The Mouse Cell Surface Protein TOSO Regulates Fas/Fas Ligand-induced Apoptosis through Its Binding to Fas-associated Death Domain*

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Mouse TOSO, the homologue of human TOSO gene, was cloned and characterized in the present study. Using immunofluorescence confocal microscopy we localized TOSO to the cytoplasmic membrane of expressing cells. Using stably transfected mouse TOSO (mTOSO)-expressing Jurkat cells, we show that TOSO protects cells from Fas/Fas ligand- and tumor necrosis factor-induced apoptosis but not from TNF-related apoptosis-inducing ligand-induced apoptosis. The Fas-induced activation of caspase-8 was significantly inhibited by the expression of mTOSO. Using deletion mutants and glutathione S-transferase pull-down approaches, we have shown that mTOSO regulates apoptosis by directly binding to Fas-associated death domain through its C-terminal domain, suggesting the disruption of death-inducing signaling complex formation as mechanism of action. Furthermore, we have expressed mTOSO in transgenic mice and show that mTOSO overexpressing primary T lymphocytes are resistant to Fas/Fas ligand-induced apoptosis. Fas (CD95) is a 45-kDa type I transmembrane protein expressed on various cells (9). FasL (CD95L) is a type II transmembrane protein of 40 kDa that can be secreted in a soluble form (26 kDa) by the action of metalloproteinases. Aggregation of Fas by FasL or agonistic anti-Fas antibodies results in recruitment of the adaptor protein (10). FADD contains two protein interaction domains: a death domain and a death effector domain. The currently accepted model is that FADD binds directly to Fas through interaction between their respective death domains. Consequently, the death effector domain of FADD interacts with the death effector domain of procaspase-8 to form the death-inducing signaling complex (DISC) (11). Juxtaposition of procaspase-8 results in auto-cleavage and activation (12). Active caspase-8 in turn activates downstream effector caspases, such as caspase-3 and -7 (12–14), causing the cell to undergo apoptosis.

Because of the importance of apoptosis in maintaining cell numbers, differentiation, and elimination of certain cells, these processes are tightly regulated. In fact, the balance between apoptotic and anti-apoptotic signals is governed by multiple interacting molecules and at multiple levels. This level of complexity is a prerequisite for proper immune defense and homeostasis. As a case in point, a novel human gene dubbed TOSO was identified as a molecule that blocks Fas-mediated apoptosis by Hitoshi et al. (15). TOSO was found to be expressed mainly by lymphocytes. These investigators also suggested that TOSO regulates apoptosis by inhibiting caspase-8 activation. Nevertheless, the relative importance of TOSO in the complex regulation of apoptosis has not been sufficiently investigated and remains largely unknown. Here we report the cloning and characterization of the mouse homologue of TOSO. We have significantly extended the knowledge about this new molecule and provide evidence for its mechanism of action.

EXPERIMENTAL PROCEDURES

Cloning the Full-length Mouse TOSO (mTOSO) cDNA—Using the sequence of human TOSO gene (15), the expressed sequence tag data bases were explored by saturated BLAST searches for mouse clones that contained human TOSO homologues. This search identified four mouse expressed sequence tag clones: AA290194, 521993, 509857, and 174968. These clones were purchased (from Research Genetics), sequenced, and analyzed; an open reading frame was identified; and the putative mouse TOSO sequence was obtained. The putative cDNA sequence was confirmed by reverse transcriptase-PCR analysis, amplifying mTOSO cDNA from mouse spleen lymphocytes using primers designed from the putative mTOSO sequence. The 5′-untranslated region (84 nucleotides) and 3′-untranslated region (139 nucleotides) were obtained by 5′- and 3′-rapid amplification of cDNA ends (Invitrogen), respectively. The obtained sequences were deposited in GenBank (accession numbers 514618 and 514626). The coding sequence cDNA of mTOSO was cloned into mammalian expression vector pRC/CMV (Invitrogen) at the HindIII/XbaI sites and named pRC/CMV-TOSO.

Plasmid Constructs—For cloning purposes all PCRs used Pfu high

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1 The abbreviations used are: TNF, tumor necrosis factor; mTOSO, mouse TOSO; FasL, Fas ligand; GST, glutathione S-transferase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; DR, death receptor; FACS, fluorescence-activated cell sorting; Ab, antibody; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline; CMV, cytomegalovirus; a.a., amino acids; pNA, p-nitroaniline.
fidelity DNA polymerase enzyme (Stratagene). The plasmid pRC/CMV-TOSO-HA, which encodes HA-tagged mTOSO at the C terminus, was constructed by PCR cloning. Using pRC/CMV-TOSO as the template, the TOSO-HA fragment was generated by amplifying with linker primers 5′/H11032-CCCAAGCTTTGTGTGTCAGCCTCACT-3′ and 5′/H11032-GCTCTAGATCAAGCGTAATCTGGTACGTCGTATGGGTATTGGCATGAAGATCTGGG-3′/H11032. The PCR product was digested with HindIII and XbaI. The vector pRC/CMV was digested with HindIII and XbaI. The digested PCR fragments of HA-tagged mTOSO was ligated into the digested pRC/CMV.

For constructions of mTOSO deletions, various fragments of mTOSO were generated by PCR cloning, and the fragments were then digested with HindIII and NotI. TOSO deletion construct 2 containing amino acids 16–422 was generated with primers 5′/H11032-CCCAAGCTTCACCATGGACAGCCACCACA-3′ and 5′/H11032-TTTTCCTTTTGCGGCCGCTCATGGGCATGA-3′/H11032. Deletion construct 3 containing amino acids 232–422 was generated by primers 5′/H11032-CCCAAGCTTCACCATGGACAGAGCCTCCT-3′/H11032 and 5′/H11032-TTTTCCTTTTGCGGCCGCTCATGGGCATGA-3′/H11032. Deletion construct 4 containing amino acids 291–422 was generated by primers 5′/H11032-CCCAAGCTTCACCATGGACAGAGCCTCCT-3′/H11032 and 5′/H11032-TTTTCCTTTTGCGGCCGCTCATGGGCATGA-3′/H11032. Deletion construct 5 containing amino acids 1–290 was generated by 5′/H11032-CCCAAGCTTTGTGTGTCAGCCTCACT-3′/H11032 and 5′/H11032-TTTTCCTTTTGCGGCCGCTCACCTCCTTTGAAAT-3′/H11032. Deletion construct 6 containing amino acids 1–231 was generated by primers 5′/H11032-CCCAAGCTTTGTGTGTCAGCCTCACT-3′/H11032 and 5′/H11032-TTTTCCTTTTGCGGCCGCTCAGTAGCTGGC-3′/H11032. The vector pRC/CMV was digested with HindIII and XbaI and ligated with digested TOSO PCR fragments.

Fig. 1. A, alignment of the predicted amino acid sequences of human TOSO and mouse TOSO gene products presented in single amino acid code. An asterisk denotes the same amino acid, and a dash denotes a gap. B, Kyte-Doolittle hydropathy plot analysis of human TOSO (390 a.a.) and mouse TOSO (422 a.a) proteins. Both proteins have a hydrophobic leader sequence and a transmembrane domain as shown in the plot.
products. All of the plasmids were propagated in Escherichia coli DH5α strain (Invitrogen) and extracted by an EndoFree Plasmid Maxi Kit (Qiagen).

**Generation of TOSO Stably Transfected Cell Lines—**TOSO-negative Jurkat cells were transfected with pRC/CMV-TOSO that contains the full-length mTOSO cDNA and a neo G418-resistant cassette by electroporation with a Gene Pulser (Bio-Rad) set at 260 V, 960 microfarads according to the manufacturer’s instructions. As a negative control, the cells were transfected with the vector pRC/CMV. To determine the mRNA expression of transfected mouse TOSO gene, reverse transcriptase–PCR was performed. The clones were selected in the presence of 800 μg/ml G418 (Invitrogen) 72 h post-transfection for 2 weeks. Individual lines were obtained by limiting dilution and screened by PCR with primers specific for mTOSO gene: 5’-CGTAAAGGATGATCTGTGTC-3’ and 5’-CTATGACTCCAGAAGCTG-3’. The correct sequence was verified in three selected clones with vector-specific primers by DNA sequencing on an ABI prism 3100 Genetic Analyzer (Applied Biosystems).

**Cell Culture and Transfection—**The human embryonic kidney (HEK) cell line 293 was propagated and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Human T lymphoma Jurkat cells were propagated and maintained in RPMI medium supplemented with 10% fetal bovine serum. All of the cells were cultured at 37 °C with 5% CO2 in humidified atmosphere. The cells were transfected with Effectin reagent (Qiagen) as directed by the manufacturer.

**Immunofluorescence Confocal Microscopy—**The HEK 293 cell line was found to be TOSO-negative but expressed the Fas cell surface protein. HEK 293 cells were transiently transfected with pRC/CMV-TOSO-HA by Effectin reagent (Qiagen) according to the manufacturer’s instructions and were cultured on six-well, covered chamber slides. At 36 h post-transfection, the cells were washed with PBS and then fixed with 3.7% formaldehyde for 15 min at room temperature. After washing with wash buffer (1% Triton in PBS), specimens were incubated with blocking solution (1%Triton, 1% nonfat milk, and 1% bovine serum albumin in PBS) for 30 min at room temperature. Specimens were incubated with either 0.1% (v/v) mouse anti-Fas antibody Clone DX2 (PharMingen), or recombinant anti-HA antibodies at 1:100 (Clontech) for 1 h at 37 °C. After three 5-min washes with wash buffer at room temperature, secondary goat anti-rabbit IgG antibodies conjugated with fluorescein isothiocyanate (Caltag) or rabbit anti-mouse IgG antibodies conjugated with Alexa Fluor 568 (Molecular Probes) were applied and incubated for 1 h at 37 °C. After washing with PBS, the specimens were subjected to confocal fluorescent microscopy.

**Apoptosis Induction and Measurement—**Transiently or stably transfected Jurkat cells and primary T cells extracted from mice spleens were treated with or without various concentrations of one or more of the following reagents: soluble recombinant human Fas Ligand (Alexis, Carlsbad, CA), the agonistic anti-Fas antibody JO2 (PharMingen), human recombinant TNF-α (Sigma), or recombinant TRAIL (Alexis) for 12 h. At fixed concentration or different concentrations for various time durations as indicated in the figures. For TNF-α- and TRAIL-induced apoptosis, the cells were co-treated with 10 μg/ml cycloheximide (Sigma). For glucocorticoid-induced apoptosis, purified primary T lymphocytes from mouse spleens were treated with dexamethasone (Sigma). 5 × 10^6 cells seeded in 96-well plates were treated with various concentrations (10^{-3}–10^{-5} M) Dexamethasone diluted from a 10^{-2} M solution stored in ethanol or with 1.10000 dilution of ethanol alone as vehicle control for 12–24 h. Following the various treatment protocols, the cells were washed in PBS, stained with propidium iodide and annexin V conjugated with fluorescein isothiocyanate in binding buffer (PharMingen) and subjected to FACS analysis on a FACSscan (Becton-Dickinson) cytometer.

**CAUTION: Activity Assay—**Caspase-8 activity was measured by ApoAlert caspase-8 Assay (Clontech) according to the manufacturer’s instructions. Briefly, cells treated with or without FasL for various periods were lysed, and the supernatant was mixed with reaction buffer containing IETD-pNA as the calorimetric substrate. Absorbance at 405 nm was determined at various time points following initiation of reaction. Fold increase in caspase-8 activity was calculated by subtracting the background (cell lysates and buffers) from the reading of induced and uninduced samples.

**In Vitro GST Pull-down Assays—**Plasmids containing full-length mTOSO or various deletions of mTOSO protein were transcribed and translated in vitro. In vitro translation was performed with rabbit reticulocyte lysate (Promega) and [35S]methionine (Promega) according to the manufacturer’s instructions. GST-FADD (gift from Dr. Marcus E. Peter, University of Chicago) was transformed into bacteria and induced by isopropyl β-D-thiogalactopyranoside. GST-FADD fusion protein was immobilized on glutathione-Sepharose beads (Sigma) and incubated with in vitro translated proteins for 2 h at 4 °C in binding buffer (PBS (1% Triton, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride)) according to the manufacturer’s instructions. The beads were then washed five times in binding buffer and analyzed by SDS-PAGE. The bands were visualized by autoradiography.

**Generation of lck-mTOSO Transgenic Mice—**mTOSO cDNA was inserted into the BamHI site of the p1017 expression vector containing the lck proximal promoter (33). The 6.9-kb NotI fragment containing lck proximal promoter, mTOSO, and human growth hormone fragment was then injected into (C57BL/6 × DBA/F1) blastocysts and implanted into pseudo-pregnant mice. Founder mice were initially screened by PCR analysis of tail DNA using primers specific for human growth hormone gene (hGH) (5’-TAGGAAGAAGCTTATATCCCAAAGG-3’ and 5’-ACAGTCTTCTAAAGTCAGTGGGG-3’). The transgene-positive lines were further confirmed by Southern blot analysis probed by 32P-labeled hGH fragment generated by PCR using the primers listed above (Random Prime Labeling; Amersham Biosciences). Expression of mTOSO in lymphocytes of transgenic mice was confirmed by Northern blot analyses. These mice were bred and maintained in the animal facility at the University of Southern California School of Medicine with the approved Animal Care and Use Committee protocol.

**Isolation of Primary T Cells—**Primary T cells from transgenic mice and nontransgenic littermates were extracted from spleens of 7–9-week-old mice by grinding the tissue with the plunger of 1-ml sterile syringes and were then suspended in complete RPMI 1640 medium (10% (v/v) fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, 1% (v/v) nonessential amino acids, 2 mM l-glutamine, 10 mM HEPES buffer, and 50 μM 2-mercaptoethanol) (Invitrogen). Spleen cells were treated with a hemolysis buffer (17 mM Tris-HCl and 140 mM NH4Cl, pH 7.2) to remove red blood cells. Adherent cells were separated by incubation for 1 h on a 10-cm dish. To prepare splenic T cells, nonadherent cells were passed through a 40 μm nylon wool column.

**Statistical Analyses—**All of the experiments were performed at least three times. The data are presented as the means ± S.E. The significance of the difference between experimental and control groups was analyzed with Student’s t test. A value of p < 0.05 was considered to be statistically significant.

**RESULTS**

The mouse TOSO cDNA obtained from mouse spleen lymphocytes encodes a 422-amino acid protein that shares a 57.7% homology with the 390-amino acid-long human TOSO protein (Fig. 1A). On the nucleotide level, a 70.2% homology exist between the mouse and the human TOSO cDNAs (data not shown). As shown in Fig. 1B, analysis of Kyte-Doolittle hydropathy plot displayed striking similarity of mouse TOSO to human TOSO. Computer analysis of mTOSO protein indicated a leader sequence corresponding to amino acids 1–17 and a transmembrane domain spreading from amino acids 258 to 286. It was also predicted by computer analysis that mTOSO is a type I membrane protein with an intracellular C-terminal domain. The predicted molecular mass of mTOSO is about 47 kDa. The cytoplasmic region has a basic amino acid-rich region and an acidic amino acid-rich region. The extracellular domain has homology to the immunoglobulin variable region (IgV)
domains. The cytoplasmic region has partial homology to FAST kinase.

Mouse TOSO Is a Cytoplasmic Membrane Protein—To determine the predicted subcellular localization of mTOSO, confocal microscopic analysis was performed by immunostaining HA-tagged TOSO. HEK 293 cells were transiently transfected with
pRC/CMV-TOSO-HA, which encodes C-terminally tagged mouse TOSO driven by CMV promoter. At 36 h post-transfection, mTOSO was detected by rabbit anti-HA Ab and visualized with anti-rabbit IgG conjugated with fluorescein isothiocyanate (green) on the cell surface (Fig. 2, middle image). As a control, the endogenous cell surface protein Fas was detected in these cells by mouse anti-human Fas Ab (DX2), and stained with Alexa Fluor 568 (red) (Fig. 2, left image). The overlapping of Fas and TOSO localizations is shown in the merged image (Fig. 2, right image). These results confirm the predicted cell surface localization of mouse TOSO. Furthermore, the superimposed images of mTOSO and the Fas protein support the co-localization of these two molecules.

Mouse TOSO Protects Jurkat Cells from Fas/FasL- and TNF-induced Apoptosis—Transfection experiments were performed to test the effects of mTOSO on apoptosis. TOSO-negative Jurkat cell line was stably transfected with pRC/CMV-mTOSO, containing the full-length mTOSO cDNA and a neo G418-resistant cassette. As a negative control, the cells were transfected with vector pRC/CMV. As shown in Fig. 3A, three positive clones (Cl-1, Cl-2, and Cl-3) express mTOSO mRNA, whereas parental Jurkat cells, and three negative clones (Cl-4, Cl-5, and Cl-6) that were transfected with the vector only do not express the transfected gene. To test whether ectopic expression of mTOSO could protect cells from apoptosis, stably transfected clones, control clones, and parental Jurkat cells were treated with various concentrations of FasL (0–100 ng/ml) for up to 24 h. As shown in Fig. 3 (B and C), clones with stable expression of mTOSO significantly protected cells from FasL-induced apoptosis as compared with parental Jurkat cells and control lines. The effect was time-dependent (Fig. 3B) and dose-dependent (Fig. 3C). Similarly mTOSO protected Jurkat cells from apoptosis induced by TNF-α in the presence of cyclohexamide (Fig. 3D).

TRAIL/Apo2L is another member of TNF superfamily that induces apoptosis through engagement of death receptors. Jurkat cells are susceptible to TRAIL-induced apoptosis. However, as shown in Fig. 3E, mTOSO expressing Jurkat cell lines were as susceptible to TRAIL-induced apoptosis as the TOSO-negative Jurkat cells.

Mouse TOSO Inhibits Caspase-8 Activation—The extrinsic apoptotic signal triggered by binding of FasL to Fas is transduced through the sequential assembly of DISC, which is composed of adaptor protein FADD and procaspase-8 (11); therefore, the activity of the apical molecule caspase-8 in this pathway was examined. Stably transfected mTOSO expressing clone (Cl-3) cells, along with the control clone (Cl-4) cells, were treated with various concentrations of FasL for different periods of time. The activation of caspase-8 was determined by enzyme assays measuring its protease activity capable of cleaving substrate IETD-pNA. As shown in Fig. 4, the expression of mTOSO significantly suppressed the activity of caspase-8, compared with control cells transfected with vector only.

Mouse TOSO Binds Specifically to FADD through Its C-terminus Domain—The activation of pro-caspase 8 is attained by being recruited to FADD molecule (13) and subsequent formation of DISC complex (11, 16). Therefore, the disruption of FADD and caspase-8 binding by mTOSO could be one of the potential mechanisms of how mTOSO executes its anti-apoptotic function. To test this hypothesis, in vitro protein binding assays were performed. As demonstrated in Fig. 5, [35S]methionine-labeled in vitro translated full-length mTOSO protein (lane 3) binds to immobilized GST-FADD protein (lane 8), whereas it did not bind to GST (lane 5). Furthermore, the addition of unlabeled mTOSO competed with the 35S-labeled mTOSO (lane 9), indicating the binding specificity between mTOSO and FADD molecules.

To further determine the specific domain in mTOSO involved in binding, various deletion mutations of mTOSO were also studied in the GST-FADD pull-down assays. As illustrated in Fig. 6A, construct 1 represents full-length protein (a.a. 1–422), construct 2 (a.a. 16–422, representing the protein without the signal peptide), construct 3 (a.a. 232–422, including the transmembrane and intracellular domain), construct 4 (a.a. 291–422, containing the C-terminal tail of the protein only), construct 5 (a.a. 1–290, lacking the C-terminal portion of the molecule), and construct 6 (a.a. 1–231, lacking the transmembrane and the entire intracellular domain of the molecule). The various deletion constructs were translated in vitro and [35S]methionine-labeled (Fig. 6B). Fig. 6C demonstrates the binding of the different constructs to immobilized GST-FADD plasmid did not compete with the 35S-labeled mTOSO (lane 9), indicating the binding specificity between mTOSO and FADD molecules.
and GST, respectively. The results indicate that the cytoplasmic C-terminal domain of mTOSO, but not the extracellular N-terminal domain, was required for binding. Even the construct expressing only the tail portion of the cytoplasmic domain (lane 6) shows specific binding to GST-FADD.

Primary T Cells Isolated from TOSO Transgenic Mice Are Resistant to Fas/FasL-induced Apoptosis—To confirm the relevance of mTOSO in regulating lymphocyte apoptosis in vivo, we produced transgenic mice that overexpress mTOSO in peripheral lymphocytes. Peripheral lymphocyte expressing mTOSO transgenic mice were established by injecting full-length cDNA of the mTOSO gene construct under the proximal lck promoter. Systematic characterization of these transgenic mice is ongoing and will be published separately. However, for the purpose of the present study, primary T cells were isolated from transgenic and nontransgenic littermates and evaluated for their capability to undergo apoptosis. Although primary mouse T lymphocytes are known to be resistant to TNF-α-induced apoptosis, they are susceptible to Fas/FasL-induced as well as glucocorticoid-induced apoptosis (17).

As shown in Fig. 7 T lymphocytes overexpressing mTOSO were significantly more resistant to FasL- or anti-Fas Ab (Jo2)-
FIG. 7. Primary T cells isolated from mTOSO transgenic mice are resistant to Fas/FasL-induced apoptosis. A, primary T lymphocytes purified from mTOSO transgenic and nontransgenic littermates were treated with FasL (40 ng/ml), agonistic anti-Fas Ab (Jo2, 100 ng/ml), TNF-α (100 ng/ml), or dexamethasone (Dex) at $10^{-6}$ M and $10^{-5}$ M for 24 h. Apoptosis was measured by staining cells with annexin V and propidium iodide and analyzed on a FACSscan cytometer. T cells were from 8–11 mice in each of the groups shown. *, statistical significance at $p < 0.001$. B, dose-dependent curves of primary T lymphocytes from mTOSO-expressing and non-mTOSO-expressing littermates treated with various concentrations of FasL as indicated. The inset shows expression of mTOSO in these seven mice depicted by PCR.
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induced apoptosis than T cells obtained from nontransgenic littermates. Fig. 7B shows dose-dependent curves obtained from four transgenic and three nontransgenic littermate mice treated with FasL. On the other hand, there was no significant difference in the susceptibility of these T cells to apoptosis induced by the glucocorticoid dexamethasone. Fig. 7A shows cell death when treated with dexamethasone at 10^{-5} and 10^{-6} m, respectively. These results support the specificity of mTOSO in regulating lymphocyte apoptosis.

DISCUSSION

Several years ago Hitoshi et al. (15) cloned a human molecule that was capable of specifically inhibiting apoptosis induced by certain members of the TNF receptor family. As a reflection of the predicted importance of this molecule in protecting lymphocytes from apoptosis, the investigators named the molecule TOSO after the Japanese liquor "that is drunk on New Year's Day to celebrate long life and eternal youth" (15). However, to our knowledge, no additional publications followed the original paper, suggesting perhaps a very short life of the molecule in the scientific literature. We have taken, therefore, a systematic approach in characterizing the mouse homologue of TOSO to first verify and confirm the suggested importance of this molecule.

In this article, we report the cloning and characterization the mouse homologue of the human TOSO gene (15). The 422-amino acid mouse TOSO has 57.7% homology with the 390-amino acid human TOSO. The analysis of Kyte-Doolittle hydrophathy plot displayed striking similarity of mouse TOSO to human TOSO, both having a hydrophilic leader sequence and a transmembrane domain. To directly demonstrate the cell membrane localization of mTOSO, we conducted immunostaining and confocal microscopy experiments, whereas the C-terminal HA-tagged mTOSO was visualized on cell membrane surfaces (Fig. 2). Also, the deletion mutation studies of mTOSO by in vitro protein binding assays confirm that mTOSO is indeed a type I membrane protein whose C-terminal is involved in FADD binding (Fig. 6).

To study the function of mouse TOSO, we established stably transfected Jurkat cell lines. Clones that contain transfected mTOSO gene significantly protected cells from FasL-induced apoptosis compared with the control Jurkat cells (Fig. 3). These results not only revealed that we had successfully cloned the functional mouse homologue of TOSO, but also it unequivocally demonstrated that mTOSO inhibits apoptosis induced by FasL.

In accordance with the notion that the Fas pathway shares some factors mediating the signal transduction with TNF-α signaling, our results show that mTOSO also protects cells from TNF-α-induced apoptosis. Because caspase-8 is the apical molecule in these pathways, we therefore examined whether the activation of this molecule was suppressed upon apoptosis triggering. Indeed, the caspase-8 activity in Jurkat cells expressing mTOSO was significantly inhibited compared with control Jurkat cells (Fig. 4). On the other hand, results indicate that expression of mTOSO in Jurkat cells does not protect cells from TRAIL-induced apoptosis. These results are somewhat surprising given that FADD and caspase 8 have been shown to associate with the endogenous TRAIL receptors DR4 and DR5 (3, 18–21), and the apparent involvement of TOSO in DISC formation is illustrated by the present study. However, other investigators have found that a FADD-independent pathway links TRAIL to caspases. Thus, ectopic expression of a dominant negative mutant of FADD, inhibited Fas-induced cell death, but did not block apoptosis triggered by TRAIL (22). Unlike Fas, TNF receptor 1, and DR3, the TRAIL receptor DR4 could not use FADD to transmit the death signal (23). Furthermore, FADD-deficient embryonic fibroblasts were susceptible to DR4-induced apoptosis but were resistant to Fas-, TNF receptor 1-, and DR3-induced cell death (24). An extra layer of complexity is added by the fact that except Fas, other TNF family receptors depend on additional intermediate proteins rather than direct binding to FADD. For instance, TNF receptor 1, DR3, and DR6 bind to the intermediate protein TRADD, which functions as the docking site for FADD (2, 25). In particular, DAP3 is the intermediate protein that links DR4 and DR5 to FADD in TRAIL-induced apoptosis (26). Therefore, our observations support the notion that multiple pathways involving other yet to be determined factors exist in TRAIL-triggered apoptosis (27).

Hitoshi et al. (15) postulated that human TOSO inhibits caspase-8 activation through increasing mRNA level of cFLIP, which disturbs DISC formation (28). However, rapid assembly of DISC complex that is detectable 60 min after Fas induction in Jurkat cells and within 30 min in SKW6.4 B lymphoblastoid cells (28, 29) argues against such mechanism given the much longer time necessary for transcription and translation of cFLIP. In contrast, a faster response mechanism in which a pre-existing factor TOSO inhibits Fas-induced apoptosis through the disruption of DISC complex formation and consequently the inhibition of caspase-8 activation is more likely to be the modus operandi.

Based on the results obtained in this study, Fig. 8 is our present model of the mechanism of how mTOSO executes its anti-apoptotic function. Thus, we propose that TOSO binds via its C-terminal tail directly to FADD and thus interferes with the recruitment of pro-caspase 8 to FADD molecule and/or its activation. Furthermore, Fas receptor DISC immunoprecipitation experiments have shown a stable ligand-receptor complex that contains FADD and caspase-8 (2). It is therefore believed that this initial caspase activation event occurs at the cell membrane. The localization of mTOSO to the cell membrane (Fig. 2) supports our proposal that the signaling event induced by TOSO occurs also at the cell membrane.

Lastly, the anti-apoptotic function of mTOSO was confirmed in primary lymphocytes from mice that overexpress mTOSO under the lck promoter. Primary T cells from mTOSO transgenic mice showed enhanced resistance to FasL or agonistic anti-Fas Ab stimulation. This effect seems to be highly specific, because these same T lymphocytes were still sensitive to glucocorticoid-induced apoptosis. The importance of the Fas/FasL apoptotic pathway in vivo is underscored by the fact that mice...
that have a recessive mutation in Fas (lpr) (30) or in the FasL (gld) (31) develop fatal systemic autoimmunity and lymphoproliferation (32). In this respect it is noteworthy that TOSO knock-out mice are embryonically lethal. Therefore, we hope that further analysis (in progress) of the TOSO overexpressing transgenic mice will yield more insight into the function of TOSO in vivo. Given the importance of maintaining a proper balance of apoptotic and anti-apoptotic signals in the immune system, these studies will be able to determine whether TOSO can be a potential therapeutic target for pharmacological manipulation of autoimmune diseases.

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