene, *toso*, that blocks Fas-mediated apoptosis. Toso expression was confined to lymphoid cells and was enhanced after cell-specific activation processes in T cells. Toso appeared limited to inhibition of apoptosis mediated by members of the TNF receptor family and was capable of inhibiting T cell self-killing induced by TCR activation processes that up-regulate Fas ligand. We mapped the effect of Toso to inhibition of caspase-8 processing, the most upstream caspase activity in Fas-mediated signaling, potentially through activation of cFLIP. Toso therefore serves as a novel regulator of Fas-mediated apoptosis and may act as a regulator of cell fate in T cells and other hematopoietic lineages.

Introduction

Apoptosis is an important homeostatic mechanism that maintains cell number, positioning, and differentiation. Several intracellular and intercellular processes are known to regulate apoptosis. One of the best characterized systems is initiated by the cell surface receptor Fas (Apo-1/CD95), which initiates apoptosis in a wide range of organisms (Yonehara et al., 1989; Itoh et al., 1991). Clustering of the Fas cytoplasmic domain generates an apoptotic signal via the "death domain" (Itoh and Nagata, 1993). Fas engagement by Fas ligand (FasL) is capable of activating cysteine proteases called caspases (Tewari and Dixit, 1995). Recent studies have implicated caspase-8 (MACH [MORT1-associated CED-3 homologue]/FLICE [FADD-like ICE]/Mch5) as linking Fas receptor signaling to downstream caspases via its association with the Fas-associated death domain (FADD)/MORT1 (Boldin et al., 1995, 1996; Chinnaiyan et al., 1995; Fernandes-Alnemri et al., 1996; Muzio et al., 1996). Several groups have reported that caspase-8 activation is inhibited by a cellular inhibitor, cFLIP (cellular FLICE-inhibitory protein)/FLAME-1 (FADD-like anti-apoptotic molecule-1)/I-FLICE (inhibitor of FLICE) (Hu et al., 1997; Irmler et al., 1997; Srinivasula et al., 1997).

A balance between life and programmed death in cells is likely to be governed by multiple interacting regulators that counteract apoptotic signals with appropriate anti-apoptotic signals. Imbalances in this regulation can result in wide variety of diseases, including cancer and immune dysfunction, and it is now clear that there are polypeptides other than Fas that contribute to dysregulation of appropriately induced apoptosis. As an example, in many tumor cell lines Fas expression does not correlate with sensitivity to Fas-induced apoptosis, implying the existence of Fas-resistance protein (Richardson et al., 1994). Also, in some types of cells, Fas-induced apoptosis requires protein synthesis inhibitors such as cycloheximide, and even in Fas-sensitive cells, protein synthesis inhibitors can play a synergistic role with cycloheximide (Yonehara et al., 1989; Itoh and Nagata, 1993). These combined observations further suggest the existence of proteins capable of suppressing Fas-generated apoptotic signaling.

In addition, in the course of a normal immune response, both cytotoxic T cell and natural killer cell activation can lead to FasL induction (Arase et al., 1995; Berke, 1995; Montel et al., 1995). Although both Fas and FasL are rapidly induced following T cell activation, activated T cells remain resistant to Fas-induced apoptosis for several days (Owen-Schaub et al., 1992; Klas et al., 1993). Thus, a mechanism exists to shield newly activated T cells from the cytotoxicity of their own FasL expression. This is likely to be an important component of T cell activation processes and protection in lymph nodes, splenic germinal centers, and other sites at which T cell activation might result in apoptosis of target cells. Therefore blockers of Fas-induced apoptosis must exist in T cells.

We therefore devised a genetic screen to identify such regulators within activated T cells. Here we report the identification and molecular cloning of a novel surface molecule, Toso, through a retroviral cDNA library–based functional screening system. *toso* is a member of the immunoglobulin gene superfamily and specifically inhibits tumor necrosis factor (TNF) receptor family member–mediated apoptosis. The results demonstrate the existence of cell surface–mediated mediated signaling pathways that lead to down-regulation of Fas-mediated apoptosis in certain cell types, and suggest that activation of T cells suppresses internal signaling systems that might lead inappropriately to T cell–induced self-killing.

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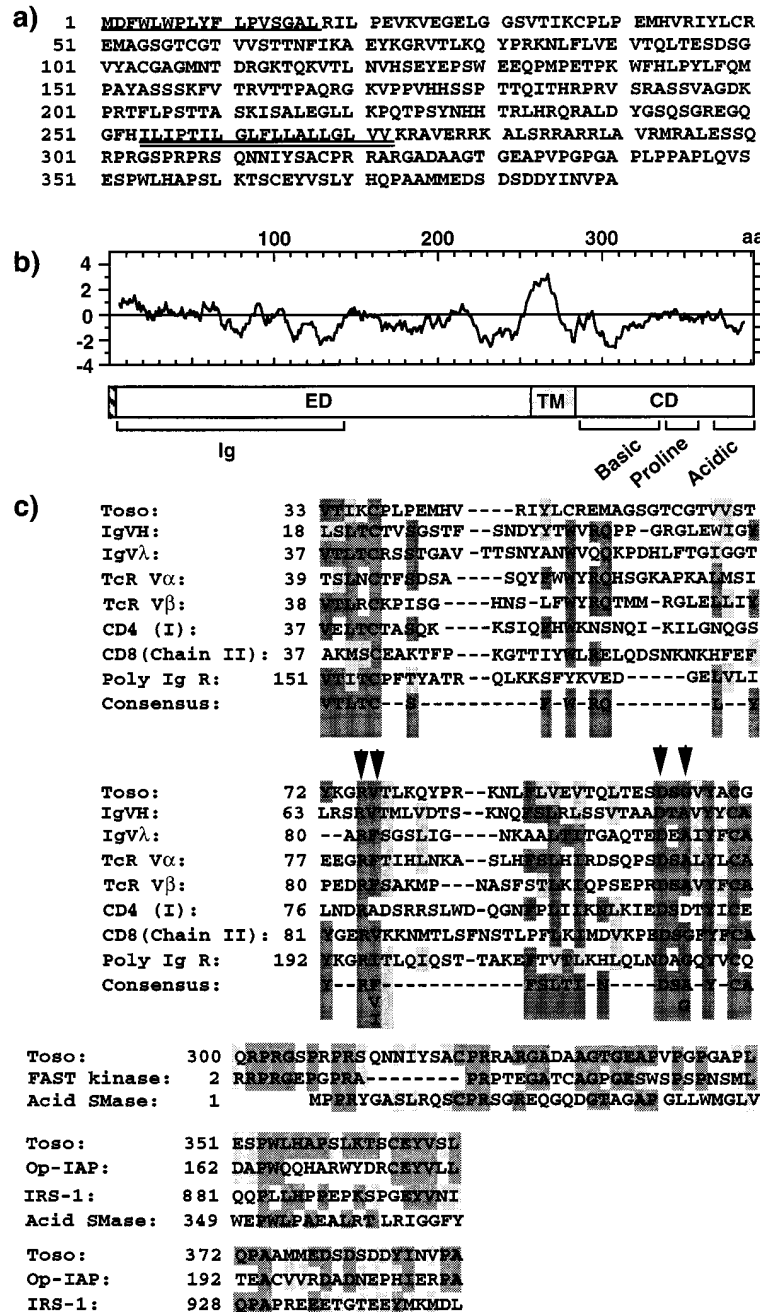[10] To whom correspondence should be addressed (gnolan@cmgm.stanford.edu).

a)

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  1  MDFWLWPLYF LPVSGALRIL PEVKVEGELG GSVTIKCPLP EMHVRIYLCR
 51  EMAGSGTCGT VVSTTNFIKA EYKGRVTLKQ YPRKNLFLVE VTQLTESDSG
101  VYACGAGMNT DRGKTQKVTL NVHSEYEPSW EEQPMPETPK WFHLPYLFQM
151  PAYASSSKFV TRVTTPAQRG KVPPVHHSSP TTQITHRPRV SRASSVAGDK
201  PRTFLPSTTA SKISALEGLL KPQTPSYNHH TRLHRQRALD YGSQSGREGQ
251  GFHILIPTIL GLFLLALLGL VVKRAVERRK ALSRRARRLA VRMRALESSQ
301  RPRGSPRPRS QNNIYSACPR RARGADAAGT GEAPVPGPGA PLPPAPLQVS
351  ESPWLHAPSL KTSCEYVSLY HQPAAMMEDS DSDDYINVPA
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b)

c)

Figure 1. Primary Structure of Toso

(A) Deduced amino acid sequence of the Toso gene product. Two hydrophobic regions are underlined: a leader sequence (single underline) and a presumptive transmembrane region (double underline).

(B) Kyte-Dolittle hydropathy plot analysis of the Toso gene product (top) and schematic presentation of Toso (bottom). The diagram shows the leader sequence (hatched bar), the extracellular domain (ED), the transmembrane region (TM, shaded bar), the cytoplasmic domain (CD), the immunoglobulin domain (Ig), the basic amino acid–rich region (Basic), the proline-rich region (Proline), and the acidic amino acid-rich region (Acidic).

(C) BLAST search results using the Toso gene product. The position of the first amino acid in each sequence is given to the left of the alignment. Dashes indicate gaps; dark and light shading refer to identical and similar residues, respectively. The aligment shows IgVH (G1HUNM), IgVλ (L1MS4E), T cell receptor (TCR) Vα (RWMSAV), TCR Vβ (RWHUVY), CD4 (U47924), CD8 chain II (X04310), Poly Ig R (QRRBG), immunoglobulin V-set consensus sequence, acid sphingomyelinase, insulin receptor substrate-1 (IRS-1), and apoptosis inhibitor, IAP, from *O. pseudotsugata* nuclear polyhedrosis virus (Op-IAP). Arrowheads indicate positions characteristic of many V-set sequences.

Results

Molecular Cloning and Chromosomal Localization of a Molecule That Inhibits Fas-Induced Apoptosis

To screen for cDNAs that encode inhibitory molecules for Fas-induced apoptosis, we infected 7.7×10^6 Jurkat cells with a retroviral Jurkat T cell cDNA library. Under these conditions, Fas activation of *lacZ* control–infected cells resulted in a spontaneous background of two or three clones per 10^6 Jurkat cells. In our library screening we obtained 26 clones that were resistant to Fas-

induced apoptosis, of which 12 carried cDNA inserts. The cDNA inserts were recloned and transduced into Jurkat cells to verify for their effect using anti-Fas monoclonal antibody (mAb). After this second round of anti-Fas screening, one clone, termed Toso, demonstrated potent inhibition of Fas-induced apoptotic signaling.

The cDNA insert of *toso* contains a 5′ noncoding region of 73 nucleotides, a coding region of 1173 nucleotides (390 amino acids), and a 3′ noncoding region of 665 nucleotides. The ATG initiation codon is contained within a standard Kozak consensus sequence. Kyte-Doolittle hydropathy plot analysis showed that Toso has

two hydrophobic regions: the amino-terminal residues from 1 to 17 correspond to the deduced signal sequence, and the residues from 254 to 272 correspond to a presumptive transmembrane region (Figure 1A, underlined and double underlined, respectively) (Hofmann and Stoffel, 1992), suggesting that Toso is a type 1 integral membrane protein. The predicted molecular weight of Toso is 41 kDa. The cytoplasmic region of Toso has a basic amino acid–rich region (K273–R323), a proline-rich region (P334–P346), and an acidic amino acid–rich region (E378–D384) (Figures 1A and 1B). Basic local alignment search technique (BLAST) analysis revealed that *toso* is a unique gene (Altschul et al., 1990).

The extracellular domain of Toso has homology to the immunoglobulin variable (IgV) domains, which are characterized by motifs in the β strand B, D, and F regions, (residues VTLTC, RV(or F,I) and DSG(or A)-Y-CA) (Williams and Barclay, 1988). Importantly, the cysteines in the IgV-like motif VTIKC at position 33 in Toso, as well as the cysteine in the IgV-like motif DSGVYAC at position 98, are appropriately distanced as in other IgV-like domains to form a disulphide bond. Toso also contains within the immunoglobulin domains two additional cysteines that are not conserved in other IgV-like domains. Thus, the presumptive extracellular domains has all of the requisite features that demarcate it as a potential IgV-like domain. The cytoplasmic region of Toso has partial homology to FAST kinase (Fas-activated serine/threonine kinase), acid sphingomyelinase, insulin receptor substrate-1, and the apoptosis inhibitor from *Orgyia pseudotsugata* nuclear polyhedrosis virus (Figure 1C), which might function to initiate some of the signaling systems acted on by Toso.

Using human × Chinese hamster hybrid cell lines and two human radiation hybrid (RH) mapping panels, the *toso* gene is mapped at 1q31-q32. This region is associated with several chromosomal abnormalities relating to leukemias, as further presented in the Discussion.

Toso Inhibits Fas-, TNFα-, and FADD-Induced Apoptosis through Inhibition of Caspase-8 Processing

To characterize the function of Toso, Jurkat.ecoR (Jurkat cells that express the receptor for ecotropic murine retroviruses) were infected with retroviruses that express Toso and with the control vectors pBabeMN-Toso, pBabeMN-lacZ, and pBabeMN-Lyt-2-α' (Tagawa et al., 1986). Jurkat.ecoR cells expressing Toso were resistant to apoptosis induced by anti-Fas mAb, whereas Jurkat cells, Jurkat.ecoR cells, and Jurkat.ecoR cells that expressed β-galactosidase (*lacZ*) or Lyt-2-α' all succumbed to apoptotic death (Figure 2A).

We then tested the effect of Toso using other apoptotic stimuli. Staurosporine, a bacterial alkaloid, is a broad-spectrum inhibitor of protein kinases and induces programmed cell death in various cell lines and dissociated primary cells in culture (Raff et al., 1993). Ceramide generation is implicated in a signal transduction pathway that mediates programmed cell death induced by Fas and TNFα (Cifone et al., 1994). Although Jurkat.ecoR cells expressing Toso were resistant to Fas-mediated apoptosis over a range of anti-Fas dilutions, these cells
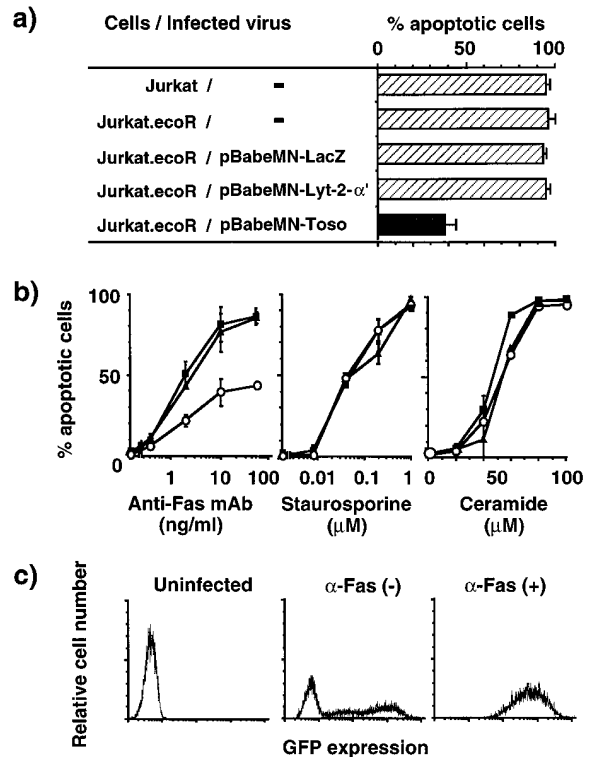
Figure 2. Differential Inhibition of Apoptosis by Toso and Relation to TNFα-Mediated Signaling

(A) Effect of Toso on anti-Fas induced apoptosis. Jurkat.ecoR cells were infected with pBabeMN-lacZ, pBabeMN-Lyt-2-α', and pBabeMN-Toso. Jurkat cells were cultured with 10 ng/ml anti-Fas mAb for 24 hr and apoptotic cells counted. The infection frequencies of pBabeMN-lacZ and pBabeMN-Lyt-2-α' were 45% and 58%, respectively. The percentage of apoptotic cells is expressed as the mean (bars) ± SD of triplicate cultures. The proportion of apoptotic cells in each culture without anti-Fas mAb was less than 2%.

(B) Effect of Toso on anti-Fas-, staurosporine-, and ceramide-induced apoptosis in Jurkat.ecoR cells (triangles) and Jurkat.ecoR cells infected with pBabeMN-lacZ (squares) and pBabeMN-Toso (circles). Cells were cultured with these reagents for 24 hr. The infection frequency of pBabeMN-lacZ was 57%. The percentage of apoptotic cells are expressed as the mean (symbol) ± SD (vertical bar) of triplicate cultures.

(C) Effect of Toso on anti-Fas mAb–induced apoptosis. Cells were infected with pBabeMN-Toso-IRES-GFP. After infection, cells were cultured with (α-Fas (+)) or without (α-Fas (−)) 50 ng/ml of anti-Fas mAb. After culture for 5 days, GFP expression in surviving cells was analyzed by FACScan.

were not resistant to any concentration of staurosporine- or ceramide-induced apoptosis (Figure 2B).

It was necessary to verify that the effect of Toso is on the cells expressing Toso and not due to effects upon bystander cells. We performed a mixing experiment in which 50% of the cells expressed Toso alone and the remaining 50% expressed the *lacZ* product. After one round of Fas stimulation, no *lacZ*-expressing cells remained, as assayed by X-Gal (data not shown). To determine further that the transduced cells are those that are protected from apoptosis, we infected Jurkat.ecoR cells with pBabeMN-Toso-IRES–green fluorescent protein (GFP). In the absence of anti-Fas mAb treatment,

we observed GFP-negative cells (46%) and GFP-positive cells (54%) in pBabeMN-Toso-IRES-GFP–infected Jurkat.ecoR cells. After 5 days of culture with anti-Fas mAb, we obtained survivors from pBabeMN-Toso-IRES-GFP–infected Jurkat.ecoR cells, but not from control pBabeMN-IRES-GFP–infected-Jurkat.ecoR cells (data not shown); 99.7% of surviving Jurkat cells expressed GFP, as shown in Figure 2C. These data indicate that Toso-expressing cells are protected from Fas-induced apoptosis and suggest that Toso does not exert its effect as a secreted form.

Since the Fas receptor has homology to the TNFα receptor, and since these two receptors share analogous signaling systems as well as several intracellular mediators (Hsu et al., 1996), we investigated the protective effect of Toso against TNFα-induced apoptosis. Jurkat.ecoR cells expressing Lyt-2-α′ or Toso were cultured with 10 ng/ml of anti-Fas mAb or 1 μg/ml of TNFα in the presence of 0.1 μg/ml of cyclohexamide for 12 hr. Toso inhibited Fas-induced apoptosis in the presence of cyclohexamide and also protected against TNFα-induced apoptosis in comparison to Jurkat.ecoR expressing Lyt-2-α′ (Figure 3A). Thus it is likely that the TNFα and Fas signaling pathways converge at a common point that can be inhibited by Toso.

Fas-mediated apoptosis is activated through FADD. We therefore examined the effect of Toso on FADD-induced apoptosis. Jurkat.ecoR cells expressing Lyt-2-α′ or Toso were infected with pBabeMN-lacZ or pBabeMN-FADD. The reinfection efficiency was approximately 40% using pBabeMN-lacZ. As shown in Figure 3B, FADD induced apoptosis in 45% of control Jurkat cells. However, FADD failed to induce apoptosis in Jurkat.ecoR cells constitutively expressing Toso. To map the downstream effects of Toso on known inhibitors of apoptosis, we investigated whether Bcl-2 and Bcl-X$_L$ expression levels were altered in Toso-expressing cells. We found that Bcl-2 overexpression can block Fas-induced apoptosis as well as staurosporine-induced apoptosis (data not shown). By Western analysis no change in the levels of expression of Bcl-2 or Bcl-X$_L$ was observed (data not shown). Thus it appears that intracellular signaling events generated by FADD can be directly and efficiently blocked by signals emanating from Toso at a point prior to engagement of Bcl-2 and Bcl-X$_L$. The results also suggest that Toso's effect is not due to down-regulation of FADD gene expression.

Our data suggest that Toso inhibits Fas-induced apoptosis downstream of FADD, which associates with caspase-8. Therefore we examined whether caspase-8 processing is inhibited by overexpression of Toso. As shown in Figure 3C, the processed form (p20) of FLICE after Fas activation was greatly reduced in pBabeMN-Toso–infected Jurkat.ecoR cells in comparison with control Jurkat.ecoR cells. These data indicate that Toso inhibits caspase-8 processing after Fas activation. Recently, several groups have reported that cFLIP is a caspase-8 inhibitor (Hu et al., 1997; Irmler et al., 1997; Srinivasula et al., 1997). We performed semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) to detect cFLIP mRNA expression. As shown in Figure 3D, cFLIP expression was induced in Jurkat.ecoR
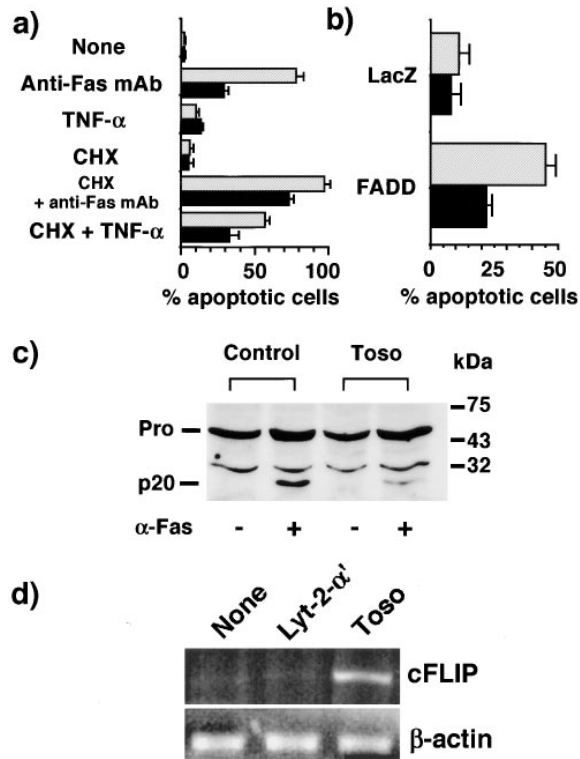

Figure 3. Toso Inhibits Caspase-8 Processing through cFLIP Expression

(A) Effect of Toso on TNFα-induced apoptosis. Jurkat.ecoR cells expressing Lyt-2-α′ (shaded bars) or Toso (solid bars) were cultured with 10 ng/ml anti-Fas mAb or 1 μg/ml TNFα in the presence of 0.1 μg/ml cyclohexamide for 12 hr, and apoptotic cells were counted. The infection frequency of pBabeMN-Lyt-2-α′ was 58%. The percentage of apoptotic cells is expressed as the mean (bars) ± SD of triplicate cultures.

(B) Effect of Toso on FADD-induced apoptosis. Jurkat.ecoR cells were infected with pBabeMN-Lyt-2-α′ (shaded bars) and pBabeMN-Toso (solid bars). The infection frequency of pBabeMN-Lyt-2-α′ was 72%. Jurkat.ecoR cells expressing Lyt-2-α′ or Toso were infected with pBabeMN-lacZ or pBabeMN-FADD, and apoptotic cells were counted at 24 hr after infection. The infection frequency of pBabeMN-lacZ in Jurkat.ecoR cells expressing Lyt-2-α′ and Toso was 39% and 43%, respectively. The percentage of apoptotic cells is expressed as the mean (bars) ± SD of triplicate cultures.

(C) Toso inhibits caspase-8 processing. Jurkat.ecoR cells (control) and pBabeMN-Toso-infected Jurkat.ecoR cells (Toso) were cultured with (+) or without (−) 50 ng/ml of anti-Fas mAb (α-Fas) for 6 hr. Caspase-8 processing was analyzed with an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science, Arlington Heights, IL) with goat anti-Mch5 p20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The positions of pro-caspase-8 (Pro), the processed form (p20), and the standard marker are indicated.

(D) RT-PCR analysis of cFLIP expression in Jurkat.ecoR cells (None), pBabeMN-Lyt-2-α′–infected Jurkat.ecoR cells (Lyt-2-α′), and pBabeMN-Toso-infected Jurkat.ecoR cells (Toso). As a control for loading, we amplified β-actin cDNA for 25 cycles.

cells that overexpressed Toso, in comparison with control Jurkat.ecoR cells and Jurkat.ecoR cells that overexpressed Lyt-2. These results suggest that Toso inhibits Fas-induced apoptosis by preventing caspase-8 processing through cFLIP up-regulation.
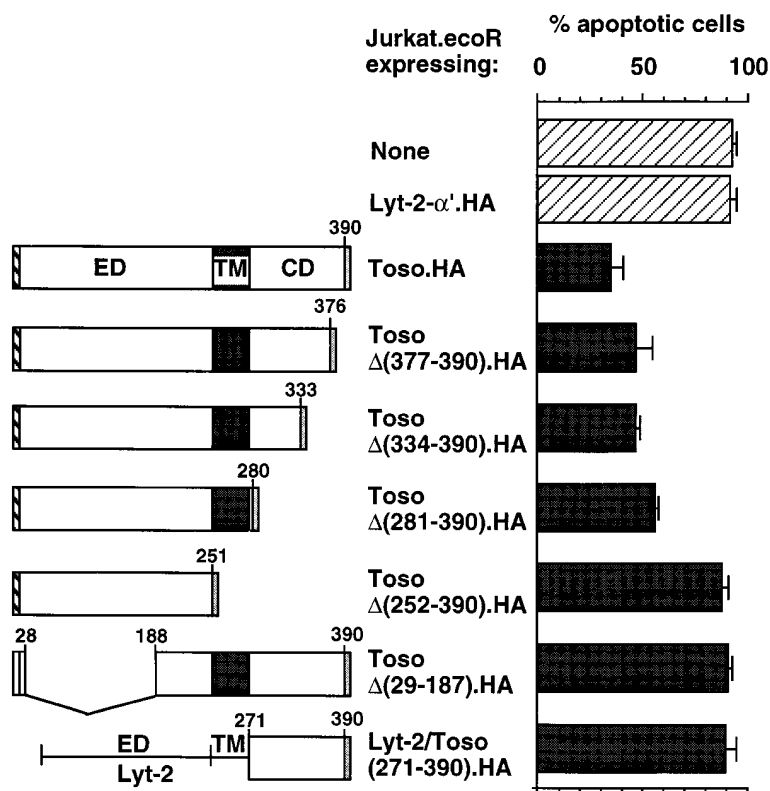
Jurkat.ecoR expressing:

% apoptotic cells

0   50   100

None

Lyt-2-α'.HA

390

| ED | TM | CD | **Toso.HA** |

376

Toso Δ(377-390).HA

333

Toso Δ(334-390).HA

280

Toso Δ(281-390).HA

251

Toso Δ(252-390).HA

28   188   390

Toso Δ(29-187).HA

271   390

ED   TM   **Lyt-2/Toso (271-390).HA**
Lyt-2

Figure 4. Functional Domain Analysis of Toso

Effect of Toso deletion mutant expression on anti-Fas mAb–induced apoptosis. The carboxy-terminal amino acid numbers of each deletion mutant are indicated; the light shaded bar represents the HA tag. Jurkat.ecoR cells expressed with mutants were cultured with 10 ng/ml anti-Fas mAb for 24 hr, and apoptotic cells were counted. The percentage of apoptotic cells is expressed as the mean (bars) ± SD of triplicate cultures. ED, extracellular domain; TM, transmembrane region.

The Immunoglobulin Domain and the Transmembrane Region of Toso Are Required for Inhibition of Fas-Induced Apoptosis

To delineate the regions responsible for anti-apoptotic signal transduction, we assayed epitope-tagged Toso (fused to the hemagglutinin [HA] tag) deletion mutants expressed in our system (Figure 4). The level of expression was similar among most cells expressing the mutants by Western blots (data not shown). Toso.HA had an apparent molecular weight of 60 kDa, suggesting that Toso is heavily glycosylated (data not shown). To explore whether Toso has an effect on Fas expression, we analyzed the cell surface expression of Fas using anti-human Fas mAb, CH11, by fluorescence-activated cell sorting. Fas was expressed at similar levels on the surface of cells expressing either full-length Toso, Toso deletion mutants, or control vector (data not shown). Thus, Toso neither down-regulates Fas nor directly interferes with the ability of the antibody to bind and presumably stimulate Fas.

Next we attempted to induce apoptosis in cells expressing Toso or these mutants using 10 ng/ml anti-Fas mAb. Apoptosis was readily induced in control Jurkat.ecoR cells and Jurkat.ecoR cells expressing Lyt-2-α'.HA, whereas apoptosis was markedly inhibited in Jurkat.ecoR cells expressing Toso.HA (Figure 4). Deletions of regions of the cytoplasmic domain of Toso from 334 to 390 still inhibited apoptosis. Moreover, a Toso deletion mutant lacking the entire cytoplasmic domain still retained substantial anti-apoptotic ability. Thus the cytoplasmic domain of Toso is not absolutely required

for the anti-apoptotic effects on Fas antibody–stimulated cells. These results indicate that the homologies observed in the cytoplasmic region of Toso, as shown in Figure 1C, are not the only sources of the anti-apoptotic signals generated by a Toso complex, although the cytoplasmic regions are required for enhancing the anti-apoptotic effects of Toso.

The Toso mutant lacking the transmembrane and cytoplasmic domains demonstrated that inhibition of Fas-induced apoptosis by Toso requires its insertion into membranes. As shown in Figure 4, TosoΔ(252–390).HA afforded no protection from apoptosis. Expression of the TosoΔ(252–390).HA protein was confirmed by Western blot analysis of culture supernatants (data not shown). We further tested whether the effect of supernatants derived from pBabeMN-TosoΔ(252–390).HA–transfected 293T cells could block Fas-induced apoptosis in Jurkat cells. The TosoΔ(252–390).HA–containing supernatants did not inhibit Fas-induced apoptosis, indicating that a membrane-proximal event dependent on *cis* localization of Toso is required for blockade of the Fas-mediated death signal.

We tested whether the immunoglobulin domain of Toso could influence the anti-apoptotic behavior of this molecule. As can be seen in Figure 4, disruption of the immunoglobulin domain completely abrogated the anti-apoptotic ability of Toso (TosoΔ(29–187).HA). Furthermore, a chimeric Lyt-2-Toso fusion protein in which the cytoplasmic domain of Toso was coupled to the extracellular and transmembrane region of Lyt-2-α' failed to inhibit Fas-induced apoptosis. We also used anti-mouse

CD8α mAb (Lyt-2) to cross-link the Lyt-2-Toso chimeras to determine whether induced multimerization of the Toso cytoplasmic domains enhanced their anti-apoptotic abilities. Jurkat.ecoR cells that express the chimeric Lyt-2-Toso fusion protein did not show any protection against anti-Fas mAb–induced apoptosis in the presence of anti-mouse CD8α mAb (data not shown).

These results suggest that some form of immunoglobulin domain–mediated dimerization of Toso is required to initiate the anti-apoptotic effect in conjunction with the cytoplasmic region of Toso or other cell surface Toso-associating proteins. To test this hypothesis, we cross-linked cell surface molecules on Toso.HA-expressing Jurkat.ecoR cells using the water-soluble cross-linker BS3 (Pierce, Rockford, IL). We detected apparent cross-linking molecular complexes at 150, 240, and 300 kDa (data not shown). This result first indicates that Toso is a surface-expressed receptor. The results are consistent with an association of Toso with another surface protein(s) of molecular weight 90 kDa. The several molecular weights observed for the cross-linked complexes are also minimally consistent with stochiometric mixtures of 60 and 90 kDa molecules.

T Cell Signaling Leading to Apoptosis Is Blocked by Activated Toso

We surveyed *toso* mRNA expression in several human tissues (Human RNA Master Blot, Clontech Laboratories) using the *toso*-coding region probe (1.2 kbp). Toso expression was observed in lymph nodes, lung, and kidney. In addition, we detected faint signals from spleen, thymus, liver, heart, and salivary gland (Figure 5A). To analyze Toso expression in immune tissues, we performed Northern blot analysis (human immune system multiple-tissue Northern Blot II, Clontech). As shown in Figure 5B, we detected endogenous *toso* mRNA species of 2.0 (major), 2.8, 3.5, and 4.3 kbp in lymph node and spleen. The nucleotide length of the cDNA was 1.9 kbp, suggesting that the additional bands might either be alternative splice products or incompletely processed messages. Toso expression was also observed in peripheral blood leukocytes (PBLs) and thymus (Figure 5B). Expression in bone marrow and fetal liver was much lower than that in lymph node and spleen, as seen after overexposure of the blot (data not shown).

To analyze which cell types express Toso, we assayed expression of Toso in several human cell lines by semi-quantitative RT-PCR involving amplification of the 1.2 kbp coding region of *toso* (Figure 5C). We detected *toso* mRNA in lymphoid cell lines such as Jurkat cells (T cell leukemia), CemT4 cells (T cell leukemia), MolT-4 cells (T cell leukemia), HB11.19 cells (B cell lymphoma), and Reh cells (acute lymphocytic leukemia; non-T, non-B). HL-60 cells (promyelocytic leukemia) displayed a consistently weak signal. In contrast, *toso* PCR products were not detected in nonhematopoietic cell lines, including HepG2 cells (hepatoblastoma), 293 cells (kidney, transformed with adenovirus), and Hela cells (cervix, adenocarcinoma). Toso therefore is expressed in cells of hematopoietic cells.

We expressed Toso in several human cell lines, including Jurkat cells, CemT4 cells, SupT1 cells (human T cell
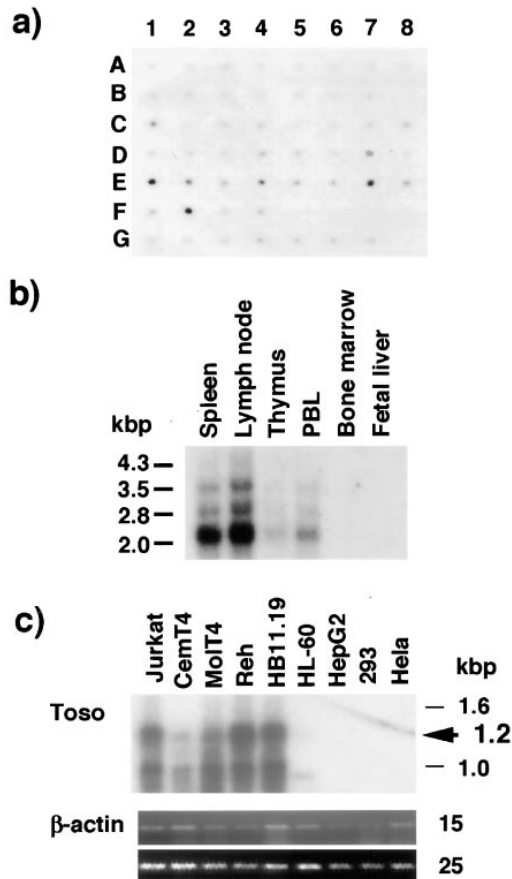

Figure 5. Expression of Toso in Several Human Tissues and Human Cell Lines

(A) mRNA dot blot analysis of the *toso* gene in several human tissues. A1, whole brain; A2, amygdala; A3, caudate nucleus; A4, cerebellum; A5, cerebral cortex; A6, frontal lobe; A7, hippocampus; A8, medulla oblongata; B1, occipital lobe; B2, putamen; B3, substantia nigra; B4, temporal lobe; B5, thalamus; B6, subthalamic nucleus; B7, spinal cord; C1, heart; C2, aorta; C3, skeltal muscle; C4, colon; C5, bladder; C6, uterus; C7, prostate; C8, stomach; D1, testis; D2, ovary; D3, pancreas; D4, pituitary gland; D5, adrenal gland; D6, thyroid gland; D7, salivary gland; D8, mammary gland; E1, kidney; E2, liver; E3, small intestine; E4, spleen; E5, thymus; E6, PBL; E7, lymph node; E8, bone marrow; F1, appendix; F2, lung; F3, trachea; F4, placenta; G1, fetal brain; G2, fetal heart; G3, fetal kidney; G4, fetal liver; G5, fetal spleen; G6, fetal thymus; G7, fetal lung.

(B) Northern blot analysis of the *toso* gene in several human immune tissues. Positions and sizes (kilobase pairs) of *toso* mRNA are indicated. The film was exposed at −70°C with an intensifying screen for 1 day.

(C) RT-PCR analysis of Toso in human cell lines (top). Positions and sizes (kilobase pairs) of Toso and standard nucleotide makers are indicated. As a control for loading, we amplified β-actin cDNA (bottom); the number of cycles is indicated at right.

leukemia), Ocl-Ly8 and AMK cells (both human B cell lines, transformed with Epstein-Barr virus), Reh cells, HL-60 cells, and HepG2 cells using pBabeMN-Toso-IRES-neo to allow cotranslational selection with Geneticin (Gibco BRL). All of the human T cell lines and one of the human B cell lines, Ocl-Ly8 cells, in which Toso was overexpressed, were inhibited for apoptosis induced by anti-Fas mAb, whereas no significant protection was observed against Fas-induced apoptosis in the

other cell lines (data not shown). Thus, the anti-apoptotic effect of Toso also is limited to certain classes of hematopoietic cells, suggesting the presence of tissue-specific mediators in these cells.

T cell activation results in increased expression of Fas and FasL on the cell surface. This is paradoxical, since it is clear that T cells do not kill themselves after such induction, whereas overexpression of Fas and FasL in other cell types does lead to cell death. In vitro, phorbol myristate acetate (PMA) and ionomycin can induce apoptosis in T cells (Oyaizu et al., 1995) by mimicking certain aspects of CD3 engagement, including up-regulation of Fas and FasL. We hypothesized that one function of Toso might be to inhibit T cell–activated self-killing and that the levels of Toso might increase following T cell activation, helping to render Jurkat cells partially resistant to up-regulated Fas and FasL. Expression of *toso* mRNA in Jurkat cells was therefore examined by Northern hybridization. As shown in Figure 6A, we detected an endogenous *toso* mRNA species of 2.8 kbp in resting Jurkat cells, although expression was seen best after overexposure of the blot (data not shown). Interestingly, we found increases in *toso* mRNA expression, including minor species (2.0, 3.5, 4.3, and 5.5 kbp), after stimulation of Jurkat cells with PMA and phytohemagglutinin (PHA) (15-fold increase) or PMA in combination with ionomycin (25-fold increase). Thus, Toso expression can be induced following T cell activation.

We hypothesized that induced endogenous Toso expression would correlate with induced resistance to Fas-mediated apoptosis. As shown in Figure 6B, Jurkat cells were susceptible to anti-Fas mAb–induced apoptosis as well as PMA/ionomycin-induced apoptosis. However, following activation with PMA/ionomycin, one third of Jurkat cells were clearly resistant to anti-Fas mAb–induced apoptosis. These results suggest that Jurkat cells activate a protective system that blocks Fas-mediated apoptosis, supporting the contention that induced protein, such as Toso, is a mediator in this protective effect.

We therefore tested whether Toso expression could rescue activation-induced programmed cell death. We randomly picked five pBabeMN-Toso–infected Fas-resistant Jurkat T cell clones and used them to assay the inhibitory effect of Toso on PMA/ionomycin-induced apoptosis. All five clones exhibited significant resistance to PMA/ionomycin-induced apoptosis, as well as continued strong resistance to Fas-induced apoptosis (Figure 6C). Control clones displayed the expected killing effect when activated with PMA and ionomycin. Toso not only inhibited apoptosis activated by Fas and TNFα, but also inhibited apoptosis induced by certain classes of T cell activation events.

Normal T cells at early stages of activation are resistant to Fas-induced apoptosis but become Fas sensitive at late stages of activation (Klas et al., 1993). We therefore examined Toso expression kinetics after PHA stimulation in peripheral blood mononuclear cells using semiquantitative RT-PCR. We observed Toso expression at day 1 and up-regulated expression at day 3 after activation. However, Toso expression was clearly decreased at days 5 and 7 (Figure 6D), correlating with Fas sensitivity studies (Klas et al., 1993). Furthermore, allogenic stimulation in mixed lymphocyte cultures was
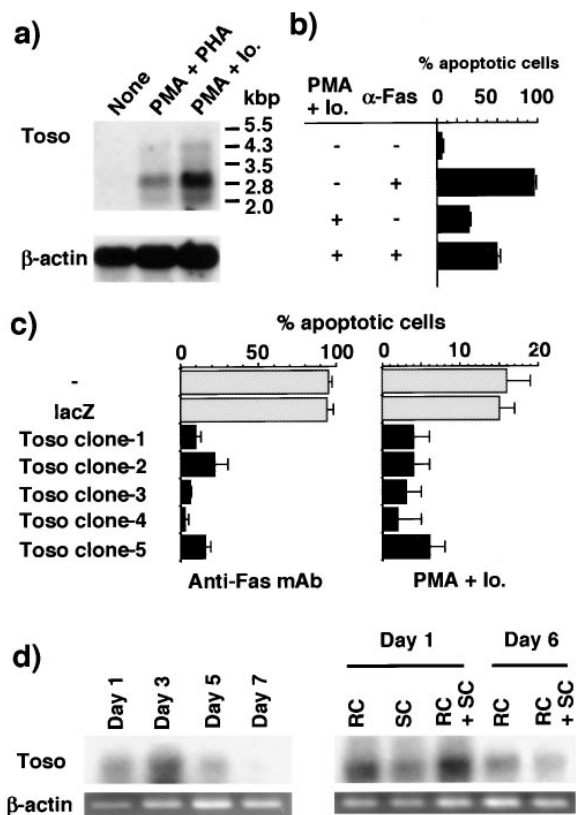

Figure 6. Inducible Expression of Toso and Resistance to Apoptosis after Stimulations with T Cell Activator

(A) Northern blot analysis of the *toso* gene in Jurkat cells (None) and Jurkat cells stimulated with PMA and PHA (PMA + PHA) or PMA and ionomycin (PMA + Io.). The positions and sizes (kilobase pairs) of *toso* mRNA are indicated.
(B) Activation-induced resistance to anti-Fas mAb–induced apoptosis in Jurkat cells. Jurkat cells were precultured with 10 ng/ml PMA and 500 ng/ml ionomycin for 12 hr and then incubated with 10 ng/ml of anti-Fas mAb for 24 hr. The percentage of apoptotic cells is expressed as the mean (bars) ± SD of triplicate cultures.
(C) The effect of Toso on PMA and ionomycin (PMA + Io.)–induced apoptosis. The infection frequency of pBabeMN-lacZ was 42%. Jurkat.ecoR cells (−), Jurkat.ecoR cells infected with pBabeMN-lacZ (lacZ), and pBabeMN-Toso-infected clones (Toso clones 1–5) were cultured with 10 ng/ml of anti-Fas mAb (left) and 10 ng/ml PMA and 500 ng/ml ionomycin (right) for 24 hr. The percentage of apoptotic cells is expressed as the mean (bars) ± SD of triplicate cultures.
(D) RT-PCR analysis of Toso (the 1.2 kbp fragment of Toso) in peripheral blood mononuclear cells after activation with PHA (top left) and in peripheral blood mononuclear cells after allogenic stimulation (top right). For allogenic stimulation, stimulator cells (SC), responder cells (RC), or mixed cells (RC + SC) were cultured for 1 day (day 1) and 6 days (day 6). As a control for loading, we amplified β-actin cDNA (bottom).

performed to determine whether Toso is activated in primary immune cells upon T cell activation. As shown in Figure 6D, Toso expression was also rapidly induced in the presence of stimulator cells on day 1; however, Toso expression in mixed lymphocyte cultures was reduced by day 6. The level of expression on day 6 was lower than the level of expression on day 1 and in responder cells alone on day 6. These results further confirm a supportive role for Toso-induced resistance to Fas-mediated death during T lymphocyte activation.
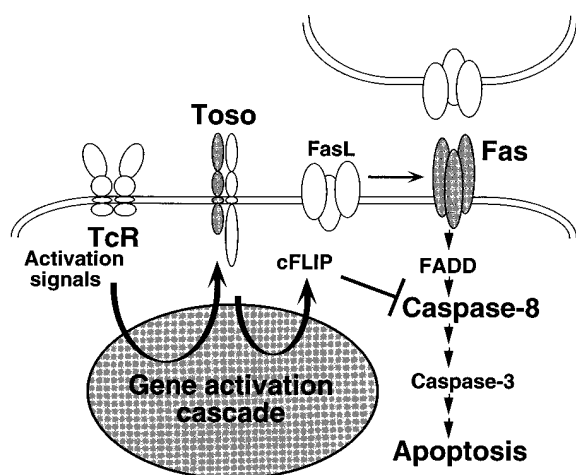
Figure 7. A Model of the Role of Toso in T Cell Activation

In this model, Toso is induced following T cell activation and protects T cells from self-induced programmed cell death. The inhibitory effects of Toso on Fas signaling maps at the level of caspase-8 through induced expression of cFLIP.

Discussion

Using a functional screen for genes that regulate Fas-mediated apoptosis, we cloned and characterized a novel immunoglobulin domain–containing polypeptide, Toso, with potent pathway-specific anti-apoptotic effects in hematopoietic cells. Toso exerted an inhibitory activity against apoptosis induced by Fas-, TNFα-, FADD-, and PMA/ionomycin but not against staurosporine- or ceramide-induced apoptosis. Mechanistically, the block to apoptotic activation, and the pathway specificity of the effect, are most likely explained by inhibition of caspase-8 processing through induction of cFLIP expression (a model summarized in Figure 7). Because Toso is expressed within lymphoid tissues and hematopoietic cells and is enhanced after T cell activation, which also transmits activation signals leading to up-regulation of Fas and FasL, this model suggests an important role for this molecule in immune system processes. As a reflection of its important protective effects, we named the molecule Toso after a Japanese liquor that is drunk on New Year's Day to celebrate long life and eternal youth.

A Specific Inhibitor of Apoptosis
in Hematopoietic Cells
Death signals through Fas and through the TNF type I receptor are mediated by adapter molecules such as FADD/MORT1 as well as by receptor-associated caspases such as caspase-8. Toso inhibited Fas-, TNFα- and FADD-induced apoptosis. Furthermore, Toso up-regulated cFLIP expression and inhibited caspase-8 processing, mapping a primary Toso anti-apoptotic effect at the level of inhibition of caspase-8 activity. Toso did not inhibit staurosporine-induced programmed cell death, and staurosporine has been shown to activate caspase-3 (Jacobsen et al., 1996). Therefore, additional Toso anti–apoptotic effects do not occur either downstream of or at the level of caspase-3. Supporting this

finding, Toso also did not inhibit ceramide-induced apoptosis, which acts downstream of or independently of caspase-3, as demonstrated in experiments using the caspase-3–specific inhibitor tetrapeptide aldehyde DEVD-CHO (Gamen et al., 1996), which does not inhibit ceramide-induced apoptosis. Overexpression of Bcl-2 or Bcl-X$_L$ is known to prevent apoptosis in response to ceramide and staurosporine (Takayama et al., 1995; Zhang et al., 1996; Geley et al., 1997; Susin et al., 1997). Toso did not change the expression levels of Bcl-2 or Bcl-X$_L$ in Jurkat cells, showing that neither Bcl-2 nor Bcl-X$_L$ was involved in the protective activities of Toso. Thus, Toso activates an inhibitory pathway that prevents caspase-8 activation following Fas stimulation, rather than blocking apoptotic signals downstream or at the level of caspase-3. This explains the apparent specificity of the blockade to TNF family–related surface receptors that use caspase-8 for apoptotic signaling.

There is some disagreement over the role of cFLIP in apoptosis. cFLIP in some studies was observed to induce apoptosis in certain situations, although cFLIP itself lacks any caspase activity (Goltsev et al., 1997; Irmler et al., 1997; Shu et al., 1997). Irmler et al. (1997) suggested that the death resulting in some cells from expression of protein is artificial and that the protein's sole function is to inhibit cell-death induction as dominant-negative form of caspase-8. In contrast, Shu et al. (1997) proposed that cFLIP acts as a death activator, prompting activation of caspase-10 upon binding to caspase-3 and also enhancing death by displacement of the putative death-inhibiting c-IAPs (cellular inhibitors of apoptosis protein) from TRAF-2. cFLIP might have multiple functions depending on the cell type and stimulus involved. Its involvment and up-regulation in Toso signaling might reflect a more complex role for Toso in the immune system that is yet to be examined.

This is consistent with the proposal that Toso expression, which transiently increased and then decreased in peripheral blood mononuclear cells after activation with PHA or allogenic stimulation, is responsible for the temporary Fas resistance in T cells. Hence, the results are consistent with the hypothesis that Toso may be involved in activation-induced resistance to apoptosis of T cells during an immune response. We conclude from the results that the inhibitory effect of Toso in activation-induced apoptosis is attributable to the inhibition of Fas-mediated signal transduction through inhibition of caspase-8, possibly mediated through cFLIP.

Domain Analysis of Toso Suggests Multiple
Interacting Partners
Deletion analysis of Toso indicated that surface expression of the IgV-like region is necessary to inhibit Fas-induced apoptosis and that the cytoplasmic domain of Toso is insufficient and indeed partly expendable for the anti-apoptotic function. Deletion of the cytoplasmic domain resulted in abrogation of only about half of the anti-apoptotic effect. This suggests that Toso must be expressed at the cell surface in a manner such that it presumably interacts other surface molecule(s) that propagate an anti-apoptotic signal. We suspect that Toso forms a heterodimer with this other surface protein

to collaborate in initiating the anti-apoptotic signal. Interactions of surface-expressed Toso complexes with ligands on or near target cells might also modulate the ability of Toso to provide anti-apoptotic signaling. We are currently investigating the existence of such ligands and contributory molecules.

Since Toso is a negative regulator of Fas-mediated cell death in lymphoid cells, it therefore could be involved in oncogenic events or resistance to chemotherapy (Friesen et al., 1996). The *toso* gene localizes within human chromosome region 1q31-q32. Chromosomal changes in 1q32 are frequently observed in human cancers, including various types of hematopoietic malignancies and solid tumors (Schmid and Kohler, 1984; Waghray et al., 1986; Jinnai et al., 1990; Yip et al., 1991; Shah et al., 1992; Mertens et al., 1997; Mitelman et al., 1997). Furthermore, studies in nude mice demonstrated that duplication of the chromosome segment of 1q11-q32 is associated with proliferation and metastasis of human chronic lymphocytic leukemic B cells (Ghose et al., 1990), suggesting the presence of dominantly acting growth-regulatory or cell survival genes. Thus, Toso is a candidate for evaluation as a proto-oncogene in several proliferative and metastatic neoplasms.

The finding that Toso can exert cell-specific and signaling pathway–specific effects on apoptosis suggests that other polypeptides exist that act on the Fas death induction cascade. Critically, the demonstration that Toso signaling induces expression of cFLIP suggests the existence of a regulatable transcription cascade that can be activated to block Fas-mediated apoptosis in some cell types. Further analysis of the Toso pathway coupled with gene disruption analysis in mice will provide clues about the overall role that Toso plays in inhibiting activation-induced T cell apoptosis in vivo.

Experimental Procedures

Screening of cDNA Library
A retroviral library containing 2×10^6 independent cDNA inserts was constructed from Jurkat cell mRNA by standard methods (Kitamura et al., 1995; Kinoshita and Nolan, unpublished data), using a retrovirus vector pBabeMN (Kinoshita et al., 1997). We transfected the library into an amphotropic virus packaging cell line, ϕNX-Ampho, which produces infectious retrovirus at up to 5×10^6 infectious units/ml, as described previously. We spin-infected Jurkat cells with the supernatant from ϕNX-Ampho cells at 2500 rpm for 1.5 hr at 32°C. Cells were then placed at 32°C for 12 hr and then incubated at 37°C for 48 hr to allow integration and expression of the retrovirus. We achieved 20%–40% infection as determined by doping of the library with marker retroviruses pBabeMN-lacZ and pBabeMN-Lyt-2-α'.

Jurkat cells were aliquoted into 96-well plates (2×10^4/well) in 100 μl of RPMI 1640 media including 10 ng/ml of anti-human Fas mAb, CH11, (Kamiya Biomedical) and 2.5% fetal calf serum for 5 days. After 5 days, we added an equal volume of fresh media supplemented with 20% fetal calf serum and cultured the cells for an additional 10 days. Jurkat cells, under conditions empirically derived, were sensitive to Fas-mediated apoptosis, with a spontaneous survival rate under our conditions of 2–3 per 10^6 cells. Cells that survived the Fas-mediated killing were identified by outgrowth in the 96-well plate format. Identified wells were expanded and total RNA was extracted. cDNA inserts were rescued using RT-PCR (AMV [avian myeloblastosis virus] reverse transcriptase from Promega and Vent DNA polymerase from New England Biolabs) with primers 5′-GCT CAC TTA CAG GCT CTC TA (LibS) and 5′-CAG GTG GGG TCT TTC ATT CC (LibA), which were located 282 and 56 bp nucleotides upstream and downstream, respectively, of cDNA insert cloning sites.

RNA Detection Using PCR
RT-PCR was performed for 35 cycles using primers 5′-AGG GGC TCT TGG ATG GAC (TosoS) and 5′-CTG GGG TTG GGG ATA GC (TosoA). Hybridization was carried out with the ^{32}P-labeled BamHI–XhoI fragment (510 bp). To detect cFLIP mRNA expression, we amplified a 1.1 kbp fragment (998–2061) of the cFLIP gene with primers 5′-GGG AGA AGT AAA GAA CAA AG and 5′-CGT AGG CAC AAT CAC AGC AT for 35 cycles.

Chromosomal Mapping
Genomic DNAs from a panel of 17 human × Chinese hamster hybrid cell lines (Francke et al., 1986) were analyzed by PCR using Toso-specific primers, 5′-AGA GGC ATA GCT ATT GTC TCG G (sense, located 369 bp downstream of the coding region) and 5′-ACA TTT GGA TCA GGG CAA AG (antisense, 508 bp downstream of the coding region). Specific PCR products were obtained from human genomic DNA and hybrid cell lines that carry human chromosome 1 (data not shown). To map the *toso* gene locus more precisely, two human RH mapping panels were typed by PCR (Cox et al., 1990; Walter et al., 1994). In the Stanford G3 mapping panel G3 (Stanford Human Genome Center), Toso cosegregated with chromosome 1 marker D1S3553, a known marker of chromosome 1 bin 115 on the SHGC RH map, on all 83 Stanford G3 panel RH cell lines. In the GeneBridge 4 mapping panel (Whitehead Institute/MIT Genome Center), Toso is located 5.4 centirays$_{3000}$ and 1.7 centirays$_{3000}$ from D1S504 and WI-9641, respectively. The order of loci in this region from centromere to qter is: D1S412, D1S306, D1S504, Toso, WI-9641, D1S491, D1S237. According to Bray-Ward et al. (1996), the yeast artificial chromosomes (YACs) containing the more proximal markers D1S412 (bin 104), D1S477 (bin 109), and D1S504 (bin 114) were mapped to 1q25-q32, 1q31-q32, and 1q25-q32 respectively, and the YACs containing the more distal markers D1S491 (bin 118), D1S414 (bin 121) and D1S237 (bin 124) were mapped to essentially the same region: 1q31-q32, 1q31-q32, and 1q32-q41, respectively.

Quantification of Apoptosis
We stained cells with 100 μg/ml ethidium bromide and 100 μg/ml acridine orange (both Sigma) in phosphate-buffered saline after incubation with several reagents such as anti-Fas mAb (Kamiya Biomedical Company), staurosporine (Sigma), ceramide (Sigma), PMA (Sigma)/ionomycin (Sigma), and human TNFα (R&D Systems)/cycloheximide (Sigma). Apoptotic cells were identified with ultraviolet microscopy as described (MacGahon et al., 1995). For FADD-induced apoptosis, mouse FADD (a gift from Dr. Angeles Estelles, Stanford University) was ligated into pBabeMN retroviral vector.

Preparation and Culture of Peripheral Blood Mononuclear Cells
PBLs from healthy volunteers were used. Adherent cells were removed by adherence to plastic culture vessels. Cells were activated with PHA-P (1 μg/ml) for 24 hr, washed, and cultured with 20 U/ml of recombinant human IL-2 (R&D Systems). To perform mixed lymphocyte culture, PBLs were treated with 20 μg/ml of mitomycin-C (stimulating cells) for 3 hr and washed. Stimulating cells were adjusted to 7×10^5 cells/ml and cultured with an equal volume and cell density of PBLs (responding cells) from another donor (Clot et al., 1975).

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