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A Novel E3 Ubiquitin Ligase TRAC-1 Positively Regulates T Cell Activation

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TRAC-1 (T cell RING (really interesting new gene) protein identified in activation screen) is a novel E3 ubiquitin ligase identified from a retroviral vector-based T cell surface activation marker screen. The C-terminal truncated TRAC-1 specifically inhibited anti-TCR-mediated CD69 up-regulation in Jurkat cells, a human T leukemic cell line. In this study, we show that TRAC-1 is a RING finger ubiquitin E3 ligase with highest expression in lymphoid tissues. Point mutations that disrupt the Zn^{2+}-chelating ability of its amino-terminal RING finger domain abolished TRAC-1's ligase activity and the dominant inhibitory effect of C-terminal truncated TRAC-1 on TCR stimulation. The results of in vitro biochemical studies indicate that TRAC-1 can stimulate the formation of both K48- and K63-linked polyubiquitin chains and therefore could potentially activate both degradative and regulatory ubiquitin-dependent pathways. Antisense oligonucleotides to TRAC-1 specifically reduced TRAC-1 mRNA levels in Jurkat and primary T cells and inhibited their activation in response to TCR cross-linking. Collectively, these results indicate that the E3 ubiquitin ligase TRAC-1 functions as a positive regulator of T cell activation. *The Journal of Immunology,* **2005, 174: 5288–5297.**

T cells rely critically on the effective engagement of their T cell surface Ag receptors (TCRs) by antigenic peptide-MHC complex to identify pathogenic challenges. The strength, quality, and duration of biochemical signals generated by stimulation of the TCR and its coreceptors are amenable to modulations by multiple regulators. Eventually, all signals converge to determine the final T cell response. During the past two decades, significant progress has been made toward understanding the mechanism of TCR signaling (1, 2).

Although components of the TCR complex have been known to undergo activation-dependent ubiquitylation (3), it is only recently that specific regulators of TCR signaling have been identified as enzymes involved in protein ubiquitylation (4–6). It is now clear that protein ubiquitylation touches upon many aspects of eukaryotic biology and is one of the most common regulatory processes (7). Its defective regulation has been implicated in diseases ranging from developmental abnormalities and autoimmunity to neurodegenerative disorders and cancer (8–11). The process of ubiquitylation involves multiclasses of enzymes known as E1s, E2s, and E3s (12). Ubiquitin (Ub)[6] is activated first by an E1 (activating enzyme) to form a high-energy thiol-ester bond between Ub and the active site cysteine of E1. It is then transferred to an E2 (conjugating enzyme), again through a thiol-ester linkage. The E3s (Ub-protein ligases) are the components responsible for recognizing both E2 (13, 14) and substrate and for promoting the formation of isopeptide bonds between Ub and lysines on the target protein or its bound multiubiquitin chain. Therefore, substrate specificity of the multienzyme complex is primarily defined by E3 (12). The human E3 protein family includes >250 RING (really interesting new gene) finger domain-containing proteins (15, 16) and numerous homologous to E6AP carboxyl terminus domain (17), plant homeodomain finger (18, 19), and U box proteins (20). The predicted existence of a large number of E3s agrees well with the finding that a diverse spectrum of proteins are regulated by Ub modification, each process presumably has its specific E3. Traditionally, ubiquitylation has been associated with protein degradation through targeting proteins to 26S proteosomes and lysosomes. Recently, however, ubiquitylation has been implicated in many other regulatory mechanisms ranging from protein kinase activation (21) to transcriptional control (22).

The Cbl protein family is the best known group of E3s involved in the regulation of TCR signaling. The protooncogene *c-Cbl* was first identified as part of an oncogenic mouse retrovirus and characterized as a multidomain signaling molecule (23, 24). More recently, Cbl proteins have been shown to function as RING finger E3s that specifically target activated receptors, receptor tyrosine kinases, and receptor-proximal molecules for ubiquitylation and to down-regulate their signaling (4, 5, 14, 25). All three mammalian Cbl proteins (c-Cbl, Cbl-b, and Cbl-3) have a unique N-terminal tyrosine kinase binding-domain, a RING finger domain that recruits E2s, and multiple proline-rich regions at the C terminus (26).

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[6] Abbreviations used in this paper: Ub, ubiquitin; GRAIL, gene related to anergy in lymphocytes; PKC, protein kinase C; AS, antisense; ctl, control; WT, wild type; Indo-1, indomethacin 1.

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Cbl proteins use either the tyrosine kinase binding or the proline-rich region domains for target protein recognization and the RING finger for ubiquitylation. Though highly related, c-Cbl and Cbl-b appear to have overlapping yet distinct functions in TCR signaling. Although c-Cbl-deficient mice exhibit increased receptor expression and enhanced signaling in thymocytes (27, 28), *Cbl-b*$^{-/-}$ results in primarily hyperactive and proliferative T and B cells in the periphery with little effect on thymocyte development (29, 30). In more recent studies, Cbl-b is shown to play a critical role in the regulation of peripheral tolerance and anergy in T cells (31). However, it is clear that both Cbl proteins play a negative role in TCR signaling, presumably by increasing the threshold for signal detection (32). Interestingly, a recent study indicates that all three mammalian Cbl proteins themselves might be regulated by ubiquitylation mediated through HECT family E3s Nedd4 and Itch (33).

Itch is another E3 Ub ligase (34) that is implicated in the negative regulation of T cell function. *Itch* deficiency leads to severe immune and inflammatory disorders and constant itching of the skin in non-agouti-lethal or itchy mice (34). *Itch*$^{-/-}$ T cells show an activated phenotype and bias toward the type Th2 cell phenotype in differentiation, possibly due to the ability of Itch to mediate JunB ubiquitylation (6). In addition, increased expression of Itch and other E3s with a negative role in T cell activation may also be part of an anergic signaling program in T cells (35).

More recently, gene related to anergy in lymphocytes (GRAIL) was described as a novel transmembrane RING finger E3 involved in the establishment of T cell anergy (36, 37). GRAIL was identified in a differential display study examining transcripts with induced expression in anergic CD4$^+$ T cells. Overexpression of GRAIL in T cell hybridomas leads to diminished cytokine IL-2 and IL-4 production. Subsequent study indicated that the Ub E3 ligase activity of GRAIL, as well as possibly its involvement in endocytic trafficking, was crucial for GRAIL's role in the induction of the anergic phenotype (37).

Many key advances have been made toward our understanding of the mechanisms of T cell activation and TCR signaling, especially in the early biochemical events initiated by TCR engagement. However, the molecular mechanisms by which these early signaling events can be regulated remains incomplete. With this in mind, we conducted a functional genetic screen in a T cell line using retroviral technology combined with a FACS-based surface activation marker enrichment strategy to identify novel proteins involved in the regulation of TCR activation (38). In this study, we describe the initial characterization of a novel protein TRAC-1 (T cell RING protein identified in activation screen), which was isolated in a truncated form from this screen. We show that TRAC-1 is a RING finger E3 Ub ligase with enriched expression in lymphoid tissues and more specifically by CD4$^+$ and CD8$^+$ T cells. Previously discovered E3 Ub ligases have all been shown to play negative roles in T cell activation. Our data demonstrate TRAC-1 as the first E3 Ub ligase that serves a positive regulatory role in T cell activation.

Materials and Methods

Cell culture

Human Jurkat T cells (clone N32H), Jurkat TAg cells (expressing SV40 large T Ag), and BJAB B cells were cultured in RPMI 1640 medium supplemented with 10% FCS (HyClone), penicillin, and streptomycin. Phoenix A cells were grown in DMEM supplemented with 10% FCS, penicillin, and streptomycin (39). The tTA-BJAB or tTA-Jurkat cell lines are described in the study by Holland et al. (40).

Reagents

The sequence of oligonucleotides 16613 (with 5′-FAM) and 13656 is 5′-CCCUCCCAUUUG-UUCAAGCAGCAUC-3′. The sequence of oligonucleotides 16615 (with 5′-FAM) and 13657 is 5′-CUAGCACGAACU UGUUUACCCUCCC-3′. FLAG- and His-tagged wild-type Ub as well as various K to R mutants of Ub were expressed and purified from *Escherichia coli* as described by Huang et al. (41).

Constructs

Wild-type TRAC-1 (aa 1–232) was cloned into the retroviral pTRA-iresGFP and pEFBOS-iresGFP vectors using standard molecular biology techniques. For generating point mutants, the targeted residues were substituted with alanine using standard PCR mutagenesis methods. The N- or C-terminal truncation mutants (TRAC-1ΔC and TRAC-1Δ76), containing aa 1–170 or aa 77–232, respectively, were generated by a standard PCR procedure. All versions of TRAC-1 were C-terminal tagged with a FLAG epitope (DYKDDDDK) and cloned into both the retroviral pTRA-iresGFP vector and the mammalian expression vector pEFBOS-iresGFP. TRAC-1WT and TRAC-1ΔC were also cloned into the pGEX-6P-3 vector (Amersham) in frame with N-terminal GST.

Stimulation and cell surface marker analysis

For CD69 up-regulation experiments, tTA-BJAB or tTA-Jurkat cells were split to 2.5 × 10^5 cells/ml 24 h before stimulation. Cells were spun and resuspended at 5 × 10^5 cells/ml in fresh complete RPMI 1640 medium in the presence of 300 ng/ml C305 (anti-Jurkat clonotypic TCR) for Jurkat, 0.3 μg/ml anti-IgM F(ab′)$_2$ (Jackson ImmunoResearch) for BJAB, or 5 ng/ml PMA for 16–20 h at 37°C. Cells were then stained with an allophycocyanin-conjugated mouse monoclonal anti-human CD69 Ab (Caltag Laboratories) at 4°C for 30 min and analyzed using a FACSCalibur instrument (BD Biosciences) with CellQuest software. Cell sorts were performed on a MoFlo (DakoCytomation). Calcium mobilization assays were performed as described elsewhere (40).

TRAC-1 quantitative PCR (TaqMan) analysis

RT-PCR or PCR was performed by the TaqMan method using the One-Step RT-PCR kit from Applied Biosystems. The following primers and probes were used: forward primer, 5′-TTACACCAGCCTGTCCGGA-3′; reverse primer, 5′-CAGACTGGTAGCAATACAGGAACG-3′; and probe, 5′-CCGCTGCGGCCACGTATTCTG-3′. Human placenta total RNA (Clontech Laboratories) was used for the standard curve and the human *RiP* gene (HuPO) or 18S RNA (Applied Biosystems) was used for normalization of cDNA or total RNA, respectively. Samples were tested in duplicate. RT-PCR was performed on an ABI PRISM 7700.

In vitro substrate-independent Ub ligase assay

Phoenix A cells were transfected with various TRAC-1 constructs by the calcium precipitation method. Twenty-four to 48 h after transfection, cells were lysed in 1 ml of lysis buffer supplemented with protease inhibitors. Cell lysates were clarified by incubating with 30 μl of protein A/G$^+$ agarose beads (Santa Cruz Biotechnology) at 4°C for 2 h. Clarified cell lysates were then incubated with anti-FLAG Ab-coated beads and tumbled at 4°C for 2 h. After incubation, the beads were washed twice with lysis buffer containing 500 mM NaCl and then equilibrated with Ub ligase buffer containing 65 mM TrisCpH 7.5), 6.25 mM $MgCl_2$, 0.75 mM DTT, and 2.5 mM ATP. The immune complexes, or alternatively 100 ng of in vitro-purified GST-TRAC-1, GST-E6AP, or GST-MDM2 fusion proteins, were resuspended in 100 μl of Ub ligase buffer plus in vitro-purified 50–100 ng E1, 50–200 ng E2 (Ubc3, Ubc4, UbcH5c, Ubc7, Ubc10, or Ubc13/Uev1A), and 100 ng Flag-Ub, or 500 ng His-Ub. As a positive control (ctl), purified Roc1/Cul-1 (100 ng) was added in a mixture containing Ub ligase buffer (100 μl) plus E1, E2, and FLAG-Ub. The reactions were allowed to proceed at room temperature or at 37°C for 1 h, followed by standard SDS-PAGE and Western blot analysis with anti-FLAG M2 Ab (Sigma-Aldrich) or polyclonal anti-Ub Ab (Boston Biochem).

Transfection of primary T lymphocytes

Freshly isolated human primary blood mononuclear cells were incubated in RPMI 1640 medium with 10% FBS at 37°C for 4 h in tissue culture flasks to allow macrophages and other adhering cells to settle down. The lymphocytes remaining in suspension were collected by centrifugation and washed one time in 1× PBS. Cells were then resuspended at 1 × 10^7/100 μl in human T cell Nucleofector solution (Amaxa Biosystems) and mixed with 5 μl of control (ctl) or TRAC-1 antisense (AS) oligonucleotide at a final concentration of 50 μM. The cell/oligonucleotide mixture was immediately subjected to an electric shock under program U-14 of Amaxa's

electroporator and transferred to a tissue culture dish with warm medium for 20 h. Since transfection efficiency using this method is routinely >95%, the cells were then sorted into pure FITC-positive populations. As a ctl, mock-transfected cells were sorted into a pure FITC-negative population under the same setting. Cells were then left untreated or stimulated with either plate-bound anti-TCR (5 μg/ml OKT3) and anti-CD28 (5 μg/ml), or PMA (5 ng/ml) and ionomycin (1 μM) at 37°C. Cell culture medium was collected at 24 and 48 h for ELISA-based assessment of IL-2 concentration. IL-2 was measured using commercial ELISA reagents (R&D Systems). At 24 h, cells were stained with anti-CD69-allophycocyanin for FACS analysis.

Results

Identification of a novel RING protein as a regulator of TCR signaling

To identify novel molecules involved in TCR-induced cell activation, we conducted a functional screen in the Jurkat T cell leukemic line using surface CD69 expression as a surrogate marker for cell activation (38). In addition to cDNAs encoding known regulators of TCR signaling, a cDNA encoding a C-terminal truncated form of an uncharacterized protein was isolated from the screen. Sequence analysis indicated that the cDNA insert was a partial sense fragment encoding the first 170 aa of a larger open reading frame encoding a protein of 232 aa (Fig. 1*A*). The corresponding translated amino acid sequence contained the myristoylation consensus sequence MGX_{1-4}(T/S) and a RING finger domain at its amino terminus (Fig. 1*A*). Other than these domains, the translated cDNA shared no sequence or structural homology with any protein of known function. We therefore named this molecule TRAC-1.

To verify that the inhibitory phenotype was caused by the truncated TRAC-1 cDNA and not by a genetic mutation or an epigenetic effect in the single-cell clone, we subcloned the sequence encoding the truncated TRAC-1 (TRAC-1ΔC) into a retroviral vector containing a bicistronically encoded GFP so that the expression of the cDNA in cells could be monitored by the concomitant expression of GFP.

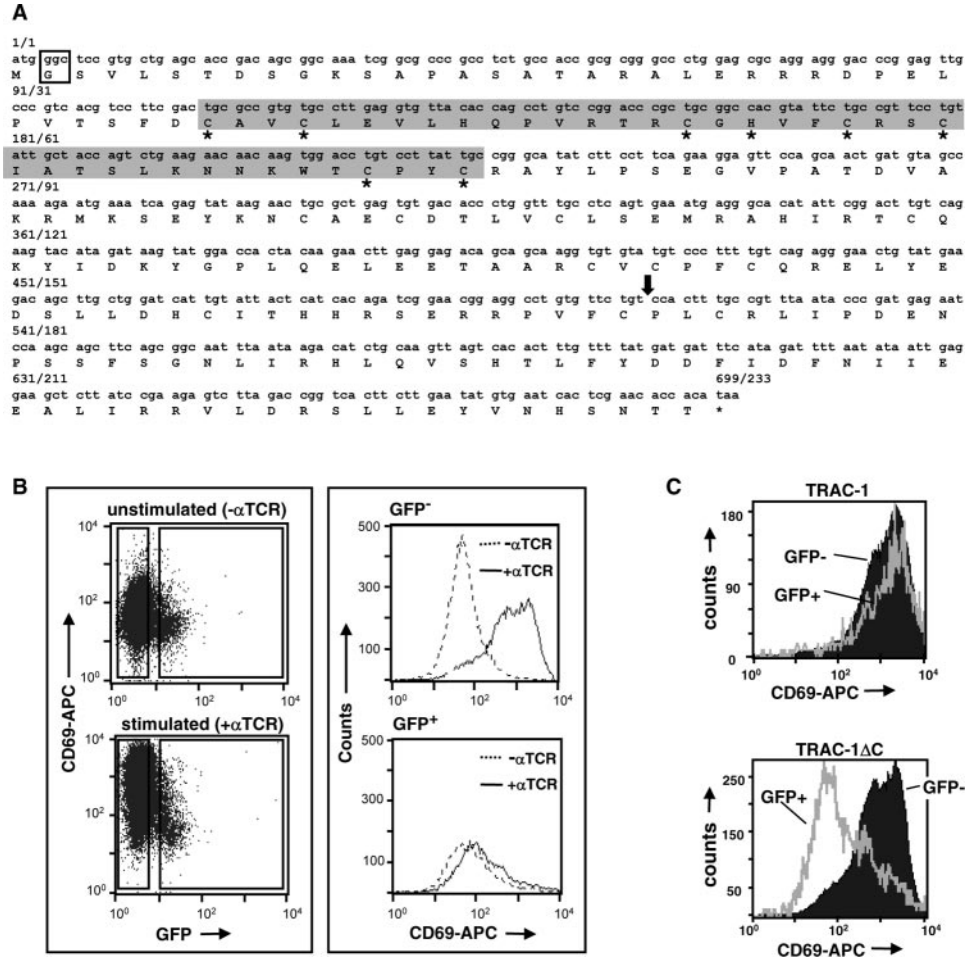

FIGURE 1. TRAC-1, a novel hit from the CD69 cell surface marker screen in Jurkat cells. *A,* Human TRAC-1 cDNA and protein sequences. The putative myristoylation site is boxed. The RING finger domain is shaded gray with consensus cysteine and histidine residues marked with ∗. The black arrow indicates the position where TRAC-1 hit was truncated. These sequence data correspond to GenBank accession number NM017831 (hypothetical protein FLJ20456). *B,* Confirmation of truncated TRAC-1 inhibitory activity by cell surface CD69 expression in naive Jurkat cells infected with pTRA-TRAC-1ΔC-iresGFP. Unstimulated cells and cells stimulated with anti-TCR were stained with allophycocyanin-conjugated anti-CD69 Ab. The *two panels* shown on the *left* are dot plot analysis showing GFP fluorescent intensity on the horizontal axis and CD69-allophycocyanin fluorescent intensity on the vertical axis. Unstimulated cells are shown in the *top left panel* and anti-TCR-stimulated cells in the *bottom left panel*. Histogram plots on the right show CD69-allophycocyanin fluorescent intensity in unstimulated (dotted line) vs anti-TCR-stimulated (solid line) cells when gated on GFP-negative (*upper right panel*) and GFP-positive cells (*lower right panel*). *C,* Effect of full-length TRAC-1 overexpression on cell surface CD69 expression. Naive Jurkat cells were infected with pTRA-TRAC-1-iresGFP or pTRA-TRAC-1ΔC-iresGFP. Unstimulated cells and cells stimulated with anti-TCR were stained with allophycocyanin-conjugated anti-CD69 Ab. The *two panels* shown are histogram analyses showing CD69-allophycocyanin fluorescent intensity on the horizontal axis and cell numbers on the vertical axis in pTRA-TRAC-1-iresGFP-infected cells (*top panel*) or pTRA-TRAC-1ΔC-iresGFP-infected cells (*bottom panel*). Both panels show cells stimulated with anti-TCR and gated on GFP^- and GFP^+ populations.

Using naive Jurkat cells infected with this construct (pTRA-TRAC-1ΔC-iresGFP), we confirmed that TRAC-1ΔC expression caused inhibition of CD69 up-regulation (Fig. 1*B*). Interestingly, expression of the full-length construct (pTRA-TRAC-1-iresGFP) failed to cause inhibition of CD69 up-regulation, suggesting that TRAC-1 itself is not a negative regulator of T cell activation (Fig. 1*C*).

TRAC-1 expression is highest in lymphoid tissues

Real-time quantitative RT-PCR analysis by the TaqMan method demonstrated that the TRAC-1 transcript is expressed at the highest levels in lymphoid tissues, including bone marrow, spleen, and thymus (Fig. 2*A*). Weak to moderate expression was also observed in most other tissues. The relative TRAC-1 mRNA level in various human cancer cell lines was also compared with that in primary blood mononuclear cells. As shown in Fig. 2*B*, TRAC-1 message was about 8-fold higher in primary blood mononuclear cells than in Jurkat cells and 293 HEK cells. The hepatocyte line Huh7 expressed little TRAC-1 message. This was consistent with our results using primary hepatocyte cells where we found that TRAC-1 mRNA was ~50-fold lower in primary cultured hepatocytes than in primary blood mononuclear cells (data not shown). The low expression level of TRAC-1 mRNA in Huh7 and primary human hepatocytes is in contrast to the moderate level of TRAC-1 expression seen in liver tissue (Fig. 2*A*). It is possible that circulating lymphocytes may have contributed to the observed TRAC-1 expression seen in some tissues.

The B cell line BJAB also expressed little TRAC-1 mRNA (Fig. 2*B*). Remarkably, TRAC-1ΔC function was also cell type specific. To test this, TRAC-1ΔC in the retroviral pTRA-ires.GFP vector was delivered by infection into either Jurkat cells or the B cell line BJAB cells (Fig. 2*C*). Two days later, the infected Jurkat and BJAB cells were stimulated with anti-TCR and anti-IgM Abs, respectively. After 20–24 h, surface CD69 expression of the GFP-positive and -negative cells was compared by FACS analysis. As shown in Fig. 2*C*, TRAC-1ΔC specifically blocked Ag receptor-mediated CD69 up-regulation in Jurkat cells upon anti-TCR stimulation, but not in BJAB cells following anti-B cell Ag receptor (BCR) challenge. Further TaqMan analysis demonstrated that TRAC-1 mRNA was more abundant in $CD4^+$ and $CD8^+$ T cells than in B cells or monocytes (Fig. 2*D*). In summary, TRAC-1 mRNA is expressed in lymphoid tissues and is particularly predominant in the $CD4^+$ and $CD8^+$ T cell populations. The results suggest that TRAC-1 function is also preferentially confined to T cells.

TRAC-1 is an E3 Ub ligase in vitro

Sequence analysis revealed that TRAC-1 contains a RING finger domain at its amino terminus. In recent years, multiple RING finger proteins have been implicated in functioning as E3 Ub ligases and in recruiting E2 proteins into the ubiquitylation machinery (7). This observation led us to investigate the possibility that TRAC-1 possesses E3 Ub ligase activity. To check for TRAC-1's E3 Ub ligase activity, we set up an in vitro substrate-independent autoubiquitylation assay using purified components. We first tested bacterially expressed, purified GST-TRAC-1 fusion protein in this assay. As expected, it demonstrated high E3 Ub ligase activity when UbcH4 was used as E2 (Fig. 3*A*, *lane 4*).

TRAC-1 cooperates with both UbcH5c and Ubc13/Uev1A to catalyze both K48- and K63-linked poly-Ub chain formation in vitro

To identify TRAC-1 interacting proteins that help to elucidate its biological function, a yeast two-hybrid screen using wild-type TRAC-1 as bait was performed (data not shown). Two closely related E2 proteins, UbcH7 (42) and UbcH8 (43), represent two of the most frequent hits. The interaction between TRAC-1 and multiple purified E2 proteins, including UbcH7 and UbcH8, has been confirmed in an in vitro E2/E3-binding assay (data not shown). Proteins can be polyubiquitylated through the formation of both K48- and K63-linked poly-Ub chains. Although the heterodimeric E2 Ubc13/Uev1A has been shown to cooperate with certain RING finger E3s (21, 44, 45) to catalyze the formation of poly-Ub chains linked through K63, UbcH5 and several other E2s have been

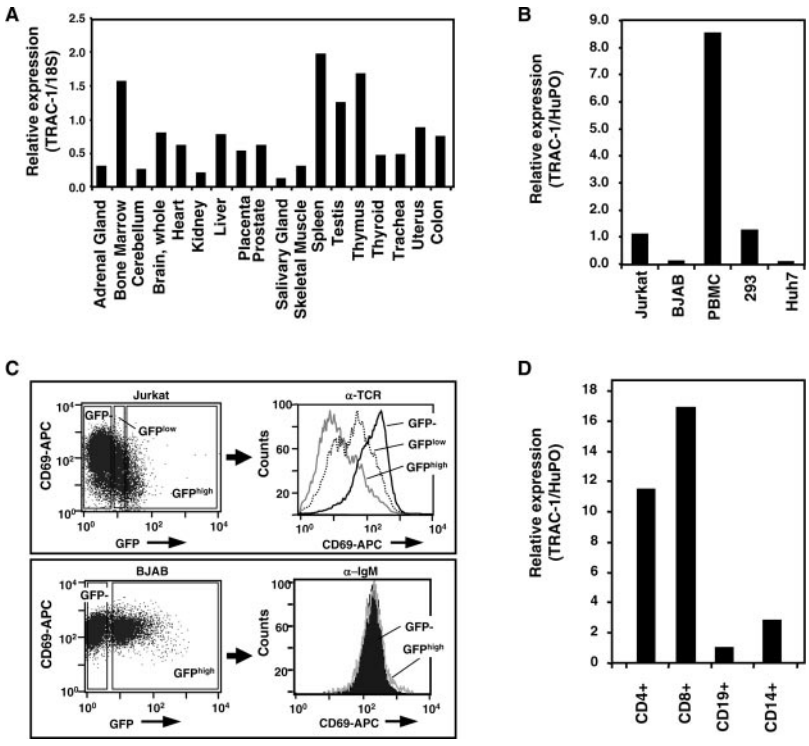

FIGURE 2. TRAC-1 is expressed in lymphoid tissues and predominantly in $CD4^+$ and $CD8^+$ T cell populations. Quantitative PCR (TaqMan) analysis of TRAC-1 expression in various human tissues (*A*), mammalian cell lines (*B*), and purified human primary cells (*D*). cDNA templates were obtained from various tissues (*A*, human tissue multiple tissue cDNA *panel I*; Clontech Laboratories) and $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, or $CD14^+$ monocytes (*C*, human blood fractions multiple tissue cDNA panel; Clontech Laboratories). Total RNA templates were purified from various cell lines as indicated (*B*). Specific TRAC-1 primers or ctl HuPO and 18S rRNA primers were used in standard TaqMan PCR or RT-PCR. *C,* Effect of TRAC-1ΔC on Jurkat and BJAB cell activation. The pTRA-TRAC-1ΔC-iresGFP vector was stably introduced into tTA- Jurkat (*top panels*) or tTA-BJAB (*bottom panels*) cells. Jurkat and BJAB cells were stimulated with anti-TCR (0.3 μg/ml C305) and anti-BCR (0.3 μg/ml anti-IgM $F(ab')_2$), respectively for 20–24 h, and Ag receptor-induced surface CD69 expression was analyzed in infected cells (GFP^{high}, gray line), GFP^{low} cells (dashed line), and uninfected cells (GFP^-, black line). Dot plots in the *left panels* show GFP fluorescent intensity on the horizontal axis and CD69-allophycocyanin on the vertical axis while histogram plots in the *right panels* show CD69-allophycocyanin fluorescent intensity.

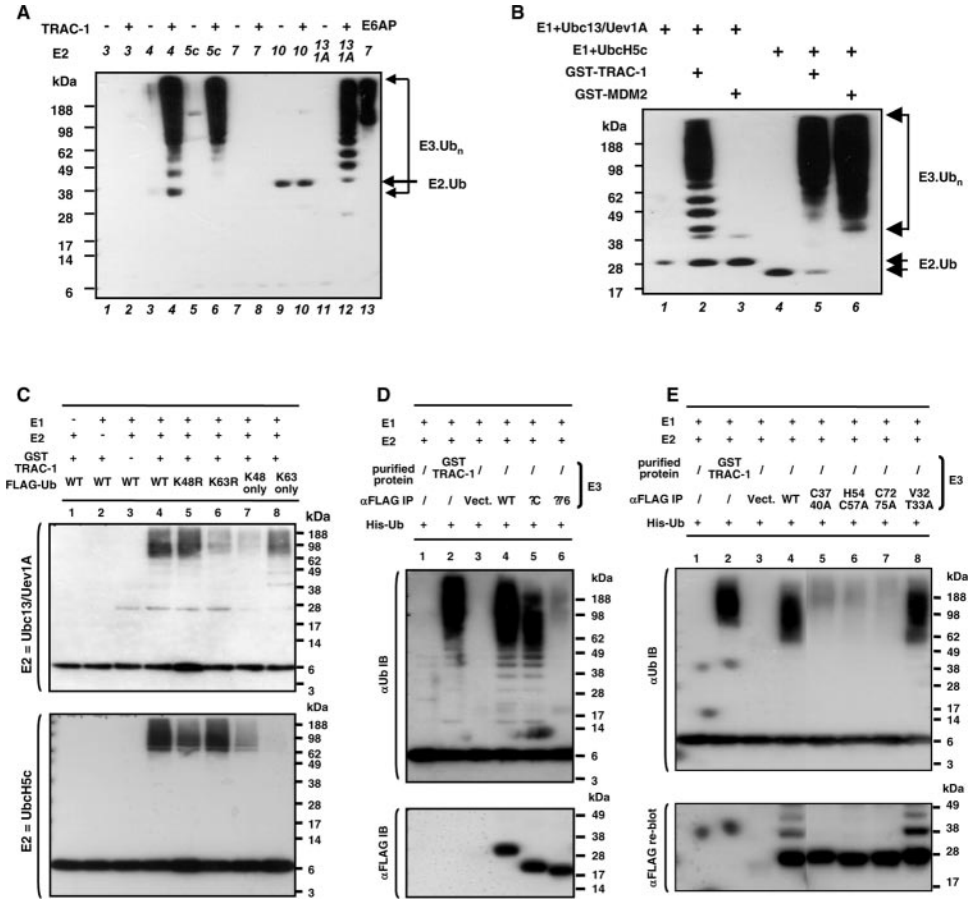

FIGURE 3. TRAC-1 is a RING-HC type E3 Ub ligase. *A,* TRAC-1 can cooperate with Ubc4, UbcH5c, and Ubc13/Uev1A to catalyze polyubiquitylation in vitro. Fifty nanograms of various purified, recombinant E2s in the absence or presence of 100 ng of GST-TRAC-1 (*lanes 1–12*) or a ctl E3 GST-E6AP (*lane 13*) was mixed with purified, recombinant E1 (50 ng), FLAG-tagged Ub (100 ng), and ATP (*lanes 1–13*). Reaction mixtures were incubated at room temperature for 1 h followed by standard SDS-PAGE and Western blot analysis with anti-FLAG Ab. *B,* TRAC-1 but not MDM2 can work with both UbcH5c and Ubc13/Uev1A to catalyze poly-Ub chain formation in vitro. One hundred nanograms of GST-TRAC-1 (*lanes 2* and *5*) or a ctl E3 GST-MDM2 (*lanes 3* and *6*) purified from insect Sf9 cells was mixed with 50 ng of purified, recombinant E2 UbcH5c (*lanes 4–6*), or Ubc13 and Uev1A (*lanes 1–3*), along with purified, recombinant E1 (50 ng), FLAG-tagged Ub (100 ng), and ATP (*lanes 1–6*). Reaction mixtures were incubated at room temperature for 1 h followed by standard SDS-PAGE and Western blot analysis with anti-FLAG Ab. *C,* TRAC-1 can catalyze both K48- and K63-linked poly-Ub chain formation in vitro. One hundred nanograms of GST-TRAC-1 was mixed with 50 ng of E1, various forms of FLAG-tagged Ub (100 ng), and ATP in the presence of either 50 ng of E2s Ubc13 and Uev1A (*top panel*) or with 50 ng of UbcH5c (*bottom panel*). Reaction mixtures were incubated at room temperature for 1 h followed by standard SDS-PAGE and Western blot analysis with anti-FLAG Ab. *D,* TRAC-1 demonstrates RING finger domain-dependent E3 Ub ligase activity in vitro. Forty micrograms of pEFBOS-TRAC-1WT-iresGFP (*lane 4*), pEFBOS-TRAC-1ΔC-iresGFP (*lane 5*), pEFBOS-TRAC-1Δ76-iresGFP (*lane 6*), and empty vector (*lane 3*) was transfected into Phoenix A cells by Ca^{2+} precipitation. The C-terminally FLAG-tagged proteins were then partially purified from cellular lysates by immunoprecipitation with anti-FLAG-coated beads and subjected to the in vitro E3 assay with purified, recombinant E1, Ubc13/UeV1A (E2), His-tagged Ub, and ATP. Reaction mixtures with either no E3 (*lane 1*) or GST-TRAC-1 purified from bacteria (*lane 2*) were included as controls. The reactions were allowed to proceed at room temperature for 1 h followed by standard SDS-PAGE and Western blot analysis with anti-Ub (*top panel*) and anti-FLAG (*bottom panel*) *Abs*. *E,* Point mutations in the RING domain abrogate or dramatically reduce TRAC-1 E3 Ub ligase activity in vitro. Forty micrograms of pEFBOS-TRAC-1WT-iresGFP (*lane 4*), pEFBOS-TRAC-1-C37,40A-iresGFP (*lane 5*), pEFBOS-TRAC-1-H54C57A -iresGFP (*lane 6*), pEFBOS-TRAC-1-C72,75A -iresGFP (*lane 7*), pEFBOS-TRAC-1-V32T33A -iresGFP (*lane 8*), and empty vector (*lane 3*) was transfected into Phoenix A cells by Ca^{2+} precipitation. The C-terminally FLAG-tagged proteins were then partially purified from cellular lysates by immunoprecipitation with anti-FLAG-coated beads and subjected to the in vitro E3 assay with purified, recombinant E150 ng, UbcH5c (E2) 100 ng, His-tagged Ub 500 ng, and ATP. Reaction mixtures with either no E3 (*lane 1*) or GST-TRAC-1 100 ng (*lane 2*) were included as controls. The reactions were allowed to proceed at 37°C for 1 h followed by standard SDS-PAGE and Western blot analysis with anti-Ub Ab (*top panel*) or anti-FLAG Ab (*bottom panel*).

shown to participate in K48-linked poly-Ub chain formation. K63-linked poly-Ub chains have been implicated in the regulation of target protein activities such as kinase activation (21), which is distinct from the traditional degradative, K48-linked ubiquitylation. To test which type of E2s TRAC-1 can cooperate with, purified GST-TRAC-1 was subjected to the in vitro autoubiquitylation assay with various purified, recombinant E2s. As shown in Fig. 3*A*, TRAC-1 demonstrated E3 ligase activity in the presence of Ubc4, UbcH5c, or Ubc13/Uev1A (Fig. 3*A*, *lanes 4*, *6*, and *12*, respectively). Interestingly, although UbcH7 interacts with TRAC-1 in vitro as well as in the yeast two-hybrid assay, it cannot cooperate with TRAC-1 to catalyze the poly-Ub chain formation in vitro (Fig. 3*A*, *lanes 7* and *8*). The purified UbcH7 protein was confirmed to be active since it can facilitate ubiquitylation when a HECT domain ligase E6AP instead of TRAC-1 is used (Fig. 3*A*, *lane 13*). Unlike Ubc4 and UbcH5c which are known to be relatively promiscuous in biochemical assays, the Ubc13/Uev1A complex has been much more selective in its ability to interact and

work with different E3s. So far, only TNFR-associated factor 2 (21), TNFR-associated factor 6 (44), Rad5 (46), CHFR (47), and poxvirus virulence factor p28 (41) were found to be able to cooperate with Ubc13/Uev1A (or Mms2). As shown in Fig. 3*B*, TRAC-1 demonstrated decent E3 ligase activity in the presence of either Ubc13/Uev1A (Fig. 3*B*, *lane 2*) or UbcH5c (Fig. 3*B*, *lane 5*). In contrast, a ctl E3 MDM2 manifested strong activity only when UbcH5c but not Ubc13/Uev1A is present (Fig. 3*B*, *lanes 3* and *6*). Collectively, these data indicate that TRAC-1 may be involved in both K48- and K63-linked poly-Ub chain formation.

Ub is one of the most conserved proteins in eukaryotes with identical sequences in humans and mice. There are seven lysine residues in human Ub, including K48 and K63. K48-linked poly-Ub chains are structurally distinctive from those linked via K63, which might be the basis for their distinctive biological functions. To further investigate whether TRAC-1 E3 ligase is capable of catalyzing K63-linked poly-Ub chain formation, an in vitro ligase assay was conducted using multiple K to R mutants of FLAG-tagged Ub. As shown in the *top panel* of Fig. 3*C* using Ubc13/Uev1A as E2, TRAC-1 catalyzed poly-Ub chain formation in the presence of either the wild-type Ub (Fig. 3*C*, *top panel*, *lane 4*) or Ub mutants with an intact K63 (Fig. 3*C*, *top panel*, *lanes 5* and *8*). In the mean time, dramatic reduction of poly-Ub chain formation was seen in reactions using mutant Ubs carrying the *K63R* mutation (Fig. 3*C*, *top panel*, *lanes 6* and *7*). In contrast, when UbcH5c was used as the E2 in the same in vitro ligase reactions, both wild-type and K63R Ubs (Fig. 3*C*, *bottom panel*, *lanes 4* and *6*) were equally efficient at being incorporated into poly-Ub chains by TRAC-1. Consistent with the indiscriminate nature of UbcH5c, it can catalyze either K48-linked poly-Ub chain formation using K48-only Ub (Fig. 3*C*, *bottom panel*, *lane 7*) or non K48-linked poly-Ub chain formation using K48R (Fig. 3*C*, *bottom panel*, *lane 5*). Yet the K63-only mutant (Fig. 3*C*, *bottom panel*, *lane 8*) was completely unable to support poly-Ub chain formation in concert with UbcH5c and TRAC-1, showing striking selectivity for Ubc13/Uev1A as E2s to catalyze K63-mediated poly-Ub linkage. Collectively these observations confirmed that TRAC-1 is capable of catalyzing both K48- and K63-linked poly-Ub formation in vitro in concert with distinct E2s. It implies that TRAC-1 can regulate target protein function through the unique K63-linked poly-Ub chain or that it can mediate its target protein's proteolytic destruction, or both. Future studies aimed at identifying TRAC-1's target protein(s) in vivo should shed light on this issue.

TRAC-1 E3 Ub ligase activity is dependent on its N-terminal RING-HC motif

We confirmed that TRAC-1 functions as an E3 in vitro, presumably through its RING finger domain at its amino terminus. The RING finger domain is a protein module involved in promoting protein-protein or protein-DNA interactions and it is defined by eight Cys/His residues distributed linearly to form either $Cys_3HisCys_4$ (RING-HC) or $Cys_3His_2Cys_3$ (RING-H2) patterns (15). These eight Cys/His residues play important structural roles by chelating two zinc atoms.

To assess the contribution of TRAC-1's RING finger and C-terminal domains to its activity, N- and C-terminal truncation mutants of TRAC-1 tagged at their C terminus with FLAG were generated. They were transiently overexpressed in Phoenix A cells and purified from cellular lysates by immunoprecipitation with anti-FLAG Ab-coated beads. The immunoprecipitates were washed and subjected to the in vitro E3 assay using purified E1, E2, and His-tagged Ub in the presence of ATP. Conjugation of multiple His-tagged Ub onto proteins can be detected in Western blots as a ladder/smear with anti-Ub Ab. As shown in Fig. 3*D*, TRAC-1 wild-type (WT) transfected cells (Fig. 3*D*, *top panel*, *lane 4*) exhibited strong E3 Ub ligase activity when compared with vector-transfected cells (Fig. 3*D*, *top panel*, *lane 3*). Consistent with the RING finger domain being required for TRAC-1 to function as an E3 Ub ligase, a TRAC-1 mutant (TRAC-1Δ76) lacking the amino-terminal 76 aa, including the RING finger domain, failed to show robust E3 activity (Fig. 3*D*, *top panel*, *lane 6*). Interestingly, TRAC-1ΔC also showed positive E3 ligase activity in this assay (Fig. 3*D*, *top panel*, *lane 5*), albeit with reduced efficiency. Collectively these results indicate that TRAC-1 has RING finger-dependent E3 Ub ligase activity and that its C-terminal amino acids are not required for this activity in vitro.

Next, we concentrated on the RING domain of TRAC-1. Previous studies indicated that the Cys/His residues play important structural and functional roles in RING finger domain activity (48). Based on the alignment of the TRAC-1 RING domain with that of the other RING finger-containing proteins, we mutated the Cys/His residues into alanine residues, making multiple double-point mutants of TRAC-1. These mutant constructs when analyzed in the in vitro E3 Ub ligase assay showed that point mutations at these key positions in TRAC-1 resulted in dramatically reduced ligase activity (Fig. 3*E*, *top panel*, *lanes 5–7*) while a ctl mutant V32T33A had no effect (Fig. 3*E*, *top panel*, *lane 8*). Thus, these results show that an intact TRAC-1 RING domain is required for its E3 Ub ligase activity.

The dominant inhibitory effect of TRAC-1ΔC on TCR stimulation requires an intact RING domain

We next tested the various TRAC-1 truncation and point mutation mutants in the anti-TCR-induced CD69 up-regulation assay in Jurkat T cells. We subcloned the constructs with a FLAG tag in the pEFBOS-iresGFP vector, transiently transfected into Jurkat cells, and checked for their effect on CD69 up-regulation. The mutations did not cause appreciable protein instability since similar levels of FLAG-tagged TRAC-1 variants were expressed as monitored by Western blots using anti-FLAG Abs (data not shown). The results are summarized in Fig. 4. As shown in the previous sections, overexpression of full-length wild-type TRAC-1 (Fig. 4, construct 1) had no effect and overexpression of TRAC-1ΔC (Fig. 4, construct 2) inhibited CD69 induction in Jurkat cells. Interestingly, overexpression of either TRAC-1Δ76 (Fig. 4, construct 3) lacking the RING finger domain or the various Cys/His point mutants (Fig. 4,

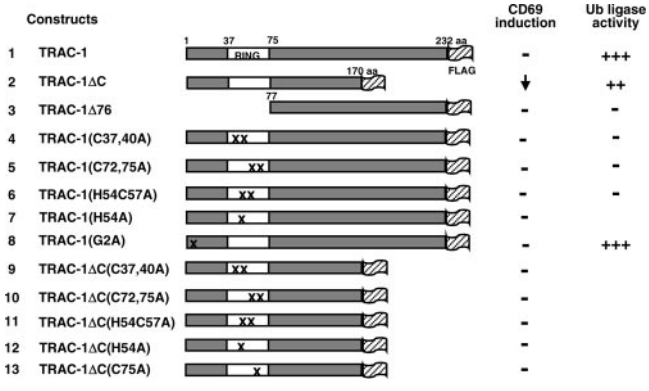

FIGURE 4. Summary of functional effects of different TRAC-1 constructs. The various constructs, shown schematically and numbered to the *left*, were cloned into pEFBOS-iresGFP vector and assayed for effects on induction of CD69 expression as in Fig. 1*C* and for Ub ligase activity as in Fig. 3*B*. The constructs are depicted as bars; the RING domain is labeled in construct 1; mutations are shown as X; and the FLAG tag is shown in dashed boxes.

constructs 4–8) did not affect CD69 up-regulation in Jurkat cells. Moreover, an intact RING finger domain with E3 activity was essential for TRAC-1ΔC's negative effect since point mutations in the RING finger's Cys/His residues abolished TRAC-1ΔC's inhibitory effect on CD69 induction (Fig. 4, constructs 9–13). Consequently, these results indicate that an intact RING finger domain is required for TRAC-1 to form meaningful interactions in a functional ubiquitylation complex.

TRAC-1 functions upstream in TCR-mediated cell signaling

To define the point of TRAC-1 action, Jurkat cells were transiently transfected with either TRAC-1ΔC or empty vector and then stimulated with anti-TCR or the phorbol ester PMA. TRAC-1ΔC specifically blocked TCR-mediated CD69 up-regulation, but not that induced by PMA (Fig. 5*A*). Since PMA treatment bypasses receptor proximal early signaling events to activate protein kinase C (PKC), the failure of TRAC-1ΔC to exert an inhibitory effect on PMA induction places TRAC-1's point of action upstream of this event.

Calcium mobilization is one of the earliest events essential for TCR-mediated gene activation. When overexpressed in Jurkat TAg cells, TRAC-1ΔC weakly inhibited anti-TCR but not ionomycin-triggered calcium mobilization (Fig. 5*B*). In contrast, TRAC-1WT demonstrated little effect on calcium influx induced by either stimulus. This is consistent with the PMA experiments showing TRAC-1 as an upstream regulator of TCR signaling, possibly acting on pathways upstream of both PKC and calcium mobilization.

TRAC-1 is a positive regulator of TCR signaling

To provide further evidence for the role TRAC-1 plays in TCR signaling, AS oligonucleotides were designed to specifically down-regulate endogenous TRAC-1 mRNA expression. Jurkat cells were transiently transfected with TRAC-1 AS 13656(AS) or inverse sequence ctl 13657 oligonucleotides. A FITC-labeled ctl oligonucleotide was spiked in the oligonucleotide samples to enable distinguishing between untransfected and transfected cell populations. After 16–24 h, a portion of the transfected cells was treated with anti-TCR Ab and the remaining cells were sorted for FITC-positive cells and harvested for total RNA extraction to measure TRAC-1 message expression by TaqMan analysis. Twenty-four hours after stimulation, cells were stained with anti-CD69-allophycocyanin for FACS analysis. As shown in Fig. 6*A*, introduction of TRAC-1 AS oligonucleotide 13656(AS) specifically inhibited anti-TCR-induced CD69 up-regulation in Jurkat cells as compared with the untransfected FITC-negative cells cultured in the same tissue culture well. This reduction of CD69 up-regulation was not observed with the inverse sequence 13657(ctl) or the FITC-labeled ctl oligonucleotide (Fig. 6*A*). As projected, the AS oligonucleotide 13656(AS) was confirmed to reduce the expression of endogenous TRAC-1 message to <20% of the ctl level (Fig. 6*B*). Taken together, these data strongly suggest that endogenous TRAC-1 functions as a positive regulator in the TCR signaling pathway.

We next evaluated the effect of TRAC-1 on primary T cell activation using TRAC-1 AS oligonucleotides. The TRAC-1 AS and ctl oligonucleotides were directly labeled with FITC for more efficient monitoring of transfection efficiency. Oligonucleotide 16613(AS) and 16615(ctl) are FITC-labeled oligonucleotides whose sequence is identical to oligonucleotides 13656(AS) and 13657(ctl), respectively. We then transfected monocyte-depleted resting primary blood mononuclear cells with FITC-labeled oligonucleotides. Transfected cells were left untreated or stimulated with either plate-bound anti-CD3(OKT3) and anti-CD28 for 24 and 48 h. Cell culture medium was analyzed for IL-2 concentration while cells were stained with anti-CD69-allophycocyanin for FACS analysis. As shown in Fig. 6*C*, in primary T cells, TRAC-1 AS oligonucleotide 16613(AS) but not its inverse ctl 16615(ctl) inhibited both CD69 up-regulation (Fig. 6*C*) and IL-2 secretion when stimulated by OKT3/anti-CD28 (Fig. 6*E*), but not by PMA/ionomycin (data not shown). TaqMan analysis revealed that TRAC-1 AS oligonucleotides efficiently down-regulated endogenous TRAC-1 message in primary T cells (Fig. 6*D*). Thus, collectively these data confirm that TRAC-1 is a necessary positive regulator required for optimal TCR signaling.

Discussion

In this report we describe the functional characterization of a novel T cell-specific E3 Ub ligase, TRAC-1, as a positive regulator of T cell activation. We have shown that a C-terminal truncated form of TRAC-1 (TRAC-1ΔC) behaves as a dominant inhibitor, blocking up-regulation of cell surface expression of the activation marker CD69 in Jurkat cells (Fig. 1). We also demonstrated that TRAC-1 possesses E3 activity in a substrate-independent Ub ligase assay in

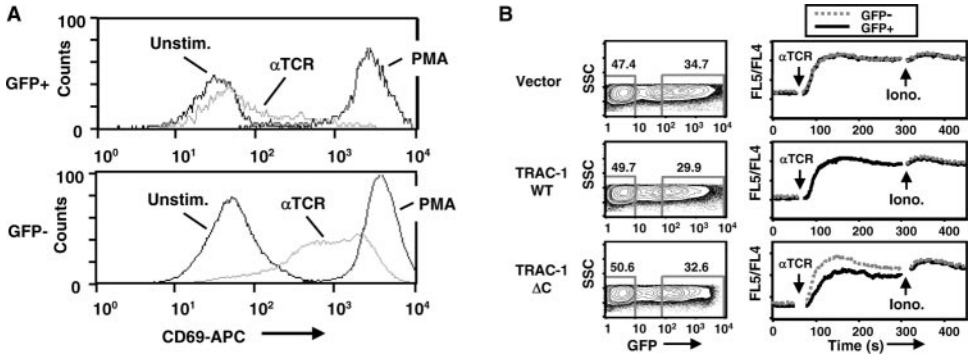

FIGURE 5. TRAC-1 is a specific modulator of TCR-mediated cell activation. *A,* The pTRA-TRAC-1ΔC-iresGFP vector was stably introduced into tTA-Jurkat 32H cells. After 48 h, cells were treated with anti-TCR or PMA for 20–24 h. The GFP-positive (*top panel*) and -negative (*bottom panel*) cells were compared for surface expression of CD69 by analytical gating. The histogram plots show CD69-allophycocyanin fluorescence intensity on the x-axis. *B,* The pEFBOS-TRAC-1-iresGFP and pEFBOS-TRAC-1ΔC-iresGFP vectors were introduced into Jurkat TAg cells. After 48 h, cells were loaded with indomethacin 1 (Indo-1), then stimulated with anti-TCR (1 μg/ml C305) during FACS analysis as described by Holland et al. (40). Intracellular calcium concentration was reflected by the ratio of geometric mean fluorescence intensity of channels FL5 (Ca^{2+}-bound Indo-1) vs FL4 (unbound Indo-1). The FL5:FL4 channel ratios were plotted vs time and subdivided by FL1 intensity to separate GFP-negative from GFP-positive cells. Ionomycin (1 μM) was added after 300 s to determine maximal responses and to control for Indo-1 uptake. SSC, Side scatter; Iono, ionomycin.

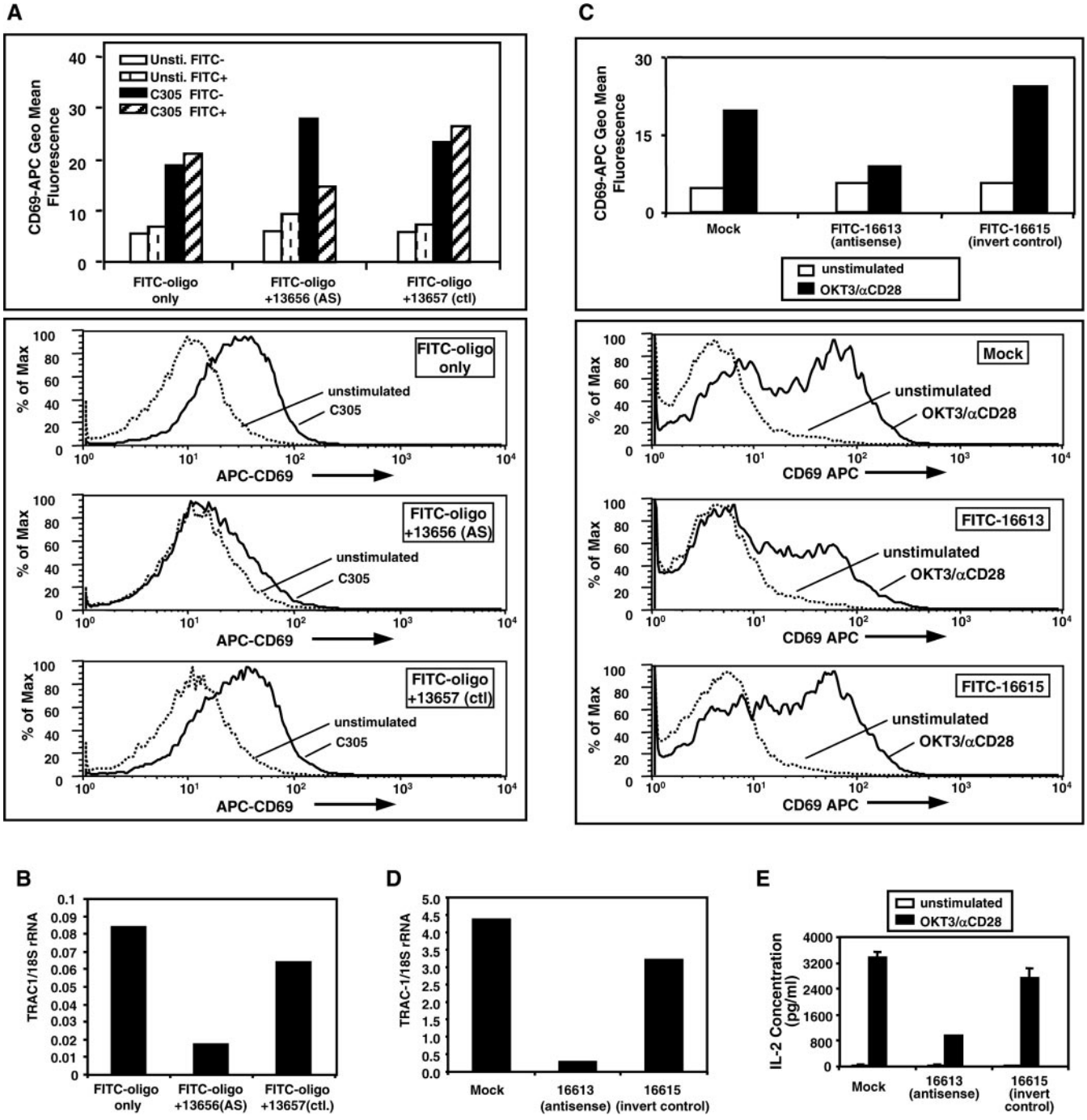

FIGURE 6. TRAC-1 is a positive regulator of TCR signaling. *A* and *B,* AS TRAC-1 oligonucleotides reduce TRAC-1 mRNA levels and CD69 expression up-regulation in Jurkat T cells. Jurkat TAg cells were transiently transfected with TRAC-1 AS 13656(AS) or ctl 13657(ctl) oligonucleotides at 50 μM plus 10 μM FITC-labeled ctl oligonucleotides by electroporation. After 16–24 h, a portion of the transfected cells was treated with anti-TCR Ab or PMA; the remaining cells were harvested for total RNA extraction to measure TRAC-1 message expression by TaqMan analysis (*B*). Twenty to 24 h after stimulation, cells were stained with anti-CD69-allophycocyanin for FACS analysis. The CD69-allophycocyanin geometric (Geo) mean fluorescence of analytically gated GFP-negative and GFP-positive cells are presented in the bar chart (*A*, *top panel*). Alternatively, the same data are presented by gating on GFP-positive cells and by plotting the CD69-allophycocyanin histogram overlay of unstimulated (dotted lines) and C305-stimulated cells (black lines) in the *bottom panel* (*A*). *C—E,* AS TRAC-1 oligonucleotides reduce TRAC-1 mRNA levels, CD69 expression up-regulation, and IL-2 production in primary blood mononuclear cells. Monocyte-depleted, resting human primary blood mononuclear cells were transfected with FITC-labeled TRAC-1 AS 16613 or invert ctl 16615 oligonucleotides at 50 μM using Amaxa Biosystems Nucleofector technology. Cells were cultured at 37°C for 20 h before being sorted into pure FITC-positive populations. Mock-transfected cells were sorted into pure FITC-negative populations under the same setting. Sorted cells were used either for total RNA extraction and TaqMan analysis (*D*) or for stimulation with medium, plate-bound anti-TCR and anti-CD28, or PMA and ionomycin for 24 and 48 h. Cell culture medium was then used for IL-2 ELISA (*E*) while cells were stained with anti-CD69-allophycocyanin for FACS analysis. The CD69-allophycocyanin geometric mean fluorescence of analytically gated GFP-negative and GFP-positive cells stimulated for 24 h are presented in the bar chart (*C*, *top panel*). Alternatively, the same data are presented by the CD69-allophycocyanin histogram overlay of unstimulated (dotted lines) and OKT3/anti-CD28-stimulated cells (black lines) in the *bottom panel* (*C*).

vitro (Fig. 3). In addition, we presented evidence showing that TRAC-1 can cooperate with Ubc4, HbcH5c, and Ubc13/Uev1A to facilitate poly-Ub chain formation, and that TRAC-1 can be involved in both K48- and K63-linked polyubiquitylation (Fig. 3). TRAC-1 mRNA was detected primarily in lymphoid tissues and particularly in the $CD4^+$ and $CD8^+$ T cell populations, suggesting a specific role for TRAC-1 in conjugation of Ub or Ub-like molecules to signaling transduction proteins in T cells (Fig. 2). Taking advantage of the availability of potent TRAC-1 AS oligonucleotides, we provided further evidence that TRAC-1 acts as a positive regulator of T cell activation in both the Jurkat T leukemic cell line and human primary T lymphocytes (Fig. 6). Taken together, the evidence presented here identifies TRAC-1 as a novel E3 Ub ligase and as a tissue-specific positive mediator of T cell activation.

The dominant inhibitory activity of TRAC-1ΔC underscores the importance of its C-terminal domain in TCR signal transduction. It is likely that TRAC-1ΔC via its RING domain is able to interact and form protein complexes, but because it lacks a complete C terminus such complexes fail to signal properly. The truncated C-terminal fragment may be required for Ub ligase activity and/or recognition and recruitment of substrates for ubiquitylation. Interestingly, TRAC-1ΔC did possess Ub ligase activity in vitro but this activity was reduced relative to wild-type TRAC-1 (Fig. 3*D*). However, the lack or reduction of ligase activity does not correlate with dominant negative function since mutations that abolish ligase activity are not dominant negative (Fig. 4). As for substrate recruitment, the C-terminal domain itself may contain substrate recognition elements in its sequence, or alternatively, it could be part of a multisubunit E3 substrate recognition complex. Identification of TRAC-1 substrates will shed light on the role of its C-terminal domain.

Importantly, the dominant inhibitory activity of TRAC-1ΔC is completely dependent on having an intact RING finger domain. We showed that point mutations of the canonical Cys and/or His residues in the RING finger domain of TRAC-1ΔC blocked the inhibitory effect on CD69 expression induced by TCR cross-linking (Fig. 4). These mutations likely cause dramatic changes in protein folding, given the critical roles of the two zinc atoms being chelated by the RING finger (14, 15). Notably, these mutations did not cause unexpected protein instability since they were expressed at similar levels as monitored by Western blots using Abs to their FLAG tags. As expected, the corresponding mutations in the context of full-length TRAC-1 also abolished or significantly reduced E3 Ub ligase activity, showing that an intact RING domain is necessary for complex formation and ubiquitylation activity (Fig. 3*E*). Taken together, the mutations on the RING finger seem to render TRAC-1ΔC unable to impede signal transduction because of failure to form defective complexes.

Interestingly, both RING-less (TRAC-1Δ76) and full-length RING point mutants failed to function as dominant inhibitors of TCR signaling (Fig. 4). That is, the RING-deficient TRAC-1 mutants by lacking or having reduced ability to interact with its endogenous protein ubiquitylation complex fail to titrate out signaling components and to dominantly inhibit signaling. It is possible that an intact RING finger may be needed to stabilize TRAC-1/effector interactions, where the effector molecule would require contacting TRAC-1 directly or indirectly at points both in the RING and the C-terminal domain. This is very plausible since ubiquitylation complexes tend to require numerous subunits involving multiple protein-protein interactions (7, 15). Also of note, overexpression of wild-type TRAC-1 in Jurkat T cells did not significantly potentiate TCR induction of CD69 expression. This suggests that TRAC-1 is not acting as a limiting component to determine the potency of TCR signaling, rather it appears TRAC-1 is playing a regulatory role. It will be interesting to assess the effect of TRAC-1 overexpression under different stimulation conditions. Relevant to the above subject, and currently being addressed, is how TRAC-1 activity is controlled and how such activity regulates T cell responses.

Finally, we showed that the phorbol ester PMA bypassed the inhibitory effects of TRAC-1ΔC on CD69 up-regulation (Fig. 5*A*). Since PMA activates the PKC enzymes including PKC-θ (49, 50), TRAC-1 action is then likely between TCR triggering and PKC activation. Similarly, TRAC-1 action appears upstream of calcium mobilization since overexpression of TRAC-1ΔC reduces calcium mobilization induced by TCR cross-linking but not by ionomycin, a calcium ionophore (Fig. 5*B*). These demarcations and the fact that TRAC-1ΔC did not inhibit CD69 up-regulation upon BCR cross-linking in the B cell line BJAB (Fig. 2*C*) limit the number of possible physiological targets for TRAC-1 in T cells.

A likely hypothesis is that by promoting the ubiquitylation and subsequent degradation of a negative regulator for T cell activation, TRAC-1 plays a necessary positive role in TCR signal transduction. Alternatively, conjugation of Ub or Ub-like molecules mediated by TRAC-1 on key signaling molecules may be used to confer additional regulatory controls to sustain and modulate T cell responses. The finding that TRAC-1 can cooperate with both Ubc4/UbcH5c and Ubc13/Uev1A types of E2s in vitro keeps the door open for both possibilities (Fig. 3, *A–C*). The identification of the cellular target(s) of TRAC-1 in the TCR signaling pathway will be needed to completely elucidate the role of TRAC-1 in T cell activation. In summary, the results indicate that TRAC-1 is the first E3 ligase identified as a positive regulator of TCR signaling and may represent a novel class of targets for therapeutic intervention to treat diseases involving aberrantly activated T cells (51, 52).

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Disclosures

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