Experimental Cell Research **252,** 96–103 (1999)
Article ID excr.1999.4631, available online at http://www.idealibrary.com on IDEAL®

Murine TRAIL (TNF-Related Apoptosis Inducing Ligand) Expression Induced by T Cell Activation Is Blocked by Rapamycin, Cyclosporin A, and Inhibitors of Phosphatidylinositol 3-Kinase, Protein Kinase C, and Protein Tyrosine Kinases: Evidence for TRAIL Induction via the T Cell Receptor Signaling Pathway

Bruce L. Musgrave, Tom Phu, Jared J. Butler, Andrew P. Makrigiannis, and David W. Hoskin[1]

Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

TRAIL (TNF-related apoptosis inducing ligand), like other members of the TNF family of proteins, is able to induce apoptosis in sensitive target cells. Recently, cell-surface TRAIL has been shown to be expressed by activated human and mouse T lymphocytes, raising the possibility that TRAIL might be involved in T cell-mediated cytotoxicity and/or immune regulation. In the present study we show by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis that activated, but not resting, mouse T cells express abundant TRAIL mRNA. TRAIL transcripts were detectable within 4 h of T cell activation. A panel of pharmacologic inhibitors was used to investigate the signal transduction pathways involved in TRAIL gene induction following T lymphocyte activation. TRAIL gene expression was sensitive to the *src*-like protein tyrosine kinase (PTK) inhibitor herbimycin A, as well as the more general PTK inhibitor genistein, suggesting the involvement of a *src* family PTK. The PKC inhibitors staurosporine and calphostin C, and the phosphatidylinositol 3-kinase (PI3-K) inhibitors wortmannin and LY294002, also prevented TRAIL mRNA transcription by activated T cells, indicating a role for PKC and PI3-K. In addition, TRAIL induction was inhibited by cyclosporin A, implicating the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin. TRAIL expression was also blocked by rapamycin, which inhibits p70 S6 kinase involved in CD28 and interleukin (IL)-2 receptor signaling. However, TRAIL mRNA expression was not induced by IL-2, suggesting that TRAIL gene induction is not coupled to the IL-2 receptor. Data obtained by RT-PCR were confirmed at the protein level by immunoblotting with TRAIL-specific antibody. We conclude that TRAIL gene induction is initiated through a T cell receptor-associated signaling pathway similar to that responsible for the expression of cytokine genes such as IL-2.

Key Words: **TRAIL; T lymphocyte; T cell receptor; signal transduction; gene expression.**

INTRODUCTION

TRAIL (TNF-related apoptosis inducing ligand) is a recently described member of the TNF (tumor necrosis factor) family which includes the cytolytic effector molecules TNFα, lymphotoxin (TNFβ), lymphotoxin β, and CD95 (Fas) ligand [1]. Like other members of the TNF ligand family (with the exception of lymphotoxin), TRAIL is a type II membrane protein, having an intracellular amino-terminal portion, an internal transmembrane domain, and a carboxyl terminus external to the cell. TRAIL mRNA is expressed in many different tissues but is especially abundant in the spleen, prostate, and lungs. TRAIL transcripts are not detectable in the brain, liver, or testis. Among the TNF family members, TRAIL shares the highest amino acid identity with CD95 ligand [2]. TRAIL, like CD95 ligand, is able to induce apoptotic cell death in sensitive target cells [1, 3]. Tumorigenic or transformed cells are particularly sensitive to TRAIL-mediated killing [1, 2]. To date, four distinct TRAIL binding receptors, termed TRAIL-R1 through -R4, have been identified [4–6]. Upon binding TRAIL, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) are able to transduce an apoptotic signal, as well as a signal leading to activation of the transcription factor NFκB [4, 5, 7]. On the other hand, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) fail to mediate apoptosis upon ligation, suggesting that these TRAIL receptors might act as nonsignaling "decoys" which protect normal cells from TRAIL-mediated killing [5, 6]. However, the recent finding that TRAIL resistance in a panel of human tumor cell lines does not correlate with TRAIL-R3/R-4 mRNA expression implies that the "decoy" hy-

[1] To whom correspondence and reprint requests should be addressed at Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7. Fax: 902-494-5125. E-mail: dwhoskin@is.dal.ca.

0014-4827/99 $30.00

pothesis may not be correct [2]. Instead, it has been proposed that differential expression of the apoptosis inhibitor FLIP (FLICE-like inhibitory protein) might account for the resistance of normal cells to TRAIL-induced apoptosis [8].

Although TRAIL mRNA has been isolated from human lymphoid tissues such as the spleen and thymus, as well as from peripheral blood lymphocytes [1], the role of TRAIL in immune function has not been determined. Constitutive expression of surface TRAIL has recently been described for transformed cells of the T and B lymphocyte lineage from both humans and mice [9]. The majority of transformed T and B cells studied also express CD95 ligand, although TRAIL and CD95 ligand appear to differ in their regulatory pathways. An examination of TRAIL expression in normal mouse lymphocytes reveals that activated, but not resting, T cells express surface TRAIL, whereas freshly isolated B cells are positive for surface TRAIL expression which is retained following activation with lipopolysaccharide [10]. Following activation, both $CD4^+$ and $CD8^+$ $\alpha\beta$-T cell receptor (TCR)-bearing T cells upregulate TRAIL expression in a fashion that involves new protein synthesis. Stimulation of peripheral human $CD4^+$ or $CD8^+$ T lymphocytes with mitogens also induces the expression of TRAIL mRNA [11]. In addition, human natural killer cells have recently been reported to express functional TRAIL [12]. Activation-induced expression of TRAIL by $CD8^+$ T cells [10, 11], coupled with the TRAIL-sensitive phenotype of certain tumor cell lines [2, 8], suggests that cell-surface TRAIL might constitute an important mechanism of T cell tumoricidal activity. In support of this, human $CD4^+$ cytotoxic T lymphocytes (CTL) have recently been shown to employ TRAIL to kill CD95 ligand-resistant melanoma cells [13]. Interestingly, activated peripheral T cells from normal healthy individuals fail to develop sensitivity to TRAIL-induced apoptosis, suggesting that, unlike CD95 ligand, TRAIL is not involved in regulating T cell expansion following activation by antigen [11]. However, freshly isolated T cells from HIV-1-infected children are sensitive to TRAIL-induced apoptosis, as are freshly isolated bone marrow cells from some patients with acute leukemia. TRAIL may, therefore, play a role in the increased apoptosis of T lymphocytes in HIV-1 infection, as well as controlling the growth of leukemic cells.

In this study we have explored the signal transduction pathways involved in TRAIL induction in mouse T lymphocytes following stimulation through the TCR/CD3 complex with anti-CD3 monoclonal antibody (mAb). Using TRAIL mRNA expression determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) as a readout system, we have employed selective pharmacologic inhibitors to demonstrate the involvement of protein tyrosine kinases (PTK), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3-K), p70 S6 kinase, and the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin in TRAIL upregulation. TRAIL expression was not induced by interleukin (IL)-2, suggesting that the IL-2 receptor (IL-2R) signaling pathway is not involved in TRAIL induction in T cells. These results were confirmed by immunoblot analysis with TRAIL-specific antibody.

METHODS

Materials. Genistein and herbimycin A were purchased from Life Technologies (Burlington, ON). Staurosporine and cyclosporin A (CsA) were obtained from Sigma Chemical Co. (St. Louis, MO). Calphostin C, wortmannin, LY294002, and rapamycin were from Research Biochemicals Int. (Natick, MA). Stock solutions of reagents were prepared according to the manufacturer's instructions and stored at −20°C. Working solutions were made in culture medium immediately prior to use. Human rIL-2 (active in the murine system) was purchased from Genzyme Corp. (Cambridge, MA) and stored at −70°C until use. Rabbit polyclonal anti-TRAIL antibody was obtained from Santa Cruz Biotechnology Inc.(Santa Cruz, CA).

Cells. Spleens from 6- to 8-week-old female C57BL/6 mice (Charles River Canada, Lasalle, PQ) were disrupted in phosphate-buffered saline (PBS; pH 7.2), and the resulting spleen cell preparation was depleted of erythrocytes by osmotic shock. Spleen cells were washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 10 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from ICN Biomedicals Canada, Mississauga, ON), 5 mM Hepes buffer (pH 7.2; Sigma), and 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (Life Technologies). Spleen cells were then passaged through a nylon wool column (Cellular Products Inc., Buffalo, NY) to remove most B lymphocytes and macrophages [14]. The resulting T lymphocyte-enriched cell preparation was counted and adjusted to a concentration of 4 to 5×10^6 cells/ml. Cell viability was typically greater than 95%. Cells (2 ml final vol) were cultured for the indicated period of time in 24-well plates in the absence or presence of anti-CD3 mAb (1/20 dilution of hybridoma 145-2C11 [15] culture supernatant), with or without drugs or rIL-2. All cultures were performed at 37°C in a humidified atmosphere containing 5% CO_2.

Semiquantitative RT-PCR. Total RNA was isolated from T lymphocytes using TRIzol reagent according to the manufacturer's instructions (Life Technologies). Single-stranded cDNA was synthesized from 1 μg RNA by reverse transcription with 200 U of Moloney murine leukemia virus reverse transcriptase in the presence of 1 μg of random hexanucleotide primers, 0.5 mM dNTPs, and 5 mCi of [^{32}P]dCTP (DuPont). Amplification of equal levels of cDNA was ensured by monitoring [^{32}P]dCTP incorporation during reverse transcription. PCR was conducted in an automatic DNA thermocycler (MJ Research, Inc., Watertown, MA). Each PCR used 5×10^4 cpm of cDNA, 2.5 units of *Taq* DNA polymerase (Life Technologies), 0.2 mM dNTPs, and 0.5 μM of each primer pair in a 1:10 dilution of PCR buffer (2 M KCl, 1 M Tris–HCl, pH 8.4, 1 M $MgCl_2$, 1 mg/ml BSA). The following primer pairs were used for PCR (amplicon size is given after the reverse primer). F refers to forward primer and R refers to reverse primer. All primers were designed to bind intron-bridging exons of the respective gene. GAPDH (F) 5′-ACTCACGGCAAATTCAACGGC-3′; GAPDH (R) 5′-ATCACAAACATGGGGGCATCG-3′ (product size: 247 bp); TRAIL (F) 5′-TCACCAACGAGATGAAGCAGC-3′; TRAIL (R) 5′-CTCACCTTGTCCTTTGAGACC-3′ (product size: 513 bp).

PCR mixtures were overlaid with 100 μl of mineral oil. The amplification protocol for GAPDH (25 cycles) and TRAIL (28 cycles) was

denaturation at 92°C for 30 s, annealing at 57°C for 30 s, and primer extension at 72°C for 1 min (GAPDH) or 2 min (TRAIL). The number of PCR cycles chosen for TRAIL and GAPDH amplifications was previously determined to generate PCR product during the exponential phase of amplification. RT-PCR performed under these conditions has been shown to be semiquantitative, providing reliable detection of twofold or greater differences in mRNA levels [16]. PCR products were visualized by electrophoresis across an ethidium bromide-stained 1.5% TAE agarose gel and the detected PCR amplicon was compared with a 100-bp ladder (Promega Corp., Madison, WI). Relative levels of PCR products were quantified by densitometric analysis of gel photographs and normalization relative to steady-state expression of GAPDH.

Immunoblotting. To prepare lysates, T cells were pelleted by centrifugation, resuspended in 0.05 ml of ice-cold lysis buffer (1% Nonidet P-40, 5 mM ethylenediaminetetraacetic acid, 0.1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin/pepstatin, and 10 μg/ml aprotinin), and incubated on ice for 30 min with vortexing every few minutes. The cell lysates were cleared by centrifugation for 10 min at 14,000*g* and the resulting postnuclear fractions were diluted 1:1 in 2× Laemmli sample buffer, heated for 5 min in a 95°C water bath, and subjected to SDS–PAGE across a 12% gel using standard procedures. Separated proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane and nonspecific protein binding sites were blocked by a 1-h incubation at room temperature in blocking buffer (3% BSA, 0.1% Tween 20, 100 mM NaCl, 10 mM Tris, pH 7.5). Next, the nitrocellulose membrane was incubated for 2 h at room temperature with antibody buffer (1% BSA, 0.1% Tween 20, 100 mM NaCl, 10 mM Tris, pH 7.5) containing 5 μg/ml rabbit polyclonal anti-TRAIL antibody. Following extensive washing with wash buffer (0.1% Tween 20, 100 mM NaCl, 10 mM Tris, pH 7.5), the nitrocellulose membrane was incubated for 1 h at room temperature with antibody buffer containing 2 μg/ml alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad Laboratories, Hercules, CA). Then the nitrocellulose membrane was again washed extensively and placed in the substrate solution (0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 0.34 mg/ml nitro blue tetrazolium, 10 mM $MgCl_2$, 200 mM Tris, pH 9.5). The color reaction was allowed to develop for 30 min before being stopped by briefly immersing the nitrocellulose membrane in distilled water. Equal protein loading was confirmed by amido black staining of a duplicate nitrocellulose membrane containing transferred proteins.

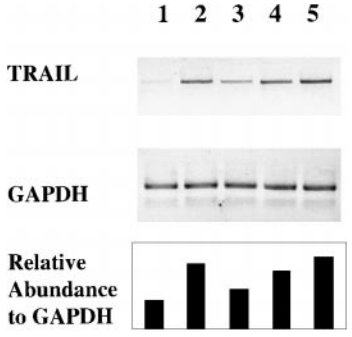

FIG. 1. Time course of TRAIL mRNA expression following T cell activation with anti-CD3 mAb. Total RNA was isolated from resting T cells (lane 1) or from anti-CD3-activated T cells at 4 h (lane 2), 12 h (lane 3), 24 h (lane 4), and 48 h (lane 5) of culture. TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods.* Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of three independent experiments.

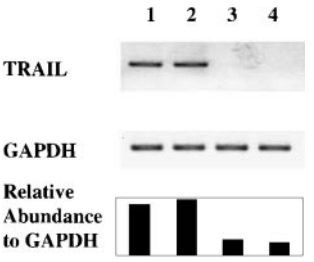

FIG. 2. Effect of PTK inhibitors on TRAIL expression. T cells were stimulated with anti-CD3 mAb in medium alone (lane 1) or in the presence of the DMSO vehicle (lane 2), 1 μM herbimycin A (lane 3), or 100 μM genistein (lane 4). Following 48 h of culture, total RNA was isolated and TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods.* Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of two independent experiments.

Densitometric analysis was used to quantify the ~34-kDa TRAIL protein band on the immunoblot.

RESULTS

Kinetics of TRAIL mRNA expression in T cells following activation with anti-CD3 mAb. To determine the kinetics of activation-induced TRAIL mRNA expression in mouse T lymphocytes, total cellular RNA was obtained from freshly isolated C57BL/6 spleen T cells prior to and at 4, 12, 24, and 48 h following stimulation with anti-CD3 mAb. TRAIL gene transcription was then assessed by semiquantitative RT-PCR. Steady-state GAPDH expression, as determined by RT-PCR, was used as a control for RNA integrity and equal loading. As shown in Fig. 1, unstimulated T cells expressed very little TRAIL mRNA. In contrast, anti-CD3 activation resulted in a marked and rapid increase in TRAIL mRNA expression which was present at high levels after only 4 h of culture. Since TRAIL mRNA expression was highest at 48 h post-stimulation, we chose 48 h as the time point at which to measure TRAIL mRNA expression in all subsequent experiments.

PTK inhibition blocks TRAIL upregulation. Since the activation of T lymphocytes through TCR signaling requires the activation of several distinct intracellular PTK [17], we employed the PTK inhibitors genistein and herbimycin A to assess the role of PTK in TRAIL induction following ligation of the TCR/CD3 complex with anti-CD3 mAb. Genistein is a potent inhibitor of all cellular PTK [18], while herbimycin A is selective for *src*-like PTK [19]. Figure 2 shows that both genistein (100 μM) and herbimycin A (1 μM) prevented the 48-h induction of TRAIL mRNA in anti-CD3-activated T cell cultures. The drug vehicle (DMSO) alone had no significant effect on the expression of TRAIL transcripts. T cell viability is not substantially altered

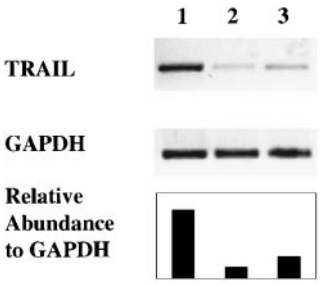

FIG. 3. Effect of PI3-K inhibitors on TRAIL expression. T cells were stimulated with anti-CD3 mAb in the presence of the DMSO vehicle alone (lane 1), 5 μM LY294002 (lane 2), or 25 nM wortmannin (lane 3). Following 48 h of culture, total RNA was isolated and TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods*. Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of three independent experiments.

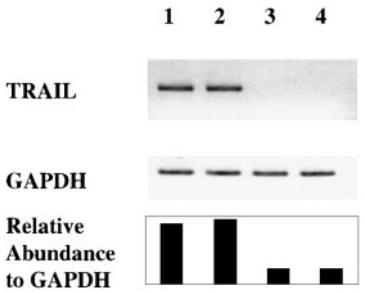

FIG. 4. Effect of PKC inhibitors on TRAIL expression. T cells were stimulated with anti-CD3 mAb in medium alone (lane 1) or in the presence of the DMSO vehicle (lane 2), 50 nM staurosporine (lane 3), or 1 μM calphostin C (lane 4). Following 48 h of culture, total RNA was isolated and TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods*. Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of two independent experiments.

in the presence of 100 μM genistein or 1 μM herbimycin A [20]. These data indicate that TRAIL upregulation in T cells involves the activation of PTK.

PI3-K inhibition prevents TRAIL induction. Wortmannin and LY294002 are potent and selective cell-permeable inhibitors of PI3-K [21, 22], a lipid/serine kinase that is involved in TCR, CD28, and IL-2R signaling in T cells [23]. To investigate the role of PI3-K in activation-induced TRAIL expression by T lymphocytes, we stimulated T cells with anti-CD3 mAb in the absence or presence of wortmannin or LY294002 and measured TRAIL mRNA expression at 48-h of culture. In comparison to control T cells stimulated with anti-CD3 mAb in the presence of the DMSO vehicle, T cells activated in the presence of 5 μM LY294002 or 25 nM wortmannin exhibited a profound reduction in TRAIL mRNA expression, suggesting that TRAIL induction depends on PI3-K activation (Fig. 3). Neither of the drugs, at the concentrations used in these experiments, had nonspecific toxic effects on T cells, as assessed by the trypan blue dye exclusion test performed at the 48-h time point (data not shown).

PKC inhibitors block TRAIL induction. We next used staurosporine and calphostin C to determine the role of PKC activation in activation-induced TRAIL upregulation. Following T cell activation through the TCR/CD3 complex, sustained PKC activation combined with increased free intracellular Ca^{2+} levels promotes the expression of several nuclear transcription factors and the induction of certain T cell genes [17]. Calphostin C is a highly selective inhibitor of PKC [24], while staurosporine is a widely used but somewhat less selective PKC inhibitor [25]. As shown in Fig. 4, TRAIL mRNA expression was abrogated in the presence of either staurosporine (50 nM) or calphostin C (1 μM), indicating that TRAIL induction is dependent upon PKC activation. Previous studies have shown that staurosporine and calphostin C, at the concentrations used in these experiments, have only a minimal effect on T cell viability [20].

CsA inhibits TRAIL gene expression. CsA is an immunosuppressive prodrug which, when complexed with cytoplasmic cyclophilins, prevents the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin from dephosphorylating the cytoplasmic component of the transcription factor NFAT, thereby blocking nuclear translocation of the cytoplasmic component of NFAT and transcriptional activation of genes such as IL-2 and CD95 ligand [26, 27]. Figure 5 shows that CsA, at 1 and 10 μM concentrations, blocks TRAIL induction in anti-CD3-activated T cells. These data indicate that TRAIL gene expression is regulated by a CsA-sensitive mechanism. In comparison to untreated control cells, the drug vehicle (ethanol) alone had no significant effect on the expression of TRAIL transcripts. T cell viability in 48-h anti-CD3-stimulated

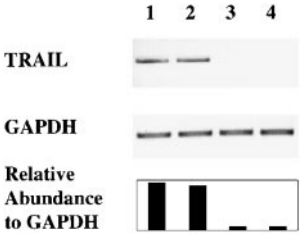

FIG. 5. Effect of CsA on TRAIL expression. T cells were stimulated with anti-CD3 mAb in medium alone (lane 1) or in the presence of the ethanol vehicle (lane 2), 1 μM CsA (lane 3), or 10 μM CsA (lane 4). Following 48 h of culture, total RNA was isolated and TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods*. Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of two independent experiments.

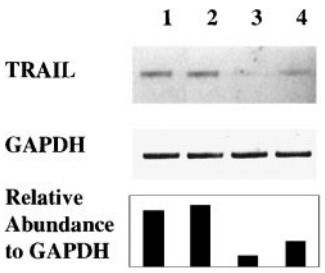

FIG. 6. Effect of rapamycin on TRAIL expression. T cells were stimulated with anti-CD3 mAb in the presence of medium alone (lane 1), the DMSO vehicle (lane 2), 1 ng/ml rapamycin (lane 3), or 10 ng/ml rapamycin (lane 4). Following 48 h of culture, total RNA was isolated and TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods*. Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of two independent experiments.

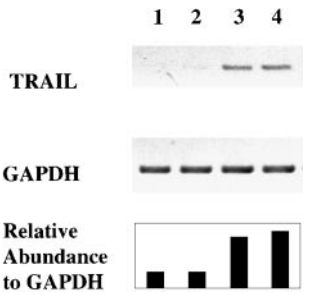

FIG. 7. Effect of IL-2 on TRAIL expression. T cells were cultured in the absence (lane 1) or presence (lane 2) of rIL-2 (250 U/ml) or with anti-CD3 mAb without (lane 3) or with 250 U/ml rIL-2 (lane 4). Following 48 h of culture, total RNA was isolated and TRAIL and GADPH mRNA levels were determined by semiquantitative RT-PCR as described under *Methods*. Densitometric analysis was performed to quantitate TRAIL expression relative to the steady-state expression of GADPH. Data are representative of two independent experiments.

cultures is unaffected by CsA at the concentrations used in these experiments [28].

TRAIL induction is rapamycin-sensitive. Rapamycin is an immunosuppressive drug which prevents p70 S6 kinase activation involved in signal transduction through both the IL-2R and the CD28 of T cells [29, 30]. To determine whether TRAIL induction requires p70 S6 kinase activation, we cultured anti-CD3-stimulated T cells in the absence or presence of rapamycin (1 and 10 ng/ml) and assessed TRAIL mRNA levels after 48 h. As shown in Fig. 6, rapamycin had an inhibitory effect on TRAIL mRNA levels in anti-CD3-activated T cells. Rapamycin, at the concentrations used in this experiment, had no significant effect on T cell viability (data not shown). These data indicate a role for p70 S6 kinase in TRAIL upregulation and suggest the involvement of CD28 and/or IL-2R signaling pathways.

TRAIL induction is IL-2-independent. The rapamycin-sensitive nature of TRAIL gene expression raised the possiblity that IL-2R signaling might contribute to TRAIL induction. IL-2 is known to induce T cells to express the cytotoxic effector molecules granzyme B, perforin, and CD95 ligand [31, 32]. We therefore cultured T cells with or without anti-CD3 mAb in the absence or presence of 250 U/ml of IL-2. As shown in Fig. 7, IL-2 alone did not induce TRAIL mRNA expression after 48 h of culture, nor did exogenous IL-2 enhance anti-CD3-induced TRAIL expression. These data suggest that TRAIL gene induction does not involve IL-2R signaling.

Immunoblot analysis of TRAIL protein expression. We next used immunoblot analysis with TRAIL-specific antibody to confirm, at the protein level, the effects of PTK , PI3-K, and PKC inhibitors, as well as CsA, rapamycin, and IL-2 on T cell expression of TRAIL. As shown in Fig. 8, unstimulated T cells failed to express appreciable levels of TRAIL protein, while T cells activated for 48 h with anti-CD3 mAb strongly upregulated expression of TRAIL protein. However, TRAIL protein levels were dramatically reduced when T cells were activated in the presence of herbimycin A (0.5 μM), LY294002 (5 μM), calphostin C (0.5 μM), CsA (10 μM), or rapamycin (10 ng/ml). T cells cultured in IL-2 alone (250 U/ml) did not upregulate TRAIL protein expression. These results are in complete agreement with the results of our RT-PCR analysis of TRAIL mRNA expression.

DISCUSSION

The recent demonstration of surface TRAIL on mouse $CD4^+$ and $CD8^+$ T cells following TCR/CD3

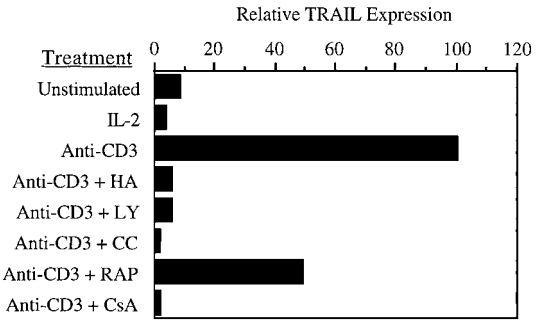

FIG. 8. Effect of IL-2 and pharmacologic inhibitors of T cell signal transduction on anti-CD3-induced TRAIL protein expression. Cell lysates were prepared from resting T cells and from T cells cultured for 48 h in the presence of IL-2 (250 U/ml) alone or anti-CD3 mAb, with or without herbimycin A (HA, 0.5 μM), LY294002 (LY, 5 μM), calphostin C (CC, 0.5 μM), CsA (10 μM), or rapamycin (RAP, 10 ng/ml) as described under *Methods*. The lysates were electrophoresed on a 12% SDS–PAGE gel and then blotted onto a nitrocellulose membrane. TRAIL (MW $\sim$ 34 kDa) was visualized with a TRAIL-specific polyclonal rabbit antibody and densitometric analysis was performed to quantitate TRAIL protein expression. Data are representative of three independent experiments.

triggering suggests possible roles for TRAIL in the immune response, including activation-induced T cell death, regulation of B cell expansion by T cells, and T cell-mediated cytotoxicity [10]. Early findings in the human system indicate that normal activated T lymphocytes fail to develop TRAIL sensitivity, arguing against a role for TRAIL in activation-induced T cell death in healthy individuals [11]. However, the same study supports a role for TRAIL in apoptosis of T cells from HIV-infected individuals, as well as antileukemic growth control. Although conclusive evidence of the involvement of TRAIL in T cell-mediated cytotoxicity must await the development of TRAIL-sensitive and -resistant target cell lines, the residual cytotoxic activity of CTL from mice deficient in both perforin and CD95 ligand is consistent with the involvement of an additional death factor such as TRAIL in CTL effector function [33]. Moreover, T cell expression of TRAIL would be expected to play an important role in antitumor immunity since TRAIL is a potent inducer of apoptosis in neoplastic cells but not normal cells [2, 8]. Evidence in support of this view comes from a recent report that TRAIL mediates killing of CD95 ligand-resistant melanoma cells by human $CD4^+$ CTL [13].

In this study we show that TRAIL mRNA expression is nearly absent in resting mouse T cells but is strongly upregulated within 4 h of TCR/CD3-induced activation and remains present at high levels in 48-h activated T cells. TRAIL protein, while almost undetectable in unstimulated T cells, was also strongly expressed by 48-h activated T cells. Our data confirm results for TRAIL protein expression by unactivated and activated mouse T cells which were recently reported by Mariani and Krammer [10]. Interestingly, the kinetics of TRAIL expression by T cells following triggering through the TCR/CD3 complex closely resemble those of CD95 ligand [27, 32], another member of the TNF family that has been implicated in immune regulation and the cytotoxic effector function of lymphocytes [34]. TCR engagement, therefore, rapidly confers upon CTL the potential to kill TRAIL- and/or CD95 ligand-sensitive target cells, as well as triggering granule-mediated cytotoxicity [35]. The ability to induce target cell death by several distinct pathways would be expected to allow CTL to kill virally infected or transformed cells which are refractory to a particular cytotoxic effector mechanism. In this regard, TRAIL expression enables T lymphocytes to eliminate tumor cells which are resistant to CD95 ligand-mediated killing [13].

We have used pharmacologic inhibitors of signal transduction processes to begin to explore the signaling pathways that are involved in activation-induced TRAIL gene expression by T lymphocytes. Both genistein and herbimycin A prevented TRAIL induction, indicating that PTKs activated in response to TCR/CD3 triggering are also involved in TRAIL gene expression. Genistein competes for the ATP-binding sites of PTK and is, therefore, broadly active against this class of kinases [18]. On the other hand, herbimycin A selectively and irreversibly degrades *src*-family PTK [19]. The failure of anti-CD3-activated T cells to transcribe TRAIL mRNA in the presence of herbimycin A therefore implicates *src*-like PTK in TRAIL induction. Since signal transduction through the TCR is known to involve the *src*-family PTK *lck* and *fyn* [17], *lck* and/or *fyn*, as well as other non-*src* PTK, may play a role in upregulating TRAIL expression by T lymphocytes.

Inhibition of anti-CD3-induced TRAIL expression by the PI3-K-selective inhibitors wortmannin and LY294002 implies a role for PI3-K in TRAIL induction. However, data obtained using wortmannin as a PI3-K inhibitor must be interpreted with caution due to the ability of wortmannin to inhibit phosphatidylinositol 4-kinase and phospholipase A_2 at concentrations that would normally be used to inhibit PI3-K [21, 36]. Nevertheless, the fact that we obtained similar results with the structurally unrelated PI3-K inhibitor LY294002 [22] strongly suggests that TRAIL upregulation was prevented due to specific PI3-K inhibition. Activation of PI3-K occurs following the activation of various PTKs associated with TCR signaling and preceeds the activation of PKC isozymes, mitogen-activated protein kinases, and p70 S6 kinase [23].

Stimulation of the TCR activates phospholipase Cγ1, resulting in the hydrolysis of phosphoinositol 4,5-bisphosphate and the production of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol [17]. Inositol 1,4,5-trisphosphate triggers the release of Ca^{2+} from intracellular stores, while diacylglycerol activates PKC. We have used the PKC inhibitors staurosporine and calphostin C to show that upregulation of TRAIL expression following T cell activation is dependent upon PKC activity and is, therefore, likely preceeded by the generation of inositol 1,4,5-trisphosphate and diacylglycerol in response to TCR signaling. Staurosporine targets the proteolytically generated catalytic domain of PKC [25] but also inhibits protein kinase A and B because of the high degree of homology which exists among protein kinase A, B, and C [37]. Calphostin C, however, is a highly selective inhibitor of PKC because it targets the regulatory domain of PKC, which is distinct from those of other protein kinases [38]. A role for PKC in TRAIL induction is indicated since both calphostin C and staurosporine were able to prevent TRAIL gene expression following T cell activation. This is consistent with previous reports that treatment with the PKC activator PMA in combination with the calcium ionophore ionomycin is sufficient to induce TRAIL expression by mouse and human T lymphocytes [10, 11]. However, at the present time we are

unable to rule out the possible involvement of protein kinase A and/or B in TRAIL gene induction.

The sustained increase in free intracellular Ca^{2+} which follows signal transduction through the TCR is critical for the activation of calcineurin [17], a Ca^{2+}/calmodulin-dependent phosphatase that is required for the nuclear assembly of the transcription factor NFAT [26]. The appearance of AP-3 and NFκB DNA binding activity is also controlled, albeit to a lesser extent, by the calcineurin pathway [39]. We have shown here that the calcineurin inhibitor CsA is able to prevent TRAIL expression by anti-CD3-activated T cells. This finding agrees with the recent suggestion that TCR/CD3-induced upregulation of surface TRAIL is governed by CsA-sensitive factors [10]. TRAIL gene expression is therefore regulated in a manner similar to CD95 ligand and T cell cytokines such as IL-2 [27, 39]. However, additional study is required to determine the relative importance of NFAT, AP-3, and NFκB in TRAIL transcription control following T cell activation through TCR/CD3 signaling.

The enzyme p70 S6 kinase is a key component of signal transduction through the IL-2R, as well as costimulation through CD28 on T cells [29, 30]. Reduced levels of TRAIL mRNA and protein in anti-CD3-activated T cells cultured in the presence of the p70 S6 kinase inhibitor rapamycin [29] therefore suggest the possible involvement of CD28 and/or IL-2R signaling in TRAIL induction. However, IL-2 failed to induce TRAIL expression in T cell cultures, and anti-CD3-induced upregulation of TRAIL gene transcription was not enhanced in the presence of exogenous IL-2, even though the addition of exogenous IL-2 strongly enhances anti-CD3-induced granzyme B and perforin expression by mouse T cells [40]. These data argue against a role for the IL-2R signaling pathway in regulating TRAIL induction. Furthermore, the IL-2-independent nature of TRAIL gene expression in T cells is consistent with the early appearance of TRAIL mRNA in anti-CD3-activated T cell cultures prior to the appearance of detectable IL-2 in culture supernatants [28]. The finding that TRAIL induction is governed by a rapamycin-sensitive signaling process in the absence of IL-2R involvement strongly suggests that ligation of costimulatory CD28 is required for optimal TRAIL expression since signal transduction through the CD28 pathway is rapamycin-sensitive [30]. In this regard, control of TRAIL gene expression differs from that of CD95 ligand gene expression which is resistant to rapamycin and, therefore, does not require p70 S6 kinase activation [40].

We conclude that the CsA- and rapamycin-sensitive, as well as PTK-, PKC-, and PI3-K-dependent, nature of the factors regulating the expression of TRAIL by anti-CD3-activated T lymphocytes is most consistent with a TCR/CD3-associated signaling pathway similar to that involved in the expression of IL-2 and other cytokines [17]. This raises the possibility that certain tumor-associated immunosuppressive molecules which affect T cell signal transduction through the TCR/CD3 complex [41] might allow tumor cells to elude TRAIL-dependent cytotoxic activity by tumor-reactive T lymphocytes.

This work was supported by a grant to D.H. from the Natural Sciences and Engineering Research Council of Canada (OGP0046295). B.M. and A.M. are recipients of NSERC Postgraduate Studentships. J.B. was supported by a CaRE (Cancer Research and Education) Nova Scotia Trainee Award with funding from the Faculty of Medicine, Dalhousie University.

REFERENCES

1. Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C.-P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and Goodwin, R. G. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3,** 673–682.
2. Griffith, T. S., and Lynch, D. H. (1998). TRAIL: A molecule with multiple receptors and control mechanisms. *Curr. Opinion Immunol.* **10,** 559–563.
3. Pitti, R. M., Marsters, S. A., Rupper, S., Donahue, C. J., Moore, A., and Ashkenazi, A. (1996). Induction of apoptosis by APO-2 ligand, a new member of the TNF cytokine family. *J. Biol. Chem.* **271,** 12687–12690.
4. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Schoenfeld, H.-J., Broger, C., Loetscher, H., and Lesslauer, W. (1997). The receptor for the cytotoxic ligand TRAIL. *Science* **276,** 111–113.
5. Pan, G., Ni, J., Wei, Y.-f., Yu, G. I., Gentz, R., and Dixit, V. M. (1997). An antagonistic decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277,** 815–818.
6. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. (1997). The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity* **7,** 813–820.
7. Schneider, P., Thome, M., Burns, K., Bodmer, J.-L., Hofmann, K., Kataoka, T., Holler, N., and Tschopp, J. (1997). TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-κB. *Immunity* **7,** 831–836.
8. Griffith, T. S., Chin, W. A., Jackson, G. C., Lynch, D. H., and Kubin, M. Z. (1998). Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. *J. Immunol.* **161,** 2833–2840.
9. Mariani, S. M., and Krammer, P. H. (1998). Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur. J. Immunol.* **28,** 973–982.
10. Mariani, S. M., and Krammer, P. H. (1998). Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. *Eur. J. Immunol.* **28,** 1492–1498.
11. Jeremias, I., Herr, I., Boehler, T., and Debatin, K.-M. (1998). TRAIL/Apo-2-ligand-induced apoptosis in human T cells. *Eur. J. Immunol.* **28,** 143–152.
12. Zamai, L., Ahmad, M., Bennet, I. M., Azzoni, L., Alnemri, E. S., and Perussia, B. (1998). Natural killer (NK) cell-mediated cytotoxicity: Differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *J. Exp. Med.* **188,** 2375–2380.

13. Thomas, W. D., and Hersey, P. (1998). TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Fas ligand-resistant melanoma cells and mediates CD4 T cell killing of target cells. *J. Immunol.* **161,** 2195–2200.
14. Julius, M. H., Simpson, E., and Herzenberg, L. A. (1973). A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3,** 645–649.
15. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E., and Bluestone, J. A. (1987). Identification of a monoclonal antibody specific to a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378.
16. Singer-Sam, J., Robinson, M. O., Bellvè, Simon, M. I., and Riggs, A. P. (1990). Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, Mtase and Zyf gene transcripts during mouse spermatogenesis. *Nucleic Acids Res.* **18,** 1255–1259.
17. Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. *Cell* **76,** 263–274.
18. Akiyama, T., Ishada, J., Nakagawa, S., Ogawara, H., Watanabe, S. I., Itoh, N., Shibuya, M., and Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262,** 5592–5595.
19. June, C. H., Fletcher, M. C., Ledbetter, J. A., Schieven, G. L., Siegel, J. N., Phillips, A. F., and Samelson, L. E. (1990). Inhibition of tyrosine phosporylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA* **87,** 7722–7726.
20. Stewart, B. H., and Hoskin, D. W. (1997). Inhibition of protein tyrosine kinases or protein kinase C prevents nonspecific killer T lymphocyte-mediated tumoricidal activity. *Biochim. Biophys. Acta* **1356,** 333–342.
21. Nakanishi, S., Catt, K. J., and Balla, T. (1995). A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc. Natl. Acad. Sci. USA* **92,** 5317–5321.
22. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **269,** 5241–5248.
23. Ward, S. G., June, C. H., and Olive, D. (1996). PI 3-kinase: A pivotal pathway in T-cell activation? *Immunol. Today* **17,** 187–197.
24. Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159,** 548–553.
25. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Norimoto, M., and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca2+ dependent protein kinase. *Biochem. Biophys. Res. Commun.* **135,** 397–402.
26. Clipstone, N. A., and Crabtree, G. R. (1992). Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. *Nature* **357,** 695–697.
27. Anel, A., Buferne, M., Boyer, C., Schmitt-Verhulst, A.-M., and Golstein, P. (1994). T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A. *Eur. J. Immunol.* **24,** 2469–2476.
28. Kaiser M., Brooks-Kaiser, J., Fitzpatrick, L., Bleackley, R. C., and Hoskin, D. W. (1993). Cytotoxic cell proteinase gene expression and cytolytic activity by anti-CD3-activated cytotoxic T lymphocytes is sensitive to cyclosporin a but is not dependent on interleukin-2 synthesis. *J. Leukoc. Biol.* **54,** 458–464.
29. Kuo, C. J., Chung, J., Florentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992). Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. *Nature* **358,** 70–73.
30. Bierer, B. E., Schreiber, S. L., and Burakoff, S. J. (1991). The effect of the immunosuppressant FK506 on alternate pathways of T cell activation. *Eur. J. Immunol.* **21,** 439–445.
31. Liu, C. C., Joag, S. V., Kwon, B. S., and Young, J. D.-E. (1990). Induction of perforin and serine esterases in a murine cytotoxic T lymphocyte clone. *J. Immunol.* **144,** 1196–1201.
32. Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K., and Nagata, S. (1995). Expression of the Fas ligand in cells of T cell lineage. *J. Immunol.* **154,** 3806–3813.
33. Braun, M. Y., Lowin, B., French, L., Acha-Orbea, H., and Tschopp, J. (1996). Cytotoxic T cells deficient in both functional Fas ligand and perforin show residual cytolytic activity yet lose their capacity to induce lethal graft-versus-host disease. *J. Exp. Med.* **183,** 657–661.
34. Nagata, S. (1997). Apoptosis by death factor. *Cell* **88,** 355–365.
35. Berke, G. (1995). The CTL's kiss of death. *Cell* **81,** 9–12.
36. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. O. (1995). Wortmannin and its structural analogue demethoxyviridan inhibit stimulated phospholipase A2 activity in Swiss 3T3 cells. Wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **270,** 25352–25355.
37. Ruegg, U. T., and Burgess, G. M. (1989). Staurosporin, K-252 and UCN-01: Potent but nonspecific inhibitors of protein kinases. *Trends Phamacol. Sci.* **10,** 218–220.
38. Tamaoki, T. (1991). Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Methods Enzymol.* **201,** 340–347.
39. Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., and Crabtree, G. R. (1989). Cyclosporin A specifically inhibits function of nuclear proteins involved in T cell activation. *Science* **246,** 1617–1620.
40. Makrigiannis, A. P., and Hoskin, D. W. (1997). Inhibition of CTL induction by rapamycin. IL-2 rescues granzyme B and perforin expression but only partially restores cytotoxic activity. *J. Immunol.* **159,** 4700–4707.
41. Correa, M. R., Ochoa, A. C., Ghosh, P., Mizoguchi, H., Harvey, L., and Longo, D. L. (1997). Sequential development of structural and functional alterations in T cells from tumor-bearing mice. *J. Immunol.* **158,** 5292–5296.

Received March 17, 1999
Revised version received August 4, 1999