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# A Novel Gene Product that Couples TCR Signaling to Fas(CD95) Expression in Activation-Induced Cell Death

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## Summary

**Cross-linking the TCR in T cell hybridomas induces cell apoptosis following activation. This activation-induced apoptosis has been used as a model for clonal deletion of thymocytes or peripheral T cells. Anti-TCR-induced apoptosis of T cell hybridomas requires de novo macromolecular synthesis, including up-regulation of Fas and FasL. The Fas–FasL interactiom then activates the apoptosis program. To study apoptosis-specific signaling processes, we generated a mutant T cell hybridoma line defective in induction of apoptosis, but competent to induce activation, upon TCR triggering. Subsequently, we cloned the gene *TDAG51*, which restored activation-induced apoptosis when transfected into the mutant cell line, and showed that *TDAG51* expression was required for Fas expression. Thus, *TDAG51* plays an essential role in induction of apoptosis by coupling TCR stimulation to Fas expression.**

## Introduction

The peripheral T cell repertoire is determined by positive and negative selection of immature thymocytes during development in the thymus (Nossal, 1994; von Boehmer, 1994). Most self-reactive immature thymocytes are eliminated during development by negative selection (clonal deletion) to establish immunological self-tolerance (Kappler et al., 1987; Sha et al., 1988; Murphy et al., 1990; Kawabe and Ochi, 1991). This process of clonal deletion is thought to be mediated by apoptosis (MacDonald and Lees, 1990; Murphy et al., 1990). Apoptosis also plays a major role in the clonal deletion of autoreactive T cells in the periphery and in the elimination of activated T cells after mounting a proper immune response (Webb et al., 1990; Kawabe and Ochi, 1991; Lenardo, 1991; Russell et al., 1991).

Much of the signaling processes during activation-induced apoptosis of T cells has been studied using T cell hybridomas (Ashwell et al., 1987; Ucker et al., 1989; Shi et al., 1992). When stimulated with antigens or anti-T cell receptor (TCR) antibodies, T cell hybridomas produce interleukin-2 (IL-2) but also undergo apoptosis (Ucker et al., 1989, 1994; Green et al., 1994). Similar to the apoptosis of normal T cells induced by anti-TCR antibody or agonistic ligands, the addition of actinomycin D, cycloheximide, or cyclosporin A inhibited anti-TCR-induced apoptosis of T cell hybridomas (Ucker et al., 1989, 1994; Green et al., 1994). In addition, glucocorticoids or irradiation also induces apoptosis in T cell hybridomas (Ucker et al., 1989, 1994; Green et al., 1994). Because of these similar properties, T cell hybridomas have been used as an in vitro model for the study of anti-TCR-induced apoptosis of thymocytes and T cells.

Using T cell hybridomas, several experiments identified a few genes that appeared to be important in the regulation of anti-TCR-induced apoptosis. It was shown that anti-TCR-induced apoptosis of T cell hybridomas is in part mediated by Fas–Fas ligand (FasL) interaction (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Nagata and Golstein, 1995; Yang et al., 1995). It was shown that inhibition of c-*myc* expression by antisense oligonucleotides blocked anti-TCR-induced apoptosis of T cell hybridomas (Shi et al., 1992; Green et al., 1994). *nur77*, a gene that is induced by anti-TCR antibody treatment of T cell hybridomas (Liu et al., 1994; Woronicz et al., 1994; Yazdanbakhsh et al., 1995), is required for induction of anti-TCR-induced apoptosis of T cell hybridomas (Liu et al., 1994; Woronicz et al., 1994; Calnan et al., 1995). In addition, the function of *nur77* was blocked by cyclosporin A, a reagent that also inhibits anti-TCR-induced apoptosis (Yazdanbakhsh et al., 1995). Furthermore, transgenic mice expressing a dominant negative form of *nur77* showed some defects in clonal deletion of self-reactive thymocytes (Calnan et al., 1995). Although it has been suggested that T cell hybridomas can only model peripheral T cell deletion because Fas–FasL plays a pivotal role in anti-TCR-induced apoptosis of T cell hybridomas, experiments on *nur77* by Calnan et al. (1995) suggest that apoptosis-regulatory genes (e.g., *nur77*) identified in T cell hybridomas may also turn out to play a role in the deletion of immature thymocytes. Therefore, results derived from experiments on T cell hybridoma cell death can parallel thymocyte and peripheral T cell death in vivo.

Despite identification of several genes involved in anti-TCR-induced apoptosis, the mechanisms of how these gene products interact to regulate the apoptosis of T cells is not well understood, which may require the identification of more apoptosis–regulatory genes. To facilitate the identification of anti-TCR-induced death signaling–specific genes, we have adopted a system involving somatic cell genetics. In this study, we generated a T cell hybridoma mutant resistant to the anti-TCR-induced apoptosis, but showing normal activation processes. By differential cDNA library screening and transfection experiments, we identified a novel gene, *TDAG51*, which complemented the defects in the mutant cell line. *TDAG51* encodes a proline–glutamine–histidine-rich protein and shows some similarity to transcription activators. Subsequent studies showed that functional *TDAG51* is required for Fas(CD95) expression, which plays a critical role in the activation-induced apoptosis of T cell hybridomas.

[‡]The first two authors contributed equally to this work.

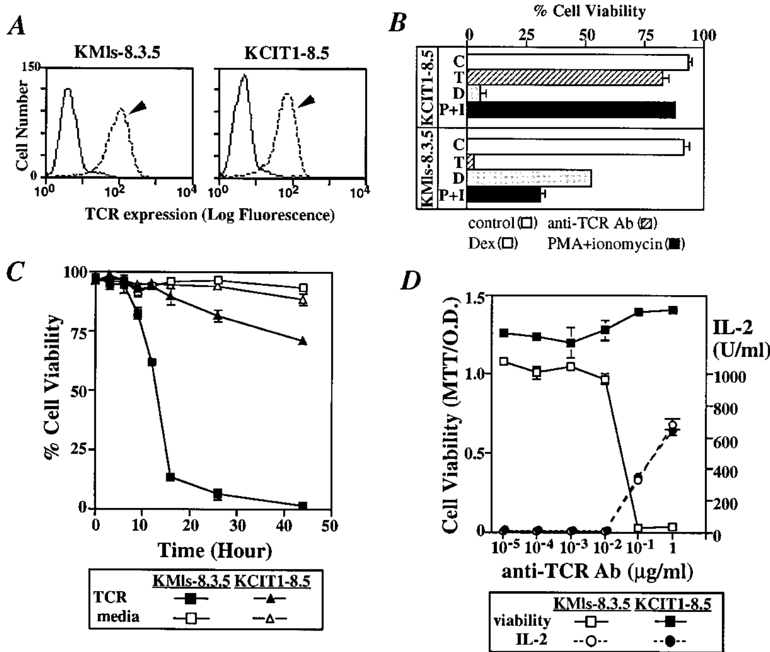


Figure 1. A Death-Resistant T Cell Hybridoma, KCIT1-8.5

(A) The mutant T cell hybridoma, KCIT1-8.5, shows surface TCR expression similar to the parental cell line, KMIs-8.3.5. Thin solid line, phycoerythrin (PE)–streptavidin alone; dotted line marked with an arrow, biotinylated anti-TCR antibody plus PE–streptavidin.

(B) KCIT1-8.5 is resistant to apoptosis induced either by anti-TCR antibody or by PMA plus ionomycin, but KCIT1-8.5 is susceptible to apoptosis induced by dexamethasone. T cell hybridomas ($4 \times 10^4$ cells/well) were cultured in media only, in wells coated with anti-TCR antibody or in media with 1 µM dexamethasone (Sigma), or in media with PMA (12 nM, Calbiochem), ionomycin (0.5 µM, Calbiochem), or both, as indicated. After 24 hr, cell viability was measured by trypan blue uptake. Representative results of at least three independent experiments are shown.

(C) Survival of KCIT1-8.5. The normal parental line (KMIs-8.3.5) or the mutant line (KCIT1-8.5) was cultured in 96-well plates ($4 \times 10^4$ cells/well) coated with the indicated amounts of anti-TCR antibody (H57–597) (Kubo et al., 1989). Cell viability was measured by trypan blue uptake at the indicated times.

(D) The mutant T cell hybridoma, KCIT1-8.5, is resistant to apoptosis, but has a normal dose–response curve for IL-2 production upon TCR stimulation. The normal parental line (KMIs-8.3.5) or the mutant line (KCIT1-8.5) was cultured in 96-well plates ($4 \times 10^4$ cells/well) coated with the indicated amounts of anti-TCR antibody (H57-597) (Kubo et al., 1989). After 24 hr, supernatants were used to measure IL-2 production as described (Yazdanbakhsh et al., 1995) and the cells were used for the MTT assay to determine cell viability (Shi et al., 1992). Representative results of at least three independent experiments are shown.

## Results and Discussion

### Isolation and Characterization of Death-Resistant T Cell Hybridoma Mutant

To study the signaling processes involved in apoptosis specifically, we generated mutant T cell hybridomas resistant to apoptosis, yet showing normal activation (i.e., IL-2 production) upon TCR stimulation. A T cell hybridoma, KMIs-8.3.5, was mutagenized with the frameshift mutagen, ICR-191, because it has been successfully used to induce recessive mutants in mammalian cells (Pellegrini et al., 1989). For mutagenesis, $5 \times 10^7$ of exponentially growing T cell hybridomas, KMIs-8.3.5, were treated with ICR191 such that 90% of the target cells were killed. After ICR191 treatment, cells were washed several times with balanced salt solution and allowed to recover. Mutagenized cells were then cultured on plates coated with anti-TCR antibody and death-resistant clones were selected. Only the clones that were resistant to anti-TCR-induced apoptosis, yet showed normal IL-2 production in response to anti-TCR antibodies, were selected. Many of the clones resistant to anti-TCR-induced apoptosis also failed to produce IL-2, and these were not further studied. Out of $5 \times 10^7$ cells, four independent death-resistant T cell hybridoma mutants were isolated (data not shown), and one of them, KCIT1-8.5, was further studied. When $1 \times 10^8$ unmutagenized KMIs-8.3.5 cells were used for the same selection procedure on anti-TCR antibody-coated plates, we were not able to isolate any clone that was resistant to anti-TCR-induced apoptosis, yet positive for IL-2 production (data not shown).

KCIT1-8.5, which expressed surface TCR similar to the parental cell line, KMIs-8.3.5 (Figure 1A), was resistant to the apoptosis induced by anti-TCR antibody and also by phorbol myristate acetate (PMA) and ionomycin (Figure 1B). The longer incubation of cells with anti-TCR antibody did not significantly affect the cell viability of KCIT1-8.5 (Figure 1C). However, both the parental line (KMIs-8.3.5) and KCIT1-8.5 had a similar dose–response curve for IL-2 production in response to the same anti-TCR stimulation (Figure 1D). To test whether defect(s) is specific to the TCR-mediated apoptosis, KCIT1-8.5 was also treated with a synthetic glucocorticoid, dexamethasone (Ucker et al., 1989; Zacharchuk et al., 1990). Different from anti-TCR antibody or PMA and ionomycin, dexamethasone induced apoptosis in KCIT1-8.5 as efficiently as in the parental T cell hybridoma, KMIs-8.3.5 (Figure 1B). These results suggest that defect(s) in KCIT1-8.5 is specific to the death-signaling pathway via the TCR.

### Isolation of *TDAG51*, Which Complements the Defect(s) in KCIT1-8.5

To identify the gene(s) responsible for the defect of KCIT1-8.5, we isolated cDNA clones that were differentially expressed in KCIT1-8.5 compared with KMIs-8.3.5. A cDNA library from TCR-stimulated KMIs-8.3.5 was differentially screened with cDNA probes made from KMIs-8.3.5 or KCIT1-8.5, both of which were stimulated with anti-TCR antibody. Since cell activation of KCIT1-8.5 upon TCR stimulation was unaffected, the differential cDNA library screening used was likely to isolate genes required for anti-TCR-induced apoptosis specifically. With this protocol, we isolated cDNA clone *TDAG51* (for T cell death–associated gene 51), whose

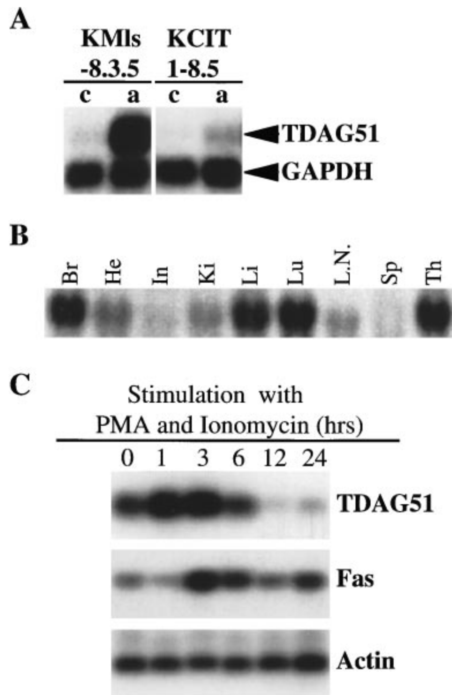


Figure 2. Isolation and Characterization of the *TDAG51* cDNA

(A) *TDAG51* is up-regulated in KMls-8.3.5 upon TCR stimulation, but not in the mutant line, KCIT1-8.5. Polyadenylated RNA purified from unstimulated (c) or TCR-stimulated (a) cells was analyzed for *TDAG51* expression by Northern blot analysis as described (Maniatis et al., 1989). After analysis, the filter was washed and hybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe to control the amounts of mRNA loaded as described previously (Yazdanbakhsh et al., 1995). All northern analyses were quantified using a Phosphoimager (Molecular Dynamics, Incorporated).

(B) *TDAG51* expression in tissues. Total RNAs from different tissues were hybridized with a *TDAG51* cDNA probe. Equal amount of RNA was loaded in each lane based on the intensity of EtBr-stained ribosomal RNA (data not shown). Abbreviations: Br, brain; He, heart; In, intestine; Ki, kidney; Lu, lung; L.N., lymph nodes; Sp, spleen; Th, thymus.

(C) *TDAG51* mRNA induction following activation. cDNAs were prepared from splenocytes stimulated with PMA (20 ng/ml) and ionomycin (2 μM) for 1, 3, 6, 12, and 24 hr. The cDNAs were then subjected to PCR using *TDAG51*-specific, Fas-specific, or actin-specific primers. Similar results were obtained when splenocytes were stimulated with plastic-bound anti-TCR antibodies (data not shown).

expression was greatly reduced in KCIT1-8.5 in comparison with KMls-8.3.5. *TDAG51* encodes a 2.0 kb mRNA whose expression in the parental line (KMls-8.3.5) was strongly induced upon TCR stimulation (Figure 2A). Although *TDAG51* mRNA was the same size in both cell lines, its level was reduced around 20-fold in KCIT1-8.5 (Figure 2A). Anti-TCR-induced up-regulation of *TDAG51* expression was not sensitive to the treatment of cycloheximide, suggesting that *TDAG51* is one of the immediate–early gene products induced during TCR stimulation (data not shown). In mice, *TDAG51* was expressed in most tissues but was more abundant in brain, lung, liver, and thymus (Figure 2B), and was also up-regulated in activated lymphocytes (data not shown).

To determine the role of *TDAG51* in anti-TCR-induced apoptosis, we infected KCIT1-8.5 with recombinant retroviral viruses carrying wild-type *TDAG51* cDNA in either sense or antisense orientation. As controls, KCIT1-8.5 was also infected with viruses containing only the *neo* gene. None of the transfectants expressing antisense *TDAG51* cDNA or vector alone became susceptible to anti-TCR-induced apoptosis (Figure 3A). However, all transfectants expressing sense *TDAG51* cDNA became susceptible to anti-TCR-induced apoptosis, although to a lesser degree than KMls-8.3.5 (Figure 3A). Anti-TCR-induced apoptosis of both KMls-8.3.5 and the *TDAG51*-transfected KCIT1-8.5 was blocked in a dose-dependent manner by soluble Fas–Fc (Ju et al., 1995) (Figure 3B.). These results establish two points. First, *TDAG51* must be essential for anti-TCR-induced apoptosis, since expression of wild-type *TDAG51* complemented the defect in KCIT1-8.5. Second, constitutive expression of *TDAG51* alone is not sufficient to induce apoptosis. Since the number of G418-resistant clones obtained from all three vectors was similar, it is unlikely that the transfectants expressing low levels of *TDAG51* were selected. Rather, it suggests that *TDAG51* is transducing the signals from TCR to the apoptotic machinery or requires additional gene products, which are induced by TCR stimulation.

DNA sequence analysis of overlapping *TDAG51* cDNA clones obtained from the mouse T cell hybridoma cDNA library revealed that *TDAG51* cDNA is 1955 nt long and ends in a canonical polyadenylation signal (Figure 4A). The longest protein that might be encoded by the *TDAG51* gene would ∼46 kDa, with a start site at nucleotides 120–122, but another potential start site (nucleotides 552–555) would encode protein of ∼30 kDa (Figure 4A). Since the original *TDAG51* cDNA clone used in the transfection experiments described above contained the sequence nucleotides 284–1769, it was likely that the start site at nucleotides 552–555 would be used to encode TDAG51 protein. To test this, full-length cDNA and two 5′ truncated *TDAG51* cDNAs were analyzed by in vitro translation assay and by transient transfection assay (Figures 4B–4D).

When it was in vitro translated, full-length *TDAG51* cDNA (51/HA-A) encoded two major proteins migrating at ∼65 kDa and ∼40 kDa, both of which migrated slower than the expected sizes (46 kDa or 30 kDa), on SDS–PAGE. Both of the 5′ truncated *TDAG51* genes that would allow initiation of protein synthesis at Met (nucleotides 552–555) produced in vitro translated proteins migrating at ∼40 kDa, which was at the same position as the smaller product from in vitro translated 51/HA-A proteins (Figures 4B and 4C).

For transient transfection analysis, full-length *TDAG51* cDNA and two 5′ truncated *TDAG51* cDNAs were hemagglutinin (HA) epitope-tagged, cloned into the mammalian expression vector, pHβAPr–*neo*, and transfected into 293 cells (Figures 4B and 4D). When transfected cell lysates were analyzed by Western blot analysis with anti-HA epitope antibody (12CA5), all three constructs produced proteins migrating at ∼40 kDa on SDS–PAGE (Figure 4D). In addition, polyclonal antibodies against the C-terminal 12 aa of TDAG51 protein also detected proteins migrating at ∼40 kDa, which are up-regulated upon TCR stimulation (Figure 4E). Therefore, the *TDAG51* gene is most likely to encode a protein of 262 aa that would start at nucleotides 552–555.

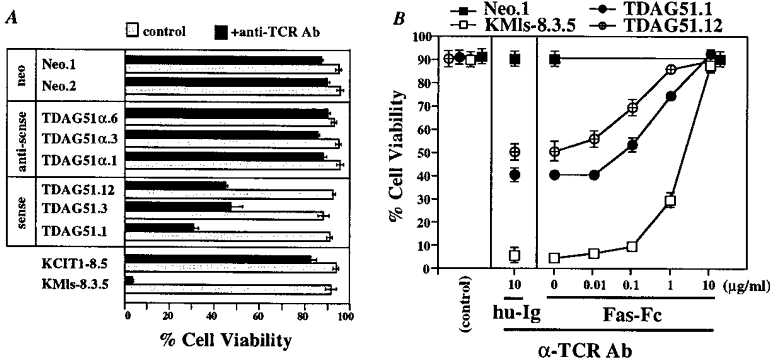


Figure 3. *TDAG51* Is Required for Anti-TCR-Induced Apoptosis of T Cell Hybridoma

(A) Expression of wild-type *TDAG51* complements the defect in KCIT1-8.5 cells. Transfectants expressing either sense (*TDAG51.1*, *TDAG51.3*, and *TDAG51.12*) or antisense (*TDAG51α.1*, *TDAG51α.3*, and *TDAG51α.6*) *TDAG51* cDNAs were tested for their susceptibility to anti-TCR-induced apoptosis as described in Figure 1, and cell viability was measured by trypan blue uptake. Only three representative clones from each transfection are shown. Transfectants expressing vector alone were also included (Neo.1, Neo.2). A total of six transfectants expressing antisense *TDAG51* and twelve transfectants expressing vector alone were tested and none of them became susceptible to anti-TCR-induced apoptosis. A total of 12 transfectants expressing sense *TDAG51* cDNA were tested and all became susceptible to anti-TCR-induced apoptosis (data not shown). All experiments were performed at least three times. All the T cell hybridoma cell lines tested expressed similar levels of TCR and produced normal amounts of IL-2 upon TCR stimulation (data not shown).

(B) Anti-TCR-induced apoptosis of both KMls-8.3.5 and *TDAG51*-transfected KCIT1-8.5 is inhibited by soluble Fas–Fc. Cells shown in (A) were stimulated with anti-TCR antibody as described in Figure 1 in the presence of the indicated concentration of either soluble Fas–immunoglobulin fusion protein (Fas–Fc; provided by Dr. S.-T. Ju, Harvard Medical School; Ju et al., 1995) or control human IgG1 (hu–Ig; Sigma). After 24 hr, cell viability was measured by trypan blue uptake. All experiments were carried out at least three times.

Sequence analysis of *TDAG51* cDNA in the mutant line, KCIT1-8.5, revealed a frameshift mutation resulting in a premature translational termination (Figure 4A). This type of mutation often causes mRNA instability due to decreased ribosomal association and may therefore explain the reduced level of mRNA (Atwater et al., 1990; Velazquez et al., 1992).

Analysis using BLAST and FASTA sequence comparison algorithms revealed that TDAG51 is very homologous to a human proline–glutamine-rich protein of unknown function that was identified from a human fetal liver cDNA library. This protein is likely to be a human homolog of TDAG51, because their amino acid sequence is about 85% identical. Both mouse and human TDAG51 did not show any significant similarities to other proteins reported previously. Two regions of the protein have highly repeated sequences; one is composed of proline–glutamine (PQ) repeats and the other of proline–histidine (PH) repeats (Figure 4A). The PQ repeats of TDAG51 showed a strong homology to herpes simplex virus UL36, a transcription activator (McNabb and Courtney, 1992). The PH repeats of TDAG51 showed a strong homology to the PRD repeat of various homeobox proteins (Gehring et al., 1994). PRD repeat in some homeobox proteins was implicated in the transcriptional activation of target genes (Han et al., 1989; Cai et al., 1994). When fused to the Gal4–DNA binding domain, the amino acid residues 154–261 of TDAG51 containing the PQ and PH repeats weakly activated a promoter containing Gal4-binding sites (data not shown), suggesting TDAG51 may work as a transcriptional activator. However, TDAG51 does not contain any obvious DNA binding motif.

### Fas(CD95) Expression Is Defective in KCIT1-8.5 and Requires Functional *TDAG51*

To determine how *TDAG51* plays a role in anti-TCR-induced apoptosis, we first examined expression in KCIT1-8.5 and the parental cell line (KMls-8.3.5) of several genes that have been reported to be essential for anti-TCR-induced apoptosis of T cell hybridomas (Shi et al., 1992; Liu et al., 1994; Woronicz et al., 1994; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Yang et al., 1995; Yazdanbakhsh et al., 1995). Neither mRNA expression of *nur77* (Figure 5A) nor its DNA binding activity (data not shown) was affected in KCIT1-8.5. In KCIT1-8.5, FasL expression was normal, but Fas expression was greatly reduced (Figure 5A). These results suggested that lack of Fas expression may be responsible for the death-resistance phenotype of KCIT1-8.5. To test this, KCIT1-8.5 was infected with recombinant retroviruses carrying Fas cDNA (Watanabe-Fukunaga et al., 1992) and the $Fas^+$ transfectants were selected by G418 resistance. When the $Fas^+$–KCIT1-8.5 transfectants were stimulated with anti-TCR antibody, all the clones became as susceptible to anti-TCR-induced apoptosis as the parental cell line, KMls-8.3.5 (Figures 5B and 5C). The results show that KCIT1-8.5 expresses functional FasL and that the defect(s) in KCIT1-8.5 is indeed located at or upstream of Fas expression.

We therefore examined whether *TDAG51* restored Fas expression in *TDAG51*-transfected KCIT1-8.5. Although constitutive expression of *TDAG51* did not restore Fas expression, both Fas mRNA and the surface Fas protein were detected upon TCR stimulation only in those cells that also expressed functional *TDAG51* (Figures 5D and 5E). Fas expression in the *TDAG51* transfectants was still significantly less than in KMls-8.3.5 or Fas-transfected KCIT1-8.5 (Figures 5B and 5E), a fact that might account for the lower susceptibility of the *TDAG51* transfectants to anti-TCR-induced apoptosis. These results show that *TDAG51* is essential, but not sufficient, for Fas mRNA expression. Another gene product or posttranslational modification of TDAG51 induced by TCR stimulation is likely to be required for Fas expression.

Fas–FasL interaction plays a critical role in the clonal deletion of autoreactive T cells in the peripheral lymphoid organs and also in the removal of activated

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   1  AGGCGGCGCGGGCGGCCGTGGGCGGTGGGGCGGGGCGGGGGCGGAGCCGGCGGCGGGGCTGGGGCGGAGCCGGCGGCGGGGGGCGTGTCCGCGGCGGGCT
 101  CTGGGAAGGCCCCAAGTTAATGAGGCGTACGCCGGCTGCCGAGCGCCTCTCGGAGCTGGGTTTTCCCCCGCGGCGCGGGCGCCAGGAGCCGCCTTTTCCG
 201  CTGGGTGTCACTCGGGGGTGGGGAGGGTGGCCCATTGAAAAGCGCCGCGAGGGGCCCCGGCCAGTGCCCTTCAGTGAGCGCTCGCCGGAGGACGGCAGAG
 301  AGCAGCCCGCTCACGGCTCCGGGATCTTGTGGCGAGTCAGGACGCGCCTGTCCCTCTGCCGGGACCCCGAGCCACCGCCGCCGCCGCCACCGCTCTGTCT
 401  CCTGCGAGTCAGCCTTCTCTGCGCGCTCCGGGCGGGCGGCCGCGGAAGCCGCTGGGGCGAGGACGGCGCAGGGCTGCTACTGCTACCCCCAGCCGGAGCG
 501  TCTGGAAGCCTGAAGGCCGAGCGGAGCAGCAGCACCCCTTATGCCGGAAGGATGCTGGAGAACAGCGGCTGCAAGGCGCTGAAGGAAGGAGTGCTGGAAA
                                                             M  L  E  N  S  G  C  K  A  L  K  E  G  V  L  E    16

 601  AGCGAAGCGACGGGCTGCTGCAGCTCTGGAAGAAAAAGTGCTGCATCCTCACCGAGGAAGGGCTACTGCTCATACCGCCCAAGCAGCTACAACAGCAGCA
      K  R  S  D  G  L  L  Q  L  W  K  K  K  C  C  I  L  T  E  E  G  L  L  L  I  P  P  K  Q  L  Q  Q  Q  Q   50

 701  GCAGCAGCAACAGCCCGGGCAGGGGACGGCCGAACCGTCCCAACCTAGTGGCCCCACCGTCGCCAGCCTTGAGCCACCAGTCAAGCTGAAGGAATTGCAC
        Q  Q  Q  Q  P  G  Q  G  T  A  E  P  S  Q  P  S  G  P  T  V  A  S  L  E  P  P  V  K  L  K  E  L  H    83
                                                         (ggctc)
                                                           G  S  P  S  P  A  L  S  H  Q  S  S  *(stop)

 801  TTTTCCAATATGAAGACTGTAGACTGCGTGGAGCGCAAGGGCAAGTACATGTACTTCACTGTGGTGATGACGGAGGGCAAAGAGATCGACTTTCGGTGCC
       F  S  N  M  K  T  V  D  C  V  E  R  K  G  K  Y  M  Y  F  T  V  V  M  T  E  G  K  E  I  D  F  R  C   116

 901  CCCAGGACCAGGGCTGGAACGCCGAGATCACGTTGCAGATGGTGCAGTACAAAAATCGCCAGGCCATACTGGCGGTCAAGTCTACCAGGCAGAAGCAGCA
      P  Q  D  Q  G  W  N  A  E  I  T  L  Q  M  V  Q  Y  K  N  R  Q  A  I  L  A  V  K  S  T  R  Q  K  Q  Q  150

1001  GCACCTGGTCCAGCAGCAGCCTCCGCAGACGCAGCAGATCCAACCCCAGCCCCAGCCGCAGATCCAACCCCAGCCCCAGCCGCAGATCCAGCCTCAACCC
        H  L  V  Q  Q  Q  P  P  Q  T  Q  Q  I  Q  P  Q  P  Q  P  Q  I  Q  P  Q  P  Q  P  Q  I  Q  P  Q  P  183

1101  CAACCCCAGCCCCAACCTCAGCCCCAACCTCAGCCCCAACCCCAACCCCAACAGCTCCACTCCTACCCGCATCCGCATCCTCATCCTTACTCTCACCCGC
       Q  P  Q  P  Q  P  Q  P  Q  P  Q  P  Q  P  Q  P  Q  Q  L  H  S  Y  P  H  P  H  P  H  P  Y  S  H  P   216

1201  ACCAGCACCCACACCCGCACCCGCACCCGCACCCTCACCCTCACCCTCACCCGTACCAACTCCAGCACGCGCACCAGCCTCTTCACTCGCAGCCGCAGGG
      H  Q  H  P  H  P  H  P  H  P  H  P  H  P  H  P  H  P  Y  Q  L  Q  H  A  H  Q  P  L  H  S  Q  P  Q  G  250

1301  TCACCGGCTTCTCCGCAGCACCTCCAACTCTGCCTGAAGAGGGCGGCACCCGGGCCACTCAAGGTTTTGAGGACTTCGGAAAGTGGGAAGAGTGCATTGC
        H  R  L  L  R  S  T  S  N  S  A  *                                                                 261

1401  TATTCAGTTCACTTGGATCAAAACCAACAGAGTCTCCCCACCCCGCGCCTGATCAAGTAGTTTGGACTTCACCCGACGGATAACGCGCGCTTGCAGTTCC
1501  CTCGTAGTTTTCTGCATAATGTTCATCACAGTTGCAGGAAGCAGATTTTGCGTCGAGATGACTTTATTTATGGACCTTTGGAAGGATCACCTAACGTAAT
1601  GGACCTTTGTAGGAACAATTTATGTACTGTAATTTTATTTTAATGTTATTTTGGATTATGATTATTTATTAGTTTTTTTATGCCTAATCTAAGACATTTC
1701  TGAATGTAGGCCACCTCCTCCCCCTCCTCGCCAAAAAGAAAAAGAAAAAAAGAAAAAAAAAAAAAGAAACTTTATTTTCAGAAACAGATTTCTTAGTAAGC
1801  CCTGATCTTGGAAGAGAAAAAGGGAAAGGGGTGGTGGAGGGGGGCAAACATGAAAAAAAAATGGGTTCAGTATTCCCAGGACATTCTCATAAAGTCGACT
1901  CTTTCATTGTATTTGAAGACGTTGAAATTAAATTCTGATATTACTTTCAGTTAaaaaaaaaaaaaaaa
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Figure 4. TDAG51 Protein

(A) Wild-type *TDAG51* cDNA sequence and its predicted amino acid sequence. The transcription initiation site (nucleotide 1) was determined by primer extension analysis (data not shown). The cDNA ends in a canonical polyadenylation signal and poly(A) tail. The GenBank accession number for *TDAG51* cDNA is U44088. The mutation in *TDAG51* cDNA in KCIT1-8.5 cells is indicated by (gg*ctc*) (nucleotides 750–755; a deletion of C at nucleotide 752 or 753 and C–T transition at nucleotide 754). This frame-shift mutation results in the premature termination of *TDAG51*, as indicated in the lower line. This was determined by sequencing *TDAG51* cDNA clones obtained from KCIT1-8.5 by PCR. Six independent clones were sequenced and all showed the same mutation.

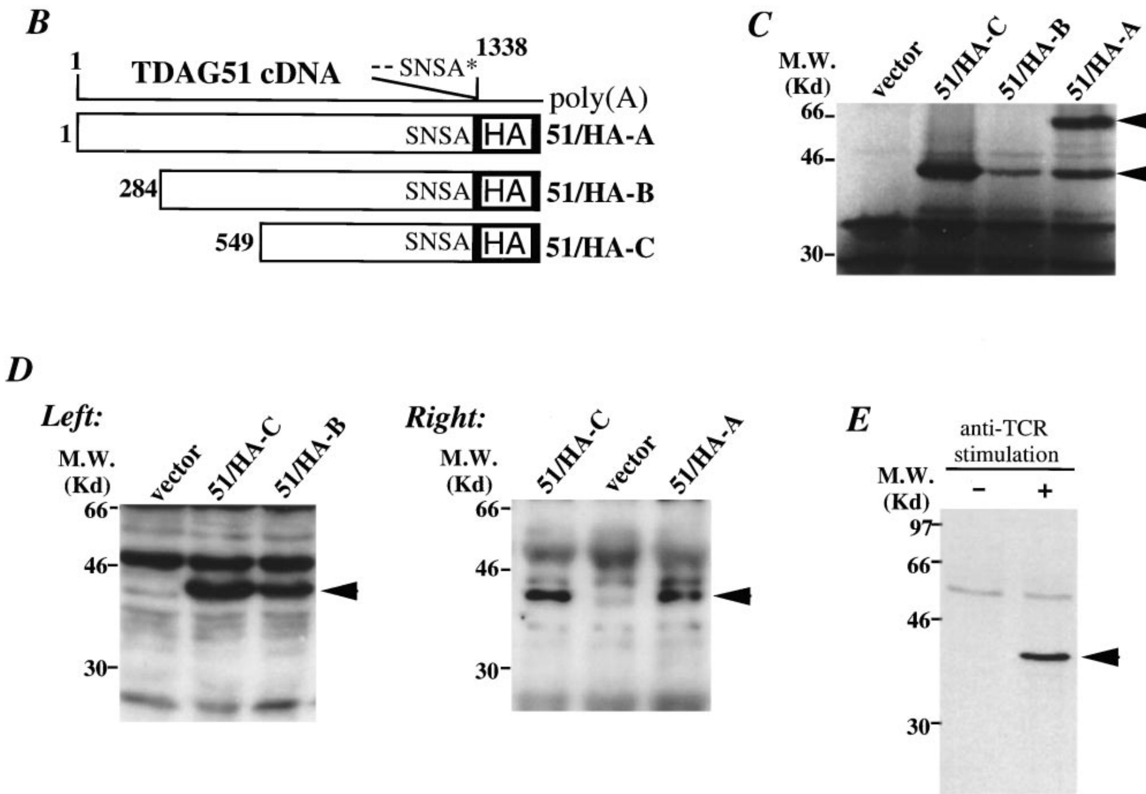
(B) Constructs of HA–epitope-tagged *TDAG51* cDNA were used to detect the TDAG51 protein. HA, HA–epitope tag. The C-terminal 4 aa residues (SNSA) of *TDAG51* prior to the HA tag are indicated. The 5′ start site (nucleotide position) of each construct is also indicated.

(C) *TDAG51* cDNA encodes a protein starting at nucleotides 552–555. Various 5′ truncated *TDAG51* cDNAs were in vitro translated and analyzed on SDS–PAGE followed by autoradiography. TDAG51-specific protein products are marked with an arrow. TDAG51 protein migrates slower than the expected size (~46 kDa or ~30 kDa) on SDS–PAGE.

(D) Expression of HA-tagged *TDAG51* in 293 cells. Western blot analysis of cell lysates transfected with expression vectors containing the constructs described in (B) were carried out with anti-HA antibody (12CA5). Either whole cell lysates (left) or lysates immunoprecipitated with 12CA5 (right) were analyzed by Western blot analysis. All the constructs (51/HA-A, 51/HA-B, and 51/HA-C) produced a protein migrating at same position (~40 K), indicating that nucleotide 552 was used as the translation initiation site. TDAG51-specific protein products are marked with an arrow.

(E) Detection of TDAG51 protein in T cell hybridomas. Whole cell lysates were prepared from control, unstimulated KMls-8.3.5 cell line (minus), or from KMls-8.3.5 cell line stimulated with anti-TCR antibody for 5 hr (plus). Rabbit polyclonal antibody generated against amino acid residues 250–261 were used for Western blot analysis. TDAG51 protein is indicated by an arrow.

(*Figure 4 continued on next page*)




T cells that have completed their immune responses (Nagata and Golstein, 1995). Defect(s) in Fas or FasL lead to a development of severe autoimmune diseases as in *gld* or *lpr* mice (Cohen and Eisenberg, 1992; Nagata and Golstein, 1995). Fas–FasL expression upon activation of peripheral T cells or T cell hybridomas is a prerequisite for the ultimate death of these cells (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Nagata and Golstein, 1995; Yang et al., 1995). In this study, we showed that TDAG51 is an immediate–early gene product required for the anti-TCR-induced apoptosis and is an intermediate that couples TCR stimulation with Fas(CD95) expression. Since TDAG51 contains a transcriptional activation domain and can be translocated into nucleus (C. G. P and Y. C., unpublished data), it is likely that TDAG51 is involved in the transcriptional control of Fas(CD95) mRNA expression. However, although genetic evidence suggested that TDAG51 regulates Fas(CD95) mRNA expression, the results in this study could not determine whether TDAG51 protein acts on the Fas(CD95) promoter directly.

The role of TDAG51 protein appears to be limited to Fas(CD95) expression, at least in T cell hybridomas, because expression of other surface receptors like TNF-R1 or OX40 was not significantly affected in the mutant cell line, KCIT1-8.5 (data not shown). Future study of how *TDAG51* regulates Fas expression will lead to the better understanding of the death-specific signaling processes of anti-TCR-induced apoptosis. Fas expression is also increased on lymphocytes during certain viral infections (e.g., HIV) (Nagata and Golstein, 1995). *TDAG51* may also have a role in the up-regulation of Fas expression and increased T cell death following viral infection.

## Experimental Procedures

### Mutagenesis and Selection of Death-Resistant T Cell Hybridomas

The culturing of T cell hybridomas was described previously (Yazdanbakhsh et al., 1995). For mutagenesis, $5 \times 10^7$ ($5 \times 10^5$ cells/ml) of exponentially growing T cell hybridomas, KMls-8.3.5, were treated with 6 μg/ml of ICR191 (Sigma) for 10 hr. These conditions for mutagenesis were chosen based on their ability to kill approximately 90% of the target cells. After ICR191 treatment, cells were washed several times with balanced salt solution and cultured in fresh medium for 2 days. Following recovery, cells were cultured on plates coated with 5 μg/ml anti-TCR antibody (Kubo et al., 1989). After 5 days, surviving cells were recovered, expanded, and recloned by limiting dilution on 96-well plates coated with anti-TCR antibody. Only the clones that were resistant to anti-TCR-induced apoptosis, yet showed normal IL-2 production in response to anti-TCR antibody, were selected. Four independent T cell hybridoma mutants described in the text were derived from independent plates during the primary selection.

### Activation and Induction of Apoptosis in T Cell Hybridomas

T cell hybridomas were activated by incubating them ($4 \times 10^4$ cells/well) in 96-well plastic tissue culture plates coated with H57-597 (Kubo et al., 1989). Stimulation of T cell hybridoma was also achieved by treating cells with PMA (12 nM, Calbiochem), ionomycin (0.5 μM, Calbiochem), or both, or by the treatment of cells with dexamethasone (1 μM) (Sigma). The activation of T cells was measured by lymphokine production, and the viability of T hybridoma cells were measured by trypan blue uptake or by MTT assay (Shi et al., 1989).

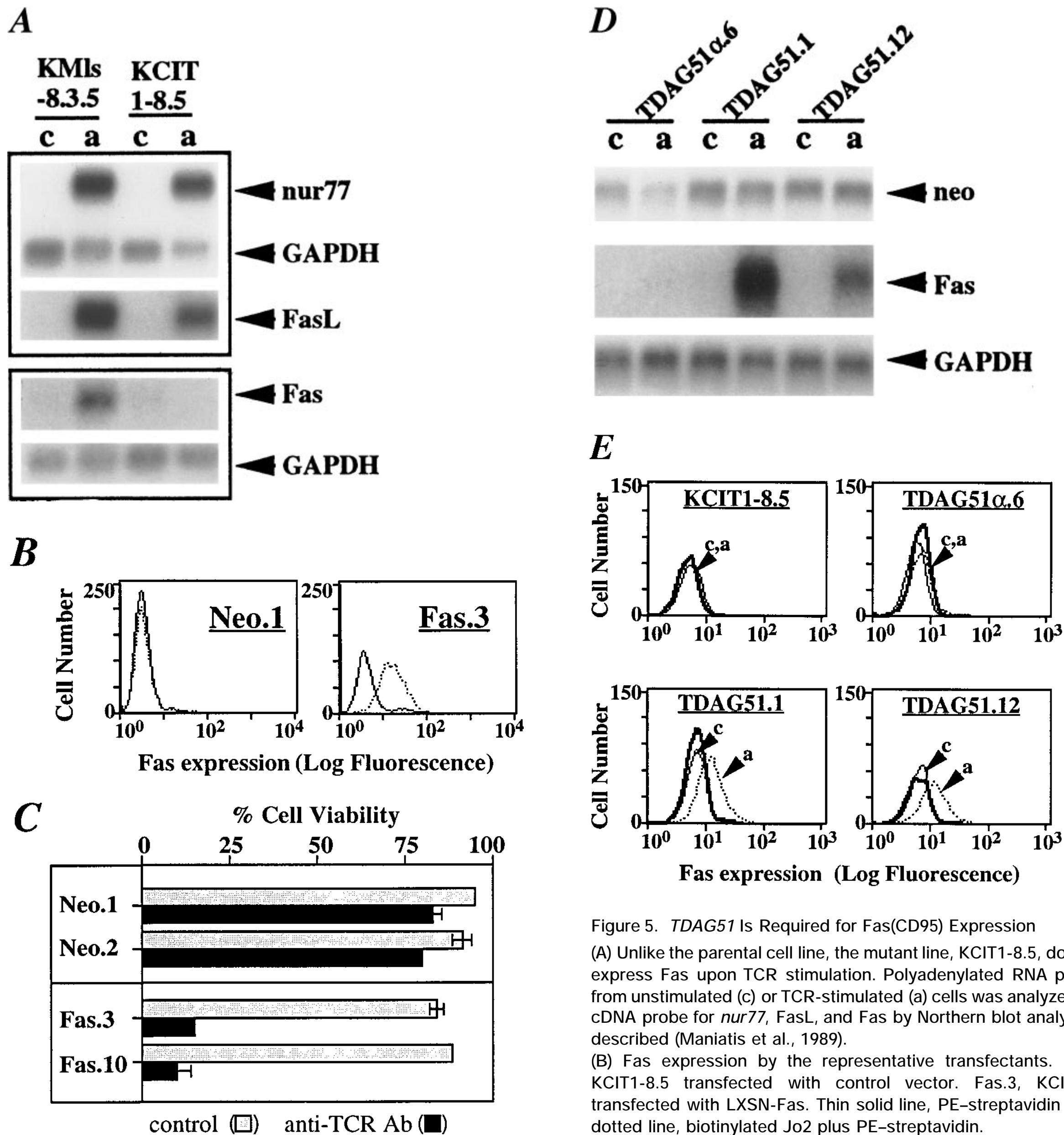



Figure 5. *TDAG51* Is Required for Fas(CD95) Expression

(A) Unlike the parental cell line, the mutant line, KCIT1-8.5, does not express Fas upon TCR stimulation. Polyadenylated RNA purified from unstimulated (c) or TCR-stimulated (a) cells was analyzed with cDNA probe for *nur77*, FasL, and Fas by Northern blot analysis as described (Maniatis et al., 1989).

(B) Fas expression by the representative transfectants. Neo.1, KCIT1-8.5 transfected with control vector. Fas.3, KCIT1-8.5 transfected with LXSN-Fas. Thin solid line, PE–streptavidin alone; dotted line, biotinylated Jo2 plus PE–streptavidin.

(C) Expression of Fas corrects the defect of KCIT1-8.5. Control or Fas$^+$–KCIT1-8.5 transfectants were cultured on anti-TCR antibody-coated 96-well plates, and cell viability was measured by trypan blue uptake. Neo.1 and Neo.2, KCIT1-8.5 transfected with control vector. Fas.3 and Fas.10, KCIT1-8.5 transfected with LXSN-Fas. Six transfectants from each transfection were tested and showed similar results.

(D–E) Expression of *TDAG51* allows Fas mRNA and surface protein expression upon TCR stimulation. The *TDAG51* transfectants described in Figure 3 were used for Northern blot and FACS analyses. *TDAG51.1* and *TDAG51.12*, transfectants expressing sense *TDAG51* cDNA. *TDAG51α.6*, transfectants expressing antisense *TDAG51* cDNA. In (D), the expression of transfected genes was determined by the presence of mRNA hybridized with bacterial aminoglycoside phosphotransferase gene probe (*neo*). Expression of Fas and GAPDH was determined as described in (A). c, control, unstimulated; a, TCR-stimulated. In (E), surface Fas expression of control, unstimulated, or TCR-stimulated cells was analyzed with biotinylated anti-Fas antibody (Jo2) using a FACScan; Thin solid line marked with c, control unstimulated cells; dotted line marked with an a, TCR-stimulated cells; thick solid lines, cells stained with PE–streptavidin alone.

**Isolation and Characterization of *TDAG51* cDNA**

A cDNA library in λZAP (Strategene) was made by standard methods (Maniatis et al., 1989) from mRNA of KMls-8.3.5, which was stimulated with anti-TCR antibody for 6 hr. To identify cDNA clones whose expression is affected in KCIT1-8.5, duplicate filters of the cDNA library were screened with $^{32}$P-labeled cDNA probes made from mRNA from anti-TCR-stimulated KMls-8.3.5 or anti-TCR-stimulated KCIT1-8.5 as described (Maniatis et al., 1989). Around $1 \times 10^5$ clones were screened and two clones (p51-2 and p43-1) were found to be differently expressed in KMls-8.3.5 and KCIT1-8.5. p51-2 was used to isolate full-length *TDAG51* cDNA. For sequence analysis of *TDAG51*, several overlapping cDNA clones were sequenced using the Sequenase Kit (United States Biochemical Company, Cleveland, Ohio). p51-2, the original cDNA clone of *TDAG51*, containing nucleotides 284–1769 of *TDAG51* cDNA, was used for the transfection experiments described in Figure 3.

For in vitro translation experiments, various *TDAG51* cDNAs described in the text were cloned into pBluescript (Strategene), which were transcribed and translated in vitro using the TNT coupled reticulocytes system (Promega) with $^{35}$S-labeled methionine.

Epitope-tagged *TDAG51* was constructed by adding nucleotides coding for the sequence LTGGGSG*FYPYDVPDYA** to the C-terminal end of the *TDAG51* open reading frame prior to the termination codon. HA epitope (Kolodziej and Young, 1991) is underlined and the asterisk indicates the stop codon. The 5′ ends of the HA-tagged *TDAG51* constructs are as follows: 51/HA-A (nucleotide 1), 51/HA-B (nucleotide 284), and 51/HA-C (nucleotide 549). The HA-tagged *TDAG51* cDNAs were cloned into an expression vector, pHβAPr-1–*neo* (Gunning et al., 1987). 293 cells were transiently transfected with vector alone or expression vectors with different HA-tagged *TDAG51* (51/HA-A, 51/HA-B, and 51/HA-C). After 48 hr, cell lysates were analyzed for HA-tagged *TDAG51* by Western blot analysis with anti-HA antibody, 12CA5, as described (Kolodziej and Young, 1991).

Rabbit polyclonal antisera recognizing *TDAG51* were prepared by Research Genetic, Incorporated (Huntsville, Alabama) using a synthetic peptide, GHRLLRSTSNSA (amino acid residues 250–261 of TDAG51 protein).

### Retroviral Infection

*TDAG51* cDNA or Fas cDNA was cloned into the retroviral vector, pLXSN (Miller and Rosman, 1989), to yield retroviral vectors. The retroviral vectors were transiently transfected into a packaging cell line, BOSC-23, by the calcium phosphate transfection method as described (Pear et al., 1993). The culture supernatants containing recombinant viruses were collected 2 days later, filtered, and used to infect KCIT1-8.5 as previously described (Pear et al., 1993). Transfectants were selected by their G418 (400 μg/ml) resistance (Yazdanbakhsh et al., 1995). Transfectants expressing either sense or antisense *TDAG51* cDNAs were confirmed by Northern blot analysis. Transfectants expressing surface Fas were isolated by staining with biotinylated anti-Fas antibody Jo2 (Pharmingen) (Ogasawara et al., 1993).

### Northern Blot Analysis

Polyadenylated RNA purified from unstimulated and stimulated cells was analyzed by Northern blot analysis as described (Maniatis et al., 1989). Stimulated cells were prepared by culturing them for 4–6 hr on plates coated with H57-597 (10 μg/ml). FasL mRNA was detected using FasL exon 1 cDNA probe (Suda et al., 1993). *nur77* RNA was detected with an exon 1–specific probe as described previously (Yazdanbakhsh et al., 1995). Fas cDNA (Watanabe-Fukunaga et al., 1992) was prepared by polymerase chain reaction (PCR). The *TDAG51* cDNA probe was prepared from p51-2 clone. After analysis, the filter was washed and hybridized with glyceraldehyde phosphate dehydrogenase (GAPDH) probe to control the amounts of mRNA loaded as described previously (Yazdanbakhsh et al., 1995). All Northern blot analysis was quantified using a Phosphoimager (Molecular Dynamics, Incorporated).

### RT–PCR Assay

Splenocytes were isolated from BALB/c mice (4–6 weeks old) and stimulated with PMA (20 ng/ml) and ionomycin (2 μM) for various periods. Total RNA was prepared from unstimulated and stimulated splenocytes using the Total RNA Isolation kit (Strategene). First-strand cDNA was synthesized from 10 μg of total RNA using MMLV reverse transcriptase and random hexanucleotides following the protocols provided by the supplier (GIBCO BRL).

Quantitative PCR was performed in the linear phase of amplification by testing PCR products from different dilutions of first-strand cDNA products. PCR amplification was performed for 35 cycles using 1 of 150 of the first-strand cDNA synthesized above. PCR products were then electrophoresed in a 2% agarose gels and subjected to Southern blot analysis as described previously (Maniatis et al., 1989). Primers used for quantitative PCR analysis are the following:
*TDAG51* (sense), 5′-gcgagtcagccttctctgcgcgct-3′; *TDAG51* (antisense), 5′-ggtgaggatgcagcactttttctt-3′; Fas (sense), 5′-agacaggatg accctgaatct-3′; Fas (antisense), 5′-ggggatccgtcgactcactccagacattg tccttcattttcatt-3′; actin (sense), 5′-atgaagatcctgaccgagcg-3′; actin (antisense), 5′-tacttgcgctgaggaggagc-3′.

### Flow Cytometric Analysis

Cells were stained and analyzed for expression of TCR and Fas on a FACScan flow cytometer (Becton Dickinson, Mountain View, California) as described previously (Park et al., 1995). Biotinylated H57-597 was prepared in our laboratory and biotinylated Jo2 was purchased from Pharmingen.

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