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Tomas Cinek; ... et. al

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Cutting Edge: Tyrosine-Independent Transmission of Inhibitory Signals by CTLA-4[1]

Tomas Cinek, Ali Sadra, and John B. Imboden[2]

CTLA-4 is an important inhibitor of T cell activation. We used Jurkat cells expressing mutants of murine CTLA-4 to study the structural requirements for inhibitory signaling. We find that signals for the inhibition of IL-2 secretion are delivered efficiently by a CTLA-4 mutant in which both cytoplasmic tyrosines have been replaced by phenylalanines. A CTLA-4 mutant that lacks the carboxyl-terminal half of the intracellular domain also retains the ability to inhibit, but deletion of an additional 11 aa completely abrogates that capability. We conclude that delivery of an inhibitory signal requires the membrane-proximal region of the CTLA-4 cytoplasmic domain and does not depend upon the tyrosine phosphorylation of CTLA-4. ***The Journal of Immunology,*** **2000, 164: 5–8.**

Engagement of CTLA-4 (CD152) on the surface of activated T lymphocytes inhibits expression of activation Ags, cytokine production, and proliferation. In vivo, a component of CTLA-4-mediated inhibition can be attributed to successful competition with the costimulatory receptor CD28 for binding to their shared natural ligands, B7-1 (CD80) and B7-2 (CD86). However, studies of cross-linking CTLA-4 by mAbs demonstrate that CTLA-4 also transduces inhibitory signals to T cells (1, 2).

The intracellular domain of CTLA-4 is highly conserved between species and has two Tyr residues. Tyr^{201} (murine sequence) is part of a motif (Tyr-Val-Lys-Met) that controls localization and trafficking of CTLA-4 (3). In the unphosphorylated state, this motif binds to AP-1 and AP-2 clathrin adaptor complexes that target CTLA-4 to intracellular compartments. Phosphorylation of Tyr^{201} releases the adaptor complexes and increases cell surface expression of CTLA-4 (4–8).

The proximal events involved in inhibitory signaling by CTLA-4 have yet to be fully elucidated. When phosphorylated, the Tyr-Val- Lys-Met motif binds the p85 subunit of phosphatidylinositol 3′-kinase (PI3K) (9), but the functional significance of this association is uncertain. CTLA-4 also can associate with the tyrosine phosphatase SHP-2 (SH2 domain-bearing protein tyrosine phosphatase).[3] Several studies indicate that phosphorylation of Tyr^{201} recruits SHP-2 to CTLA-4 (10–12), but one recent report observed tyrosine-independent association of CTLA-4 and SHP-2 (13). CTLA-4-associated SHP-2 has been reported to dephosphorylate the ζ-chain of the TCR complex (13) or to inhibit the TCR signaling pathway by dephosphorylating several of its other key components: Fyn, Lck, ZAP-70, and the Ras regulator $p52^{SHC}$ (10). However, not all studies have found an inhibition of early TCR signaling events. Revilla Calvo et al. (14) did not observe an effect of CTLA-4 ligation on the phosphorylation of TCR ζ and ZAP-70 but did find inhibition of more downstream events: the activation of extracellular signal regulated kinase and Jun N-terminal kinase.

To explore the nature of CTLA-4 signaling and to define its structural requirements, we studied the effects of cytoplasmic domain mutations on the ability of CTLA-4 to inhibit IL-2 production by Jurkat T cells stimulated through CD3 and CD28. Our results demonstrate that tyrosine phosphorylation of CTLA-4 is not necessary for inhibitory signaling in this system and establish an important role for the membrane-proximal region of the cytoplasmic domain.

Materials and Methods

Abs and reagents

cDNA encoding wild-type (WT) murine CTLA-4 (mCTLA-4) was provided by Dr. Peter Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). UC10-4F10-11 (hamster anti-mCTLA-4 mAb) hybridoma was a gift of Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL). Purified mAb was obtained from ascitic fluid using protein A agarose (Life Technologies, Gaithersburg, MD). Control hamster mAb G235-2356 (anti-trinitrophenol), and murine mAbs UCHT1 and CD28.2 specific for human CD3ϵ and CD28, respectively, were purchased from PharMingen (San Diego, CA).

Generation and expression of mCTLA-4 mutants

All mutant cDNAs were generated from WT mCTLA-4 by established PCR methods as described previously (15) and confirmed by double-stranded sequencing. The mutants were subcloned into the expression vector pBSRαEN and stably transfected into Jurkat E6.1 cell line (Ref. 15; Fig. 1).

Department of Medicine, Rosalind Russell Research Laboratory, San Francisco General Hospital, and University of California, San Francisco, CA 94143

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[2] Address correspondence and reprint requests to Dr. John Imboden, Box 0868, University of California, San Francisco, CA 94143. E-mail address: imboden@itsa.ucsf.edu

[3] Abbreviations used in this paper: SHP-2, SH2 domain-bearing protein tyrosine phosphatase; mCTLA-4, murine CTLA-4; WT, wild type.

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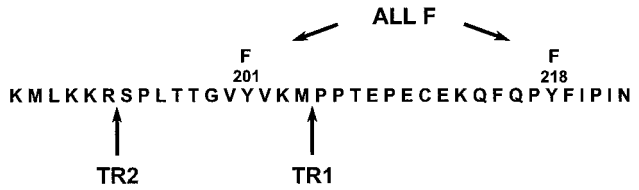

FIGURE 1. A scheme of truncation and substitution mutants of mCTLA-4. Shown is the amino acid sequence of murine CTLA-4 cytoplasmic domain depicted in one-letter code. Arrows below the sequence mark the sites of truncation in TR1 and TR2 mutants, whereas the two Tyr → Phe substitutions in the ALL F mutant are indicated above the sequence.

Flow cytometry

Transfected Jurkat cells were stained for 1 h with FITC-labeled UC10-4F10-11 mAb on ice or at 37°C and were analyzed on a FACSort instrument (Becton Dickinson, Mountain View, CA) using CellQuest software.

Preparation of mAb-coated microspheres

Aldehyde/sulfate latex microspheres (5 μm; Interfacial Dynamics, Portland, OR) were coated with mAbs in a following manner: 2×10^7 beads/ml were incubated in PBS containing 4 μg/ml of anti-human CD3 mAb and 20 μg/ml of anti-mouse CTLA-4 mAb at 37°C for 1.5 h. The unoccupied binding sites were blocked by 2% BSA in PBS for 30 min at room temperature, followed by two washes with complete RPMI medium (RPMI 1640 with glutamine supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 55 μM 2-ME, all from Life Technologies). The beads were then resuspended at the density of 2×10^7 beads/ml in the same medium, and the exact number was verified by counting with Coulter Counter ZBI (Coulter Electronics, Hialeah, FL). The control beads were coated with anti-human CD3 mAb and control hamster IgG in the same way except the ratio of coating mAbs was adjusted to compensate for differences in binding capacities between hamster IgG and anti-mouse CTLA-4 mAb. This was done to obtain approximately equal stimulation of untransfected cells by the same number of anti-CD3/mCTLA-4 and anti-CD3/control beads. Usually several batches of slightly differing control beads were prepared and the best matching batch was used for experiments.

Jurkat cell culture and IL-2 assay

All cells were maintained at 37°C in a 5% CO_2 incubator in complete RPMI medium, supplemented for passage of transfected clones with 2 mg/ml G418 (Life Technologies). For IL-2 assays, cells were cultured in 96-well U-bottom plates at 2×10^5 cells per well in complete RPMI without G418, with addition of soluble anti-human CD28 mAb to a final concentration of 1 μg/ml. Latex microspheres coated with anti-human CD3 mAb and either anti-mouse CTLA-4 mAb or control hamster mAb were added in indicated amount (from 5 to 40 μl of bead suspension, representing 1×10^5 to 8×10^5 beads) to each well. The volume in all wells was brought to 200 μl with complete RPMI. After 16 h, supernatants were harvested and assayed for IL-2 using a human IL-2 ELISA kit from Immunotech (Westbrook, ME). Optical density of the samples was determined on a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA), and data analysis was performed with accompanying software. Because Jurkat subclones differ in the magnitude of IL-2 produced (15), the data are presented as the percent of the IL-2 response to anti-CD3/control beads when two or more clones are compared.

Results and Discussion

Following its induction on activated T cells, the cell surface expression of CTLA-4 is limited and tightly regulated, with the majority of CTLA-4 localized to intracellular stores. Similarly, after stable transfection with a cDNA encoding WT mCTLA-4, Jurkat T cells expressed only low levels of mCTLA-4 on the cell surface, as detected by staining on ice with an anti-mCTLA-4 mAb (Fig. 2*A*). However, staining at 37°C revealed considerably higher levels of cellular mCTLA-4 (Fig. 2*B*). The enhanced staining at 37°C is consistent with the behavior of CTLA-4 in activated T cells and likely reflects trafficking of mCTLA-4 between the cell surface and intracellular stores, with the consequent intracellular accumulation of the staining mAb (16).

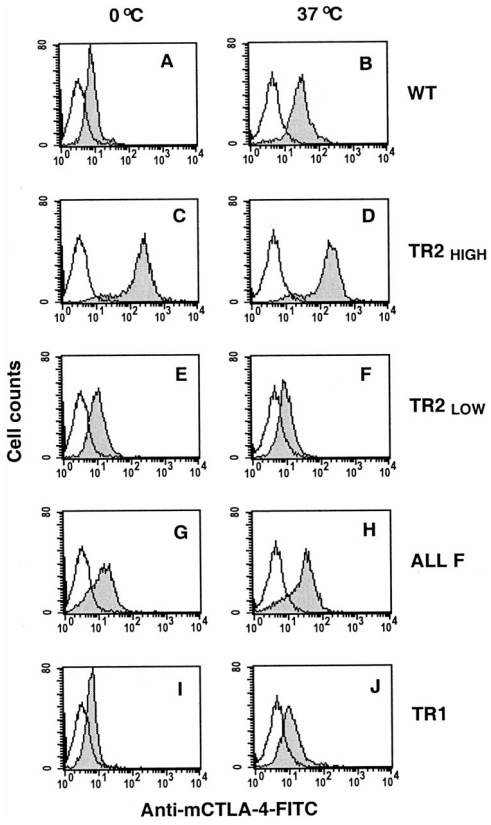

FIGURE 2. Expression of mCTLA-4 mutants in Jurkat cells. Jurkat cells were stably transfected with expression plasmids encoding WT or indicated mutants (Fig. 1) of mCTLA-4. Clones were established and stained either on ice (*left panels*) or at 37°C (*right panels*) for 1 h with FITC-labeled UC10-4F10-11 mAb. After washing, cells were analyzed by flow cytometry (shaded peaks). Unshaded histograms represent untransfected cells stained in an equal manner.

Jurkat cells produce readily detectable levels of IL-2 in response to the combination of anti-CD3 and anti-CD28 mAbs. To determine whether mCTLA-4 can deliver an inhibitory signal to Jurkat, we stimulated Jurkat cells with varying amounts of beads coated either with anti-CD3 mAb plus anti-CTLA-4 mAb (anti-CD3/CTLA-4 beads) or with anti-CD3 mAb plus a control hamster mAb (anti-CD3/control beads). Soluble anti-CD28 mAb was added to all cultures to provide costimulation.

As expected, untransfected Jurkat cells produced comparable levels of IL-2 in response to the two types of beads (Fig. 3*A*). In marked contrast, WT mCTLA-4-expressing Jurkat cells secreted substantially lower amounts of IL-2 when stimulated with anti-CD3/CTLA-4 beads than with anti-CD3/control beads (Fig. 3*B*). The differences were reproducible and significant (see below, Fig. 5). The inhibitory effect of anti-mCTLA-4 was most pronounced at the smallest doses of beads, suggesting that greater engagement of CD3 molecules overcomes the inhibitory signaling of mCTLA-4. Consistent with the findings of Fraser at al. (17), inhibition required immobilization of the anti-CD3 and anti-CTLA-4 mAbs on the same bead. When we performed these experiments using a mixture of beads coated separately with anti-CD3 and anti-mCTLA-4 mAbs, we observed either no effect of anti-mCTLA-4 or a slight increase in IL-2 production (data not shown).

To determine whether the inhibitory effect on IL-2 production requires the mCTLA-4 cytoplasmic domain, we created a truncation mutant (TR2) that deletes all but 6 aa of the intracellular

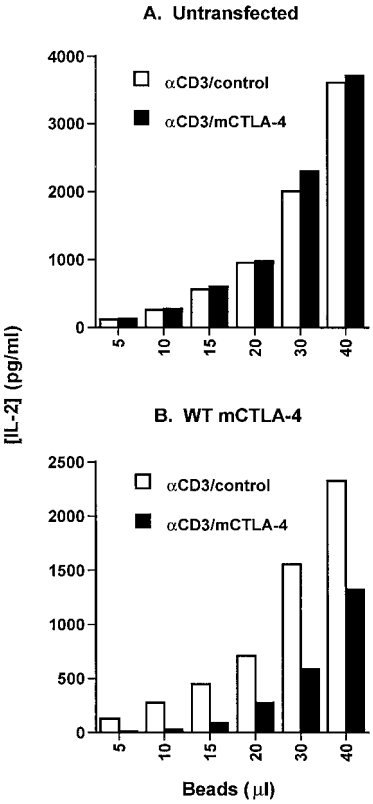

FIGURE 3. WT mCTLA-4-mediated inhibition of IL-2 production in Jurkat cells. Untransfected (*A*) or WT mCTLA-4-transfected (*B*) Jurkat cells were cultured either with anti-CD3/mCTLA-4 beads (■) or anti-CD3/control beads (□) as described in *Materials and Methods*. To provide costimulation, soluble anti-human CD28 mAb at a final concentration of 1 μg/ml was added to all cultures. After 16 h, supernatants were harvested and assayed for IL-2. Bars show the IL-2 levels produced in response to the indicated doses of beads. These data are representative of seven independent experiments.

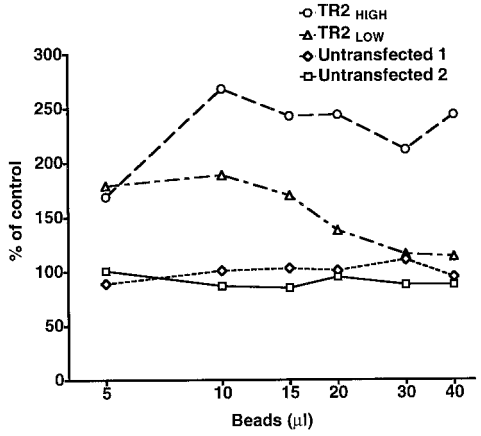

FIGURE 4. Deletion of all but 6 aa from cytoplasmic tail of mCTLA-4 abrogates delivery of the inhibitory signal. The Jurkat cells, either transfected with the TR2 mutant of mCTLA-4, or untransfected (as indicated), were stimulated, and the IL-2 assays were performed as described in *Materials and Methods*. The results are presented as the percent of the IL-2 level achieved with anti-CD3/CTLA-4 beads relative to IL-2 induced by anti-CD3/control beads for each dose of beads. $TR2_{HIGH}$ and $TR2_{LOW}$ designate clones with high (Fig. 2, *C* and *D*) and low (Fig. 2, *E* and *F*), respectively, expression of TR2 mCTLA-4. Two different batches of untransfected cells used are marked as Untransfected 1 and 2. Shown is a typical experiment of four independent experiments performed.

region (Fig. 1). This truncation removes the motif responsible for the intracellular localization and trafficking of CTLA-4 (3). As expected with deletion of that motif, the staining of TR2 was bright and was comparable on ice and at 37°C (Fig. 2, *C* and *D*), indicating that the great majority of TR2 was expressed on the cell surface. In marked contrast to WT mCTLA-4-expressing Jurkat cells, TR2-expressing cells produced more IL-2 in response to anti-CD3/CTLA-4 beads than anti-CD3/control beads (Fig. 4). The levels of cell surface TR2 were considerably higher than the levels of WT mCTLA-4 expressed by Jurkat cells (Fig. 2, *A* and *C*). To be certain that TR2 does not transmit inhibitory signal when expressed on the cell surface at levels comparable to those of WT mCTLA-4, we isolated a clone with low expression of TR2 (Fig. 2, *E* and *F*). The enhanced stimulatory effect of the anti-CD3/CTLA4 beads was less pronounced with this clone but was still apparent (Fig. 4, $TR2_{LOW}$). Thus, either TR2 transmits a positive signal, or, more likely, its expression increases the avidity of the interaction between Jurkat cells and the beads, thereby facilitating engagement of CD3. In either event, however, the ability of mCTLA-4 to inhibit IL-2 production clearly depends upon sequences that are deleted by the TR2 truncation.

To determine whether CTLA-4 signaling requires its two cytoplasmic Tyr residues, we created a mutant (ALL F mCTLA-4) in which both Tyr^{201} and Tyr^{218} are replaced with Phe (Fig. 1). Mutation of Tyr^{201} also affects the motif involved in the intracellular localization and trafficking of mCTLA-4, and the ALL F mutant was expressed at higher levels on the cell surface than WT mCTLA-4 (Fig. 2, *G* and *H*). Mutation of the two cytoplasmic Tyr had little effect on the ability of mCTLA-4 to transmit an inhibitory signal (Fig. 5). At low doses of beads, the inhibitory capacity of ALL F was comparable to that of WT mCTLA-4 ($p > 0.1$ for 5 and 10 μl of anti-CD3/CTLA-4 beads). At higher doses of the anti-CD3/CTLA-4 beads, ALL F continued to inhibit, but not as effectively as WT mCTLA-4.

Pro-X-X-Pro motifs can interact with Src homology 3 domains and constitute a Tyr-independent mechanism for the recruitment of

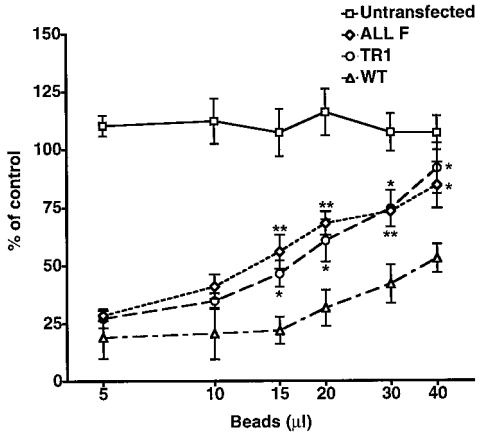

FIGURE 5. Substitution of Tyr^{201} and Tyr^{218} for Phe or truncation of the carboxyl-terminal half of the cytoplasmic domain does not eliminate the inhibitory capacity of mCTLA-4. The Jurkat cells transfected with the WT, ALL F, and TR1 mutants of mCTLA-4, or untransfected (as indicated), were stimulated, and the IL-2 assays were performed as described in *Materials and Methods*. For each dose of beads, the graph shows the percent of the IL-2 level achieved with anti-CD3/CTLA-4 beads compared with the IL-2 level achieved with anti-CD3/control beads. The data represent mean ± SE of four separate experiments. Points on ALL F and TR1 curves found to be significantly different from WT are marked with one ($p < 0.04$) or two ($p < 0.01$) asterisks.

signaling molecules. The cytoplasmic domain of mCTLA-4 contains one Pro-X-X-Pro sequence. To examine the possible significance of that sequence, we generated a Jurkat clone that expressed a truncation mutant of mCTLA-4 (TR1) that deleted the carboxyl-terminal half of the cytoplasmic domain, including the Pro-X-X-Pro sequence (Fig. 1). The cell-surface expression of TR1, which retains the motif responsible for intracellular localization and trafficking, was comparable to that of WT mCTLA-4 (Fig. 2, *I* and *J*). Deletion of the distal half of the intracellular region had no significant effect on the ability of mCTLA-4 to transmit an inhibitory signal when the lower doses (5 or 10 μl) of the anti-CD3/CTLA-4 beads were used (Fig. 5). As was observed with the ALL F mutant, TR1 was less effective than WT CTLA-4 with higher doses of the beads.

Our results demonstrate that engagement of mCTLA-4 by mAb down-regulates the production of IL-2 by Jurkat cells stimulated through CD3 and CD28. The inhibitory signal delivered by mCTLA-4 in this system requires neither cytoplasmic Tyr residues nor the carboxyl-terminal half of the cytoplasmic domain. The ALL F and TR1 mutants appear somewhat less efficient in inhibiting IL-2 than WT CTLA-4. These mutations may alter the overall structure of the molecule so that signaling is less efficient or may affect directly sites for coupling to signaling pathways. Regardless, however, the more striking finding is the extent to which inhibitory signaling is preserved despite mutation of all tyrosines or deletion of the carboxyl-terminal half of the cytoplasmic domain.

Our findings argue against the paradigm of tyrosine-dependent recruitment of signaling molecules to CTLA-4 but are in accord with the recent finding that tyrosine phosphorylation is not required for CTLA-4 to associate with SHP-2 and TCR ξ (13). Despite the presence of readily detectable levels of SHP-2 in Jurkat and despite considerable effort, we have not been able to demonstrate coimmunoprecipitation of SHP-2 with WT mCTLA-4 in these cells. This may simply reflect technical difficulties, but we cannot exclude the alternative possibility that, in Jurkat, mCTLA-4 signaling is independent of stable interactions with SHP-2.

The findings here present a sharp contrast with similar studies of CD28. Despite considerable sequence similarities, the two molecules appear to use distinct regions of their cytoplasmic domains for signaling. Tyr^{201}, Tyr^{218}, and the two Pro residues of the Pro-X-X-Pro sequence in CTLA-4 are conserved in CD28, but, unlike CTLA-4, each of these appears important for CD28 signaling (18–21). In contrast to the preservation of CTLA-4 function observed with TR1, the analogous truncation of CD28 eliminates costimulation (18). Comparison of our two mCTLA-4 truncation mutants suggests a critical role for the membrane-proximal region spanning residues 194–204. Deletion of this region, which occurs with truncation from TR1 to TR2, completely abrogates delivery of an inhibitory signal by mCTLA-4. Thus, these 11 aa either are directly involved in signal transduction or are required for the structural integrity of a more proximal signaling region.

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