T Cell Receptor-dependent Cell Death of T Cell Hybridomas Mediated by the CD30 Cytoplasmic Domain in Association with Tumor Necrosis Factor Receptor-Associated Factors

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Summary

CD30 is a member of the tumor necrosis factor receptor superfamily and a surface marker for Hodgkin's disease. Normal activated T cells and several virally transformed T or B cell lines also show CD30 expression. The interaction of CD30 with its ligand induces cell death or proliferation, depending on the cell type. In this report we characterize the signals mediated by the intracellular domain of CD30 and show that, in combination with signal(s) transduced by the T cell receptor, the multimerization of CD30 cytoplasmic domain induces Fas(CD95)-independent cell death in T cell hybridomas. Deletion analysis shows that the COOH-terminal 66 amino acids of CD30 are required to induce cell death. Using the yeast two-hybrid system, we have identified that the same region of CD30 interacts with tumor necrosis factor receptor-associated factor (TRAF)1 and TRAF2. These results indicate that TRAF1 and/or TRAF2 play an important role in cell death in addition to their previously identified roles in cell proliferation.

C D30 is a member of the TNF/nerve growth factor (NGF) receptor superfamily which includes TNF-R1, TNF-R2, the low affinity NGF receptor, CD40, CD27, Fas, 4-1BB, and OX40 (1–10). All of these cell surface receptors show similarity characterized by repeating cysteine-rich motifs in their extracellular domains. The expression of these receptors is tightly regulated during activation and differentiation. For example, the expression of these receptors is upregulated upon antigen specific activation of T cells. The interaction of these receptors with their respective ligands induces cell death or proliferation, depending on the cell type (1–5, 10, 11). Despite their diverse effects on cells, the signaling processes of these receptors are only beginning to be unveiled. In contrast to their extracellular domains, the cytoplasmic domains of these receptors contain no significant homology within the superfamily or to other known proteins, suggesting that they may use distinct signaling pathways.

Three members of the TNF family (TNF-R1, Fas, and CD30) have been shown to induce cell death upon their interaction with their ligands (1–3, 12). A region of weak homology has recently been identified between the cytoplasmic domains of TNF-R1 and Fas (13, 14). This region, now referred to as the death domain, is required for the induction of cell death by these receptors. Proteins interacting with the cytoplasmic domains of these receptors also contain similar death domains (15–17). However, CD30 does not possess the death domain in its cytoplasmic do-

main, despite the fact that interaction of CD30 with its ligand induces cell death in certain cells, suggesting that CD30 uses a different signaling pathway for cell death induction.

In this report we characterize the signals mediated by the intracellular domain of CD30 and show that the multimerization of the CD30 cytoplasmic domain, in combination with a concomitant signal(s) via the TCR, induces Fas(CD95)-independent cell death of T cell hybridomas. Deletion analysis shows that the COOH-terminal 66 amino acids of CD30 are required to induce cell death. Using the yeast two-hybrid system, we have identified that the same region of CD30 interacts with TNFR-associated factor (TRAF)1 and TRAF2 (18). These results strongly suggest that TRAF1 and/or TRAF2 are involved in CD30-mediated cell death induction in T cell hybridomas.

Materials and Methods

Construction of Chimeric Receptors. The extracellular and transmembrane portion of CD8α (residues 1–187; 19) were amplified by PCR from pMV7F1.2 (kindly provided by Dr. Dan Littman, The Skirball Institute, New York University, New York, NY) with primers 5'CD8 (5'-CGCGTCGACATGGCCTTACCA-GTG-3') and 3'CD8 (5'-ACGTCTTGAATTCCTGTGGTT-GCA-3'). The amplified fragments were cloned into SalI and EcoRI sites of pBluescript (Strategene Inc., La Jolla, CA) to give pCD8Ext. Cloning of foreign cDNA into the EcoRI site at the 3' end of CD8 in pCD8Ext will give in-frame fusion proteins.

5'CD30 (5'-GGGAATTCCACCGGAGGGCCTGCAGGAAGC-GGATTC-3') and 3'CD30 (5'-AGAGGATTCAGCCCAGGC-CTCACTTTCCAGAGG-3') primers were used to amplify the cytoplasmic domain of CD30 (amino acid residues 408–595; 4) from the cDNA of activated peripheral T cells. Similarly, chimeric receptors with COOH-terminal deletions of the CD30 cytoplasmic domain were made using different 3' primers, 5'-GCAGGATTCTCACAGTGGCTGGCTCATTAACCC-3' for CD30(−127) and 5'-AGCGGATCCTCACCCCACGATCAC-GGTGTCAGC-3' for CD30(−66). Fas cytoplasmic domain (amino acid residues 177–319; 20) was also obtained by PCR. The amplified fragments were cloned into pBluescript and sequenced. Fragments with correct sequences were inserted in-frame into the EcoRI site of pCD8Ext. The resulting chimeric receptors and wild-type CD8 were excised from the plasmid and ligated into the XhoI and BamHI sites of the retroviral expression vector, pLXSN, for transfection experiments (21).

Transfection and Retroviral Infection. The retroviral vectors expressing wild type CD8 (CD8) and chimeric receptors (CD8/CD30, CD8/C2, CD8/C4, and CD8/Fas) were transiently transfected into a packaging cell line, BOSC-23, as previously described (22). 2 d after transfection, recombinant viruses were collected and used to infect the T cell hybridomas KMls-8.3.5 or KCIT1-8.5. Transfectants were selected by G418 resistance. The surface expression of CD8 and chimeric receptors was determined by immunofluorescence analysis with OKT8 and FITC-conjugated goat anti–mouse Abs. The transfectants showing similar surface expression of CD8 and chimeric receptors were isolated and used for further analysis. For induction of cell death, the transfectants (5 × 10⁴/well) were plated on 96-well plates coated with purified H57-597 (10 μg/ml) and/or OKT8 (10 μg/ml) for 24 h. The purified anti-Fas MAb Jo2 (PharMingen, San Diego, CA) was used at 1 μg/ml. The production of IL-2 was measured as described previously (23). Cell viability was measured by trypan blue uptake. The percentage of cell viability indicated is the number of live cells per total cell number in the well. All are represented as an average of at least three different experiments.

Interaction of CD30 Cytoplasmic Domain with TRAF1 and TRAF2. PCR was used to obtain the entire cytoplasmic domain or different deletions of CD30 as described in Fig 1. Fragments with correct sequences were fused in-frame to glutathione S-transferase (GST) by cloning into the EcoRI and NotI sites of pGEX-5X-1 (Pharmacia Inc., Piscataway, NJ). The plasmids were transformed into *Escherichia coli* XL-1 blue (Strategene Inc.). Protein induction, purification, and coprecipitation experiments were done essentially as described previously (24). For coprecipitation experiments in 293 cells, pHβactin-1-neo plasmids expressing the epitope tagged TRAF1 (12CA5 epitope; 25) or TRAF2 (1D4 epitope; 26) and the eukaryotic GST expression vector pEBG (kindly provided by Dr. E. Spanopoulou, The Rockefeller University) expressing GST-CD30 or GST were cotransfected in various combinations as indicated in Fig. 3 *b*. Approximately 24 h after transfection, the cells were lysed, and lysates were subjected to purification with glutathione beads (24). Purified proteins were analyzed by Western blot analysis with mAbs 12CA5 and 1D4 as described (25, 26). For in vitro association experiments, full-length murine TRAF1 and TRAF2 cDNA in pBluescript were transcribed and translated in vitro using the TNT®-coupled reticulocytes system (Promega Corp., Madison, WI) with ³⁵S-labeled methionine. Equal amounts of in vitro–translated TRAF1 or TRAF2 were incubated in binding buffer (PBS containing 0.1% NP-40, 0.5 mM dithiothreitol, 10% glycerol, 1 mM PMSF, 2 μg/ml aprotinin) with ∼1 μg of fusion protein bound to glu-
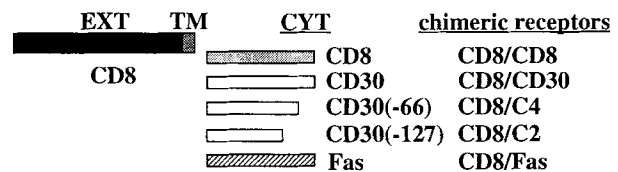
tathione beads for 45 min at 4°C. After washing five times with binding buffer, the proteins were eluted by boiling in SDS sample buffer for 5 min and subsequently analyzed by SDS-PAGE.

Northern Blot Analysis. 2 μg of poly(A)⁺ RNA, purified from unstimulated and stimulated KMls-8.3.5 T cell hybridoma, was analyzed by Northern blot analysis as described (23). Cells were stimulated with anti-TCR Ab (on plates coated with 10 μg/ml H57-597 overnight) for 4–6 h (23). 2.1-kb mRNA was detected by hybridization with TRAF1 cDNA probe, and 2.2-kb mRNA was detected with TRAF2 cDNA probe as previously described (18). After analysis, the filter was washed and rehybridized with glyceraldehyde phosphate dehydrogenase (GAPDH) probe to control for the amount of mRNA loaded as described (23).
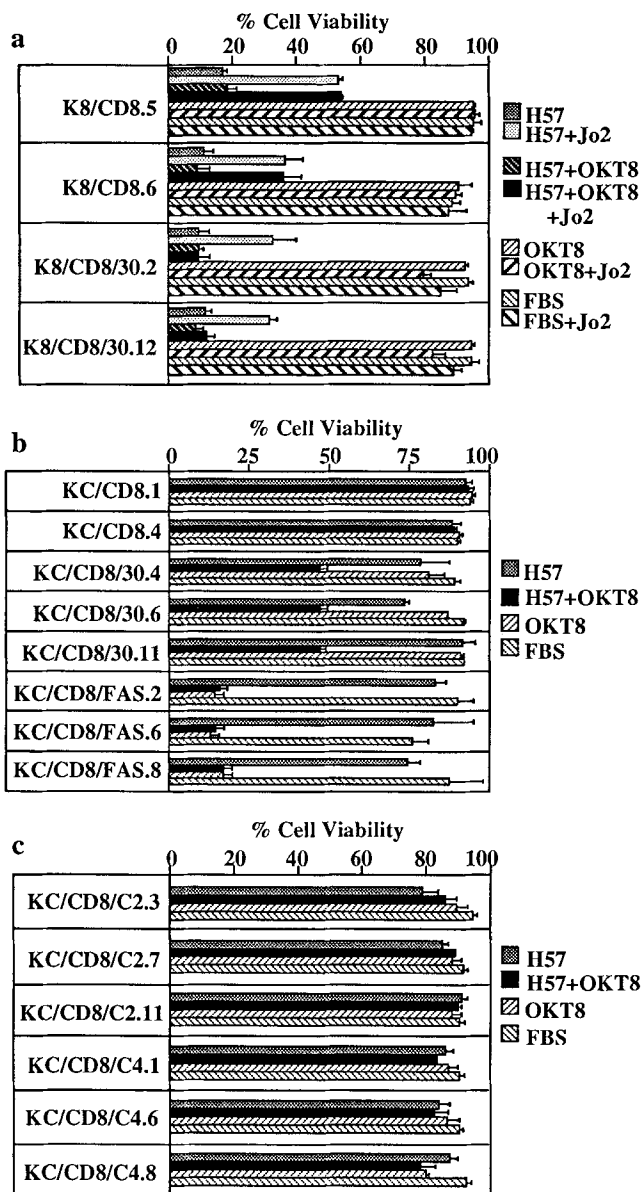
Results and Discussion

TCR-dependent Cell Death of T Cell Hybridomas Mediated by Multimerization of CD30 Cytoplasmic Domains. Receptor multimerization is a common signal transduction mechanism in the TNF receptor superfamily (1–3). Therefore, we have analyzed the signaling processes mediated by CD30 with a chimeric receptor (CD8/CD30) that links the extracellular and transmembrane domains of CD8 to the cytoplasmic domain of CD30 (4, 19) (Fig. 1). Either the chimeric receptor or wild-type CD8 was transfected into the T cell hybridoma KMls-8.3.5. Transfectants expressing similar surface receptors were selected by immunofluorescence analysis with the anti-CD8 (Ab) OKT8 (data not shown). OKT8 treatment of CD8⁺ or CD8/CD30⁺ transfectants changed neither IL-2 production nor cell viability. Treatment with anti-TCR Ab (H57-597) induced significant cell death in both transfectants as previously described (23). Combined treatment with H57-597 and OKT8 resulted in a similar degree of cell death (Fig. 2 *a*). OKT8 did not affect IL-2 production induced by H57-597 (data not shown).

CD8⁺ and CD8/CD30⁺ transfectants incubated with both H57-597 and Jo2 (an anti-Fas Ab; 27) showed partial inhibition of TCR-mediated cell death as previously described (28), suggesting that the anti-TCR Ab–induced cell death was mediated via Fas(CD95) (Fig. 2 *a*). However, when both transfectants were treated with H57-597, OKT8, and Jo2, cell death of CD8/CD30⁺ transfectants was not inhibited by Jo2, whereas that of CD8⁺ transfectants was (Fig. 2 *a*). Similar results were also obtained with

EXT	TM	CYT	chimeric receptors
		CD8	CD8/CD8
CD8		CD30	CD8/CD30
		CD30(-66)	CD8/C4
		CD30(-127)	CD8/C2
		Fas	CD8/Fas

Figure 1. Construction of various chimeric receptors. The extracellular and transmembrane portions of CD8α were fused to the various regions of the CD30 or Fas cytoplasmic domain. CD30, the entire region of CD30 cytoplasmic domain; CD30(−66), deletion of the COOH-terminal 66 amino acid residues of CD30; CD30(−127), deletion of the COOH-terminal 127 amino acid residues of CD30; Fas, the entire Fas cytoplasmic domain. The resulting chimeric receptors are designated as CD8/CD30, CD8/C4, CD8/C2, and CD8/Fas on the right.

% Cell Viability

a

	H57, H57+Jo2, H57+OKT8, H57+OKT8+Jo2, OKT8, OKT8+Jo2, FBS, FBS+Jo2
K8/CD8.5	
K8/CD8.6	
K8/CD8/30.2	
K8/CD8/30.12	

b — % Cell Viability (legend: H57, H57+OKT8, OKT8, FBS)

KC/CD8.1, KC/CD8.4, KC/CD8/30.4, KC/CD8/30.6, KC/CD8/30.11, KC/CD8/FAS.2, KC/CD8/FAS.6, KC/CD8/FAS.8

c — % Cell Viability (legend: H57, H57+OKT8, OKT8, FBS)

KC/CD8/C2.3, KC/CD8/C2.7, KC/CD8/C2.11, KC/CD8/C4.1, KC/CD8/C4.6, KC/CD8/C4.8

Figure 2. (*a*) Combined treatment with anti-TCR and anti-CD8 Abs induces cell death of the CD8/CD30+ KMls-8.3.5 transfectants, which is not blocked by the addition of anti-Fas Ab. The wild-type CD8+ KMls-8.3.5 transfectants are indicated as K8/CD8.5 and K8/CD8.6. The CD8/CD30+ KMls-8.3.5 transfectants are K8/CD8/30.2 and K8/CD8/30.12. The transfectants were treated with the Abs indicated in the legend: H57 (anti-TCR Ab, H57-597), OKT8 (anti-CD8 Ab), Jo2 (anti-Fas Ab), and FBS (fetal bovine serum). Cell viability was determined after 24 h by trypan blue uptake. (*b*) Both anti-TCR and anti-CD8 Abs are required to induce cell death in CD8/CD30+ KCIT1-8.5 transfectants. Transfectants expressing CD8/Fas undergo cell death by the treatment of anti-CD8 Ab alone. CD8+ KCIT1-8.5 transfectants are indicated as KC/CD8.1 and KC/CD8.4. The CD8/CD30+ KCIT1-8.5 transfectants are KC/CD8/30.4, KC/CD8/30.6, and KC/CD8/30.11. The CD8/Fas+ KCIT1-8.5 transfectants are KC/CD8/FAS.2, KC/CD8/FAS.6, and KC/CD8/FAS.8. Ab treatments of the transfectants are indicated on the legend: H57 (anti-TCR Ab, H57-597), OKT8 (anti-CD8 Ab), and FBS (fetal bovine serum). Cell viability was determined after 24 h. (*c*) The COOH-terminal 66 amino acid residues of CD30 are required for the induction of cell death. The KCIT1-8.5 transfectants expressing CD8/C2 (deletion of the COOH-terminal 127 amino acid residues) are KC/CD8/C2.3, KC/CD8/C2.7, and KC/CD8/C2.11. The KCIT1-8.5 transfectants express-

another T cell hybridoma, DO11-10/S4.4 (data not shown), demonstrating that multimerization of both the CD30 cytoplasmic domain and the TCR transduced a cell death signal that is independent of Fas. This Fas independence was confirmed by transfection experiments in the T cell hybridoma KCIT1-8.5, which does not undergo cell death upon TCR cross-linking due to deficient Fas(CD95) expression (Park, C.G., S.Y. Lee, and Y. Choi, manuscript submitted for publication). As shown in Fig. 2 *b*, combined treatment with H57-597 and OKT8 induced significant cell death of CD8/CD30+ KCIT1-8.5 transfectants, which was not affected by the addition of Jo2 (data not shown). To determine the functional regions within the CD30 cytoplasmic domain responsible for induction of cell death, further transfection experiments were performed with CD8/CD30-chimeric receptors encoding COOH-terminal deletions of CD30 (Fig. 1); the results indicate that the COOH-terminal 66 amino acids of CD30 are required to induce cell death (Fig. 2 *c*).

These results establish that multimerization of CD30 cytoplasmic domains in T cell hybridomas induces cell death, similar to what has been observed in certain other cell lines (1–3, 5, 11). The CD30-mediated cell death of T cell hybridomas, however, requires a concomitant signal(s) via the TCR, which differs from the TCR-independent cell death induced by multimerization of Fas cytoplasmic domains (Fig. 2 *b*).

TRAF1 and TRAF2 Are Involved in the Death-signaling Processes via CD30. It has been previously reported that the cytoplasmic region of Fas(CD95) contains a death domain, crucial for cell death induction, that mediates protein–protein interactions with other death domain–containing signal transducers (12, 13, 16, 17). The cytoplasmic domain of CD30 does not have an apparent death domain, suggesting that different signaling processes are responsible for cell death induction. To further characterize the cell death–signaling processes mediated by CD30, we used the yeast two-hybrid system (29) and identified two clones (clones 9 and 35) that interact specifically and equally well with the cytoplasmic domain of CD30 (data not shown). Sequence analysis of cDNAs of these clones revealed that clone 9 contained amino acid residues 204–409 of TRAF1, and clone 35 contained amino acid residues 241–501 of TRAF2, which were previously identified by their association with p75 TNF receptor (TNF-R2) (18; data not shown).

The specific interaction of TRAF1 and TRAF2 with the CD30 cytoplasmic domain was confirmed by coprecipitation experiments using a series of GST fusion proteins with the cytoplasmic domain of CD30 and other cell surface receptors as controls. Both TRAF1 and TRAF2 were coprecipitated with GST–CD30 after coexpression in 293 cells

ing CD8/C4 (deletion of the COOH-terminal 66 amino acid residues) are KC/CD8/C4.1, KC/CD8/C4.6, and KC/CD8/C4.8. The transfectants were treated with the Abs indicated on the legend: H57 (anti-TCR Ab, H57-597), OKT8 (anti-CD8 Ab), and FBS (fetal bovine serum). Cell viability was determined after 24 h.
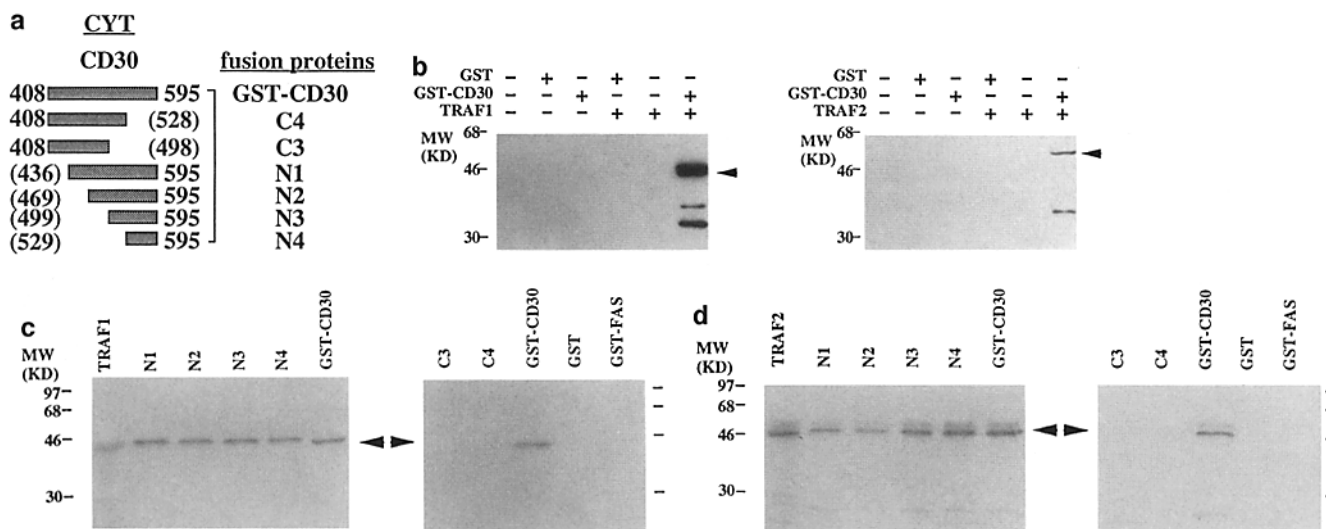
Figure 3. The cytoplasmic domain of CD30 interacts with TRAF1 and TRAF2. (*a*) GST fusion proteins containing various portions of the CD30 cytoplasmic domain. The GST fusion proteins with COOH-terminal deletions of CD30 are C3 (deletion of the COOH-terminal 97 residues) and C4 (deletion of the COOH-terminal 66 residues). The GST fusion proteins with NH₂-terminal deletions of the CD30 cytoplasmic domain are N1 (deletion of the NH₂-terminal 28 residues), N2 (deletion of the NH₂-terminal 61 residues), N3 (deletion of the NH₂-terminal 91 residues, and N4 (deletion of the NH₂-terminal 121 residues). Amino acid residue numbers of CD30 are also indicated. (*b*) Coprecipitation of TRAF-1 and TRAF-2 by GST–CD30 in 293 cell extracts. (*c* and *d*) Association of in vitro translated TRAF1 or TRAF2 with GST–CD30. The COOH-terminal 66 amino acid residues of CD30 are sufficient to interact with TRAF1 and TRAF2. Different deletions of the CD30 cytoplasmic domain are described in *a*. In vitro–translated TRAF1 and TRAF2 products before the coprecipitation experiments are indicated as TRAF1 and TRAF2, respectively. TRAF1 and TRAF2 are marked with arrows. *GST–FAS*, GST fusion protein with the Fas cytoplasmic domain.

(Fig. 3 *b*). In vitro–translated TRAF1 and TRAF2 showed independent binding to GST–CD30 (Fig. 3, *c* and *d*). Neither TRAF1 nor TRAF2 bound to GST or GST–Fas (Fig. 3. *c* and *d*).

Further experiments with a series of NH₂- and COOH-terminal deletion mutants of CD30 showed that the same COOH-terminal 66 amino acid residues of CD30 necessary for cell death induction are sufficient for interaction with either TRAF1 or TRAF2 (Fig. 3, *c* and *d*). This correlation strongly suggests that TRAF1 and/or TRAF2 are involved in the CD30-mediated cell death of T cell hybridomas described above. Interestingly, although TRAF2 expression was not significantly altered, the level of TRAF1 mRNA was dramatically increased in T cell hybridomas upon activation via the TCR (Fig. 4). This raises the possi-

bility that the upregulation of TRAF1 expression may be part of the TCR-mediated signal(s) required for CD30-mediated cell death induction described above. Future experiments with dominant-negative forms of TRAF proteins are required to show whether TRAF proteins are indeed mediating signals for observed cell death.

TRAF1, TRAF2, and other TRAF proteins were previously shown to interact with TNF-R2, CD40, and the EBV transforming protein LMP1, all of which play important roles in cell proliferation (18, 30–32). The results presented here show that TRAF1 and/or TRAF2 also interact with CD30. However, the interaction of TRAF proteins with CD30 appears to induce cell death rather than cell proliferation of T cell hybridomas. Since CD30 directly interacts with either TRAF1 or TRAF2, whereas TNF-R2 interacts with TRAF1 indirectly via TRAF2 (18), it suggests that the manner by which TRAF proteins interact with cognate cell surface receptors may result in different effects on cells. However, it is also equally possible that different outcomes of TRAF-mediated signals, cell proliferation or death, may be dependent on cell type rather than its cognate cell surface receptors.

Fas has been shown to be a key molecule in maintaining peripheral T cell tolerance. However, the negative selection of immature thymocytes is not affected in Fas-deficient mice, suggesting that a Fas-independent pathway must operate in the clonal deletion of self-reactive thymocytes (12). The result that cell death induced by TCR and CD30 is independent of Fas suggests that CD30 may play an important role during negative selection of immature thymocytes.
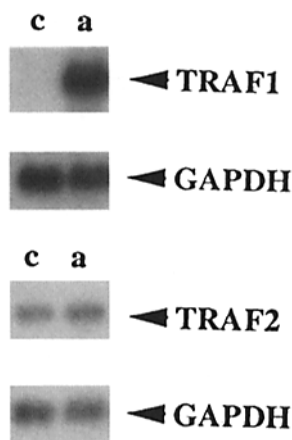
Figure 4. TRAF1 expression in T cell hybridomas is dramatically upregulated upon activation via the TCR. Similar results were obtained with RNA from KCIT1-8.5 (data not shown). *c*, control unstimulated RNA; *a*, TCR-activated RNA.

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