A Potential Role for Tyrosine Phosphatases

Phosphorylation:319

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J Immunol 2002; 168:4420-4429; doi: 10.4049/jimmunol.168.9.4420
http://www.jimmunol.org/content/168/9/4420

CTL-A suppresses proximal TCR signaling in resting human CD4+ T cells by inhibiting ZAP-70 Tyr 319 phosphorylation: a potential role for tyrosine phosphatases.
CTLA-4 Suppresses Proximal TCR Signaling in Resting Human CD4+ T Cells by Inhibiting ZAP-70 Tyr319 Phosphorylation: A Potential Role for Tyrosine Phosphatases

Christine Guntermann and Denis R. Alexander

The balance between positive and negative signals plays a key role in determining T cell function. CTL-associated Ag-4 is a surface receptor that can inhibit T cell responses induced upon stimulation of the TCR and its CD28 coreceptor. Little is known regarding the signaling mechanisms elicited by CTLA-4. In this study we analyzed CTLA-4-mediated inhibition of TCR signaling in primary resting human CD4+ T cells displaying low, but detectable, CTLA-4 cell surface expression. CTLA-4 coligation with the TCR resulted in reduced downstream protein tyrosine phosphorylation of signaling effectors and a striking inhibition of extracellular signal-regulated kinase 1/2 activation. Analysis of proximal TCR signaling revealed that TCR ζ-chain phosphorylation and subsequent ζ-associated protein of 70 kDa (ZAP-70) tyrosine kinase recruitment were not significantly affected by CTLA-4 engagement. However, the association of p56lck with ZAP-70 was inhibited following CTLA-4 ligation, correlating with reduced actions of p56lck in the ZAP-70 immunocomplex.

Moreover, CTLA-4 ligation caused the selective inhibition of CD3-mediated phosphorylation of the positive regulatory ZAP-70 Y319 site. In addition, we demonstrate protein tyrosine phosphatase activity associated with the phosphorylated CTLA-4 cytoplasmic tail. The major phosphatase activity was attributed to Src homology protein 2 domain-containing tyrosine phosphatase 1, a protein tyrosine phosphatase that has been shown to be a negative regulator of multiple signaling pathways in hematopoietic cells. Collectively, our findings suggest that CTLA-4 can act early during the immune response to regulate the threshold of T cell activation.


O ptimal activation and differentiation of naive CD4+ T cells to cytokine-producing effector cells requires engagement of the TCR/CD3 complex and costimulatory molecules (1). Two well-characterized receptors belonging to the Ig superfamily are CD28 and CTLA-4. Whereas CD28 acts as a positive costimulator for T cell activation, resulting in augmented and sustained IL-2 secretion, proliferation, and induction of survival factors, such as Bcl-xL, (2), the function of CTLA-4 has been more controversial. With the exception of one report (3), recent evidence indicates that CTLA-4 plays an inhibitory role in regulating T cell responses. This idea was suggested by the observation that cross-linking of CTLA-4 in conjunction with anti-CD3/CD28 mAb inhibits extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase pathways (4), attenuates NF-κB and AP-1 activation (5), and inhibits cell cycle progression (6), IL-2 secretion, and T cell proliferation (7). In addition, CTLA-4 blockade in vivo increases T cell responses to antigenic challenges (8), enhances T cell-mediated tumor rejection (9), and exacerbates autoimmune disease (10). The most compelling evidence for a negative regulatory function for CTLA-4 has come from CTLA-4−/− mice that develop fatal lymphoproliferative disease, a phenotype that results from polyclonal activation of peripheral T cells that infiltrate and cause multiorgan destruction (11). Like CD28, CTLA-4 shares the same ligands, namely B7-1 (CD80) and B7-2 (CD86). However, CTLA-4 binds with an affinity 10- to 20-fold higher than that of CD28 (12). Because CTLA-4 is not readily detectable on resting T cells and is up-regulated after activation, there has been a prevailing idea that CTLA-4 terminates on-going T cell responses, possibly by opposing CD28-mediated costimulation by competing for the CD80/CD86 ligands and/or by actively blocking CD28-induced signals (13). However, other reports have emphasized a role for CTLA-4 during the first 24 h of activation, suggesting that CTLA-4 may be important for setting thresholds for T cell responses by down-regulating early manifestations of T cell activation, including CD69 and CD25 expression as well as IL-2 secretion (6).

The molecular basis for these outcomes and the signaling mechanisms induced by CTLA-4 remain poorly understood. CTLA-4 has a 36-aa cytoplasmic tail that is 100% conserved in mammalian species. It contains two tyrosines at positions Y201 and Y218, which are present in the YVKM and YFIP motifs, respectively. In addition, it possesses one proline-rich motif that could recruit SH3 domain-containing signaling molecules, suggesting that the CTLA-4 cytoplasmic tail may play a role in signaling and be important for its function.

Phosphatidylinositol 3-kinase (14) and the protein tyrosine phosphatase (PTPase) Src homology protein 2 domain-containing tyrosine phosphatase 2 (SHP-2) (15) become recruited to the phosphorylated pY201VKM motif in the CTLA-4 tail. In addition, interaction of CTLA-4 with the TCR ζ-chain (16) and with the serine/threonine phosphatase PP2A (17) have been described. However, despite the identification of associated kinases/PTPases...
with CTLA-4, the mechanism of negative signaling by CTLA-4 remains elusive. One model that explains the inhibitory function of CTLA-4 proposes that recruitment of SHP-2 to CTLA-4 mediates dephosphorylation of the CD3 \( \zeta \)-chains, thereby interfering with the phosphorylation of crucial effector molecules essential for TCR signaling (15, 16). However, this model has not been validated in normal T cells and has been challenged by the observation that SHP-2 acts as a positive regulator of the Ras/mitogen-activated protein kinase pathway of the TCR signaling cascade (18). Thus, the biochemical events that mediate CTLA-4 inhibitory function remain controversial.

Little information is available about CTLA-4 signaling in primary human T cells, and the present study was designed to address this question. We found that CTLA-4 engagement inhibited TCR-stimulated events, as evidenced by reduced protein tyrosine phosphorylation and inhibition of ERK-1/2 activation. Analysis of proximal TCR signaling revealed that both TCR \( \zeta \)-chain phosphorylation and \( \zeta \)-associated protein of 70 kDa (ZAP-70) recruitment remained intact upon CTLA-4 ligation, whereas there was a marked inhibition of the association of p56\(\text{Lck}\) tyrosine kinase with ZAP-70. Furthermore, CTLA-4 engagement resulted in marked inhibition of CD3-induced phosphorylation of tyrosine 319 in the interdomain B of ZAP-70. Because this phosphorylation site constitutes a binding site for the SH2 domain of p56\(\text{Lck}\) and because p56\(\text{Lck}/\text{ZAP-70}\) association is thought to be critical in promoting TCR coupling to intracellular signals (19), our findings provide a novel molecular explanation for attenuated TCR signaling and subsequent inhibition of IL-2 production resulting from CTLA-4 engagement. The inhibitory effects of CTLA-4 were evident in resting CD4+ T cells, suggesting that CTLA-4 can act early during the immune response to regulate the threshold of T cell activation. In addition, phosphorylated versions of the cytoplasmic domain of CTLA-4 were used as affinity reagents to screen lysates for PT-Pase activities. We demonstrate in in vitro studies that SHP-1, but not SHP-2, accounts for the major PT-Pase activity associated with CTLA-4 peptides containing both the phosphorylated Y218 and the doubly phosphorylated Y201 and Y218 motifs. Because SHP-1 has been implicated in negatively regulating the signaling thresholds that influence TCR signaling and activation (20–22), the potential formation of a CTLA-4/SHP-1 signaling complex may provide an explanation for CTLA-4-mediated T cell suppression in human T cells.

Materials and Methods

Antibodies

The following Abs were used in this study: CD3 mAb (UCHT-1; Serotec, Oxford, U.K.); CD28 mAb (clone 9.3; Dr. M. Glennie, Tenvosus Laboratory, Southampton, U.K.); CTLA-4 mAb (clone B13; Immunotech, Marseille, France); isotype control trinitrophenol (TNP), PE-labeled-TNP, and CTLA-4 mAbs (BD PharMingen, San Diego, CA); rabbit antiserum to the p56\(\text{Lck}\) substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Roche).

Preparation of CD4+ T lymphocytes

CD4+ T cells were obtained from buffy coats originating from healthy donors (National Blood Transfusion Center, Brentwood, U.K.). PBMC were isolated by density gradient centrifugation using lymphoprep (Nycomed, Oslo, Norway) and washed three times with PBS. Adherent cells were removed by incubation of cells in plastic culture flasks for 60 min at 37°C. Nonadherent cells were incubated for 20 min at 4°C in RPMI 1640 with saturating concentrations of CD8, CD11, CD14, CD16, CD19, CD33, and glycoporphin mAbs. Cells were washed and incubated with sheep anti-mouse IgG-coupled magnetic Dynabeads (Dynal Biotech, Oslo, Norway) for 45 min. CD4+ T cells were isolated by removing the supernatant containing nonmagnetized cells. Cells were rested for 1 h at 37°C in RPMI/2% FCS before stimulation.

Preparation of mAb-coated microspheres

Latex microspheres (1 \( \mu \)m in diameter; Sigma, Poole, U.K.) were coated with CD3, CD28, CTLA-4, or mouse isotype control IgG (TNP) mAb in place of anti-CTLA-4. For beads used in IL-2 cytokine assays, 10\(^6\) beads/ml PBS were coated with CD3 mAb at 1 \( \mu \)g/ml, CD28 mAb at 2.5 \( \mu \)g/ml, and CTLA-4 or TNP mAb at 10 \( \mu \)g/ml in the presence of 1 mg/ml BSA. Washed beads were resuspended in 100 \( \mu \)l complete RPMI 1640 and added to 100 \( \mu \)l of the CD4+ T cell suspension. For stimulating cells before biochemical analysis, beads (10\(^6\)) were coated with CD3 mAb or CTLA-4/TNP mAb (all at 20 \( \mu \)g/ml) for 1 h, washed twice with PBS, and then resuspended in 2 ml serum-free RPMI 1640. FACS analysis of beads was used to demonstrate that the CTLA-4 and TNP mAbs bound at comparable levels and did not reduce the binding of the CD3 mAb (data not shown).

II-2 cytokine assay

CD4+ T cells (2 \( \times \) 10\(^6\) cells/well in complete RPMI 1640) were cocultured with mAb-coated microbeads (bead/cell ratio, 5:1) in round-bottom 96-well plates in a total volume of 200 \( \mu \)l complete RPMI 1640. Supernatants from duplicate cultures were collected after 24 h and assayed for IL-2 using an ELISA kit (BioSource, Nivelles, Belgium).

Activation of CD4+ T cells for biochemical analysis, immunoprecipitation, and immunoblotting

Resting CD4+ T cells (10\(^6\) per stimulation) were activated with TNP, CD3 plus TNP, or CD3 plus CTLA-4 mAb-coated beads (bead/cell ratio, 100:1) in 3 ml serum-free RPMI 1640 for 5 min at 37°C. Cells were centrifuged and lysed in ice-cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM Na\(_3\)VO\(_4\), 1 mM 4-[2-aminoethanesulfonyl]fluoride (AEBSF), 1 mM EGTA, 1 mM EDTA, and protease inhibitor cocktail (Roche, Lewes, U.K.). After 15 min of incubation on ice, lysates were prepared by centrifugation and preincubated by incubation with protein G-Sepharose (Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 45 min at 4°C. Lysates from 3 \( \times \) 10\(^7\) cells were boiled in 3 \( \times \) SDS sample buffer and subjected to immunoblotting with phospho-ERK and phosphotyrosine mAbs following transfer to polyvinylidene difluoride membranes (Millipore, Bedford, U.K.). For immunoprecipitations, cleared cell lysates were incubated for 1–24 h at 4°C with TCR-\(\zeta\), p56\(\text{Lck}\), ZAP-70, or phosphospecific (Y319) ZAP-70 Abs bound to protein G-Sepharose. The immune complexes were washed three times in Nonidet P-40 lysis buffer and either subjected to SDS-PAGE for immunoblotting or prepared for an immunocomplex in vitro kinase assay. Bands were visualized by ECL (Amersham Pharmacia Biotech) or by the addition of the alkaline phosphatase-specific substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Roche).

Immunocomplex in vitro kinase assays

TCR-\(\zeta\), ZAP-70, and p56\(\text{Lck}\) immunoprecipitates were prepared as described above and washed in buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM Na\(_3\)VO\(_4\), and 1 mM AEBSF). To preincubate lysates of p56\(\text{Lck}\), unstimulated samples were lysed and subjected to three consecutive rounds of immunoprecipitation using p56\(\text{Lck}\) Ab. Depleted samples were then used for ZAP-70 immunoprecipitation, washed, and subjected to in vitro kinase assays. Precipitates were resuspended in 40 \( \mu \)l kinase assay buffer (50 mM Pipes (pH 7.4), 10 mM MgCl\(_2\), 10 mM MnCl\(_2\), 1 mM Na\(_3\)VO\(_4\), 1 mM AEBSF, 2 mM DTT, 4.5 \( \mu \)M cold ATP, and 5 \( \mu \)M \( c^{32}\)P[ATP], and kinase reactions were performed for 10 min at room temperature. The reaction was stopped by adding 50 \( \mu \)l of 3X SDS reducing sample buffer. Samples were boiled, and proteins were resolved on 7–15% SDS-PAGE, followed by autoradiography and immunoblotting.
Peptides representing the complete CTLA-4 cytoplasmic tail were synthesized and HPLC-purified by the Biochemical Facility (Babraham Institute, Cambridge, U.K.). The amino acid sequences of the peptides used were as follows: KMLKRRSLTTPGTVKMPTEPECEKFQFYIPIN (unphosphorylated peptide), KMLKRRSLTTPGTVKMPTEPECEKFQFYIPIN (pY201 peptide), KMLKRRSLTTPGTVKMPTEPECEKFQFYIPIN (pY201 peptide), and KMLKRRSLTLPGTVKMPTEPECEKFQFYIPIN (pY218 peptide). Peptides were covalently bound to cyanogen bromide-activated Sepharose beads (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Peptides were subsequently used to precipitate PTpase activities from whole cell lysates originating from DT40 and SHP-2-deficient DT 40 cells (from Prof. T. Kurosaki, Kansai Medical University, Japan), from primary human CD4+ T cells, and from motheaten (me/me) mice (from Dr. J. Matthews).

PTpase activity was measured using the Malachite green PTpase assay kit (Upstate Biotechnology). Postnuclear lysates from 4–6 × 10^6 cell equivalents were prepared as described above, but without the PTpase inhibitor Na3VO4, and were incubated with 30 µl Sepharose slurry for 1 h at 4 °C. Samples were incubated for 1 h at 4 °C with 30 µl CTLA-4 tail peptide Sepharose slurry. To deplete lysates of SHP-1, samples originating from human CD4+ T cells were subjected to four consecutive rounds of SHP-1 immunoprecipitation using SHP-1 Ab coupled to protein G-Sepharose beads. SHP-1-depleted lysates were then exposed to CTLA-4 tail hydrolysis. Precipitates were washed three times with lysis buffer, followed by three washes in PTpase assay buffer containing 25 mM HEPES (pH 7.2), 50 mM NaCl, 5 mM DTT, 2.5 mM EDTA, and 1 mg/ml BSA. Samples were resuspended in PTpase assay buffer and incubated for 10 min at 37 °C with 250 µM phosphopeptide (TSTEPQpYQPGENL) as the substrate. After centrifugation, the supernatants were transferred to microtiter wells and incubated with 100 µl Malachite Green solution for 15 min, and PTpase activity was quantitated by measuring the absorbance at 630 nm using a microtiter plate reader. All assays were performed in duplicate in parallel with assay blanks containing CTLA-4 synthetic tail peptide beads, PTpase assay buffer, and phosphopeptide substrates. PTpase activity was calculated from standard curves after subtracting background values. The remaining peptide pellets were resuspended in 3× SDS sample buffer, boiled, and subjected to SDS-PAGE and Western blot analysis as described above.

Flow cytometry
Cells were incubated for 30 min at 4°C with PE-conjugated CTLA-4 or isotype-specific TNP mAb in PBS/1% BSA/0.1% sodium azide or fixed with 1% paraformaldehyde and permeabilized with 0.3% saponin before incubation with mAb for intracellular CTLA-4 staining. Cells were analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

Results
Colligation of CTLA-4 down-regulates IL-2 cytokine production
Freshly purified human CD4+ T cells were stimulated with CD3/CD28 and CTLA-4 mAbs coimmobilized on microbeads, and culture supernatants were assayed for IL-2 cytokine production. Stimulation using CD3 mAb alone caused little IL-2 secretion, but this was greatly increased upon CD3 and CD28 costimulation (Fig. 1). Concomitant ligation of CTLA-4 with CD3/CD28 consistently resulted in a striking reduction (50–70% inhibition) of IL-2 secretion (Fig. 1). This effect was specific to CTLA-4, because the irrelevant TNP control mAb had no effect when co-cross-linked with CD3/CD28 (data not shown). These results confirm an earlier report (23) and establish that in our experimental system engagement of human CTLA-4 exerts an inhibitory effect on CD3/CD28-mediated T cell activation events resulting in diminished IL-2 production.

CTLA-4 is expressed constitutively on resting, primary human CD4+ T cells
It has been suggested that CTLA-4 is expressed at detectable levels at the T cell surface only after activation (24). Previous studies have shown that CTLA-4 is located primarily in endosomal vesicles, from where it cycles continuously to and from the cell surface (25). To measure cell surface and cytoplasmic CTLA-4 expression, FACS analysis was conducted on intact and permeabilized resting, purified human CD4+ T cells. Table I lists the 23 blood donors who were analyzed for CTLA-4 expression, and Fig. 2 shows a representative FACS profile of CTLA-4 receptor staining. In most instances (17 of 23 donors) flow cytometric analysis of CD4+ T cells revealed low, but detectable, levels of surface CTLA-4 (7 ± 1% positive cells; Table I and Fig. 2A). As expected, a larger proportion of CTLA-4 could be detected intracellularly.
after treatment of cells with saponin (34 ± 5% positive cells; Table I and Fig. 2B). However, we noticed considerable variation in CTLA-4 expression levels among individual CD4+ T cell preparations originating from various blood donors (Table I), presumably due to the different immune histories of the donors. The observation that CTLA-4 was detectable on resting CD4+ T cells raises the possibility that it may also be operational and functional, resulting in inhibition of the very earliest stages of T cell activation.

CTLA-4 engagement inhibits TCR-induced tyrosine phosphorylation of proteins and ERK-1/2 activation

The aim of our investigation was to elucidate the molecular mechanism of action by which CTLA-4 engagement inhibits TCR-mediated activation signals in primary cells.

It is unknown whether CTLA-4 interferes with TCR-mediated protein tyrosine phosphorylation in primary resting human CD4+ T cells. Therefore, we evaluated the effects of CTLA-4 ligation on TCR signaling by analyzing changes in tyrosine phosphorylation in whole cell lysates. Fig. 3A shows that TCR stimulation caused increased protein tyrosine phosphorylation of proteins migrating in the range of ~34–40 and ~75–150 kDa and that most phosphorylation events were much reduced upon CD3/CTLA-4 coligation. Stripping and reprobing the membrane with p95vav mAb demonstrated comparable loading between lanes. Minor qualitative variations in tyrosine-phosphorylated substrates were noted between different donors, but CTLA-4-mediated inhibition of phosphorylation was consistently noted in five separate experiments. Intriguingly, as Fig. 3A illustrates, basal tyrosine phosphorylation was also reduced by CTLA-4 ligation in T cells prepared from several different donors. The striking generalized reduction in protein tyrosine phosphorylation suggests that CTLA-4 interferes with TCR signal transduction at a very early stage in the initiation of the signaling cascade.

It was reported that CTLA-4 ligation attenuates activation of the MAPK and c-Jun N-terminal kinase pathways in activated murine T cells and in Jurkat T cells stably transfected with CTLA-4 (4, 26). Fig. 3B shows that coengagement of CTLA-4 with the TCR in primary resting CD4+ T cells also results in significant inhibition (~80%) of CD3-stimulated ERK-1/2 activation. Subsequent reprobing of the membrane with an ERK-2 Ab revealed comparable loading of protein between lanes. CTLA-4-mediated inhibition of ERK-1/2 activity was also demonstrated at later time points, excluding the possibility of a modified kinetic response induced by CTLA-4 (data not shown). Thus, our results suggest that coengagement of TCR and CTLA-4 perturbs proximal TCR signal transduction independently of CD28 engagement and upstream or at the level of ERK-1/2 activation.
TCR ζ-chain tyrosine phosphorylation and ZAP-70 recruitment are not inhibited by CTLA-4 engagement

To investigate whether CTLA-4 counteracted the most proximal step in the TCR signaling cascade, we measured TCR ζ-chain tyrosine phosphorylation following CTLA-4 coengagement. Phosphorylation of TCR-ζ by p56<sup>ικκ</sup> causes binding of ZAP-70 tyrosine kinase via its SH2 domains, leading to its phosphorylation and activation (27). Stimulation of the TCR complex resulted, as expected, in generation of the TCR-ζ p21 and p23 phosphoisomers, but coengagement of CTLA-4 did not significantly change their phosphorylation status (Fig. 4A, upper panel). Likewise, CTLA-4 did not interfere with ZAP-70 recruitment to the TCR complex, as assessed by immunoblotting TCR ζ-chain immunoprecipitates for ZAP-70 (Fig. 4B). In addition, ZAP-70 immunoprecipitates were analyzed for coassociated TCR-ζ. Fig. 4C shows that CTLA-4-TCR coligation did not interfere with the ability of ZAP-70 to interact with the TCR-ζ p23-kDa phosphoisoform. Thus, TCR ζ-chain phosphorylation and ZAP-70 recruitment to the TCR ζ-chain remained unaltered by CTLA-4 engagement.

**FIGURE 4.** CD3-induced TCR-ζ tyrosine phosphorylation and ZAP-70 recruitment to TCR-ζ are not affected by CTLA-4. A, TCR-ζ immunoprecipitates from resting CD4<sup>+</sup> T cells were immunoblotted for phosphoryrosine (upper panel) and TCR-ζ (lower panel). B, TCR-ζ immunoprecipitates from stimulated cell lysates were immunoblotted for ZAP-70. C, ZAP-70 was immunoprecipitated from stimulated lysates and immunoblotted with TCR-ζ Ab (upper panel). The blot was stripped and reprobed for ZAP-70 (lower panel). All results are representative of at least three experiments.

**CTLA-4 perturbs p56<sup>ικκ</sup>-ZAP-70 coassociation**

To investigate whether CTLA-4 cross-linking interfered with the recruitment and/or activation of kinases within TCR signaling complexes, in vitro kinase assays were conducted on ZAP-70 immunoprecipitates originating from TCR/CTLA-4-stimulated cells. Fig. 5A shows that CTLA-4 engagement caused a striking reduction in the phosphorylation of several proteins migrating at 70, 56, 28, and 25 kDa in the immunocomplex. Immunoblot analysis revealed, as expected, that the 70-kDa protein was ZAP-70 (Fig. 5A, middle panel). We speculated that the ZAP-70-associated 56-kDa protein was p56<sup>ικκ</sup> based on previous work (28); however, we were unable to identify this protein unambiguously as p56<sup>ικκ</sup> by Western blotting due to the presence of the IgG heavy chain. Instead we immunoprecipitated ZAP-70 from p56<sup>ικκ</sup>-depleted lysates and performed in vitro kinase assays in the ZAP-70 immunocomplex and in p56<sup>ικκ</sup> immunoprecipitates as a control. The results confirmed that the 56-kDa protein in the ZAP-70 immunocomplex was indeed p56<sup>ικκ</sup>, as this protein was undetectable in the immunoprecipitates prepared from p56<sup>ικκ</sup>-depleted lysates (Fig. 5A, lower panel). Our results show that CTLA-4 perturbs phosphorylation of ZAP-70 and/or recruitment of several proteins interacting with ZAP-70, including p56<sup>ικκ</sup> as well as pp28 and pp25.

Because p56<sup>ικκ</sup> was identified as one of the major hypophosphorylated proteins in ZAP-70 immunocomplex kinase assays following CTLA-4 engagement, we analyzed more directly whether interaction of p56<sup>ικκ</sup> with ZAP-70 was perturbed after CTLA-4 engagement. Analysis of p56<sup>ικκ</sup>-immunoprecipitates for ZAP-70 by Western blotting revealed that TCR/CTLA-4 ligation reduced the binding of ZAP-70 to p56<sup>ικκ</sup> by 47 ± 3% (n = 3; Fig. 5B, upper panel). Both ZAP-70 and p56<sup>ικκ</sup> were at comparable levels in the lysates used for immunoprecipitation (Fig. 5B, lower panels). To probe the regulation of ZAP-70 more selectively, we examined the phosphorylation status of the particular pool of ZAP-70 found in TCR-ζ immunocomplexes by in vitro kinase assay. Fig. 5C shows that TCR-induced phosphorylation of a 70-kDa protein (upper panel), established by immunoblotting to be ZAP-70 (middle panel), was reduced by 48 ± 10% (n = 3) after co-cross-linking with CTLA-4 mAb. Immunoblotting confirmed the findings shown in Fig. 4A that CTLA-4 engagement reduced neither TCR-ζ phosphorylation nor ZAP-70 recruitment per se (lower panels). Thus, reduced interaction of ZAP-70 with p56<sup>ικκ</sup> correlates with inhibited ZAP-70 phosphorylation.

**CTLA-4 ligation selectively inhibits phosphorylation of ZAP-70 Y319 but does not affect p56<sup>ικκ</sup> kinase activity**

One mechanism that could explain the reduced ZAP-70-p56<sup>ικκ</sup> association is that CTLA-4 might inhibit the kinase activity of p56<sup>ικκ</sup>. Alternatively, CTLA-4 could directly interfere with the phosphorylation state of tyrosine residues present in ZAP-70. In particular, phosphorylated Y319, which was shown to be a docking site for the SH2 domain of p56<sup>ικκ</sup> (19), could be a target for CTLA-4. To distinguish these possibilities, in vitro kinase assays were performed on p56<sup>ικκ</sup>-immunoprecipitates originating from CD3/CTLA-4-stimulated lysates. As is shown in Fig. 6A, p56<sup>ικκ</sup> kinase activity is not reduced following CTLA-4 engagement, indicating that the activation of p56<sup>ικκ</sup>, an upstream regulator of the TCR/ZAP-70 signaling complex, is not affected by CTLA-4. We next
addressed whether CTLA-4 interferes with phosphorylation of the ZAP-70 Y319 site. Lysates from stimulated samples were subjected to immunoprecipitations using an Ab specific for phosphotyrosine 319 in ZAP-70 and the amount of pY319-ZAP-70 was then detected by ZAP-70 immunoblotting (Fig. 6B). Whereas CD3 stimulation induced phosphorylation of Y319 in ZAP-70, CTLA-4 engagement resulted in a striking inhibition of that phosphorylation site. The same results were obtained in independent experiments in which the phospho-ZAP-70 Ab was used to immunoblot pY319-ZAP-70 immunoprecipitates (Fig. 6C). Reprobing of whole cell lysates used for the immunoprecipitations were immunoblotted for ZAP-70 (middle panel) and p56\(^{\text{Lck}}\) (lower panel) to demonstrate comparable levels. C. In vitro kinase assays were performed in TCR \(\zeta\) chain immunocomplexes. Membranes were blotted for ZAP-70 (middle panel) and TCR-\(\zeta\) (lower panel). Results are representative of at least three separate experiments.

**SHP-1 constitutes the major PTPase activity associated with the cytoplasmic tail of CTLA-4**

Signaling pathways responsible for CTLA-4-mediated inhibition are largely unknown. Examination of tyrosine phosphorylation levels in T cells from CTLA-4\(^{-/-}\) mice revealed that a number of proteins implicated in TCR signaling, such as CD3-\(\zeta\), ZAP-70, Shc, Lck, and Fyn, are hyperphosphorylated and hyperactive (15). These observations led to the suggestion that tyrosine phosphatases may be involved in the inhibitory signal transduced by CTLA-4. One candidate PTPase is SHP-2, which has been shown to be recruited to CTLA-4 through the phosphorylated Y201VKM motif in the cytoplasmic tail (15). However, the involvement of SHP-2 in mediating CTLA-4-mediated inhibition and SHP-2 binding to CTLA-4 has been challenged in other studies (29, 30). Our observation that phosphorylation events were inhibited after CTLA-4 cross-linking prompted us to investigate whether PTPases could be recruited to CTLA-4. The complete CTLA-4 cytoplasmic tail was synthesized in nonphosphorylated, doubly phosphorylated, or mono-tyrosine-phosphorylated versions. These were used as affinity reagents to precipitate PTPases from cellular lysates that were subsequently analyzed for associated PTPase activity. Using
Jurkat cell lysates it was possible to detect PTPase activity associated with the phosphorylated CTLA-4 tail containing the pY218 motif and the doubly phosphorylated pY201 and pY218 motifs (data not shown). Comparable results were obtained when the chicken DT40 cell line was used (Fig. 7A). Blotting the precipitates for the presence of SHP-2, we found that SHP-2 associated predominantly with the tyrosine-phosphorylated versions of the CTLA-4 tail (Fig. 7A). To further analyze the contribution of SHP-2 activity associated with CTLA-4, we made use of a SHP-2-deficient DT40 cell line. As expected, no SHP-2 was precipitated from the deficient cells (Fig. 7A). Interestingly, however, there was no difference in CTLA-4-associated PTPase activity between CTLA-4 tail precipitates derived from wild-type and SHP-2-deficient DT40 cells (Fig. 7A). Similar results were obtained when the phosphorylated TCR-ζ peptide immunoreceptor tyrosine-based activation motif was used as a substrate (data not shown). These findings suggest that SHP-2 does not contribute to the PTPase activity associated with the cytoplasmic tail of CTLA-4.

SHP-1 represents another candidate PTPase that could contribute to the observed CTLA-4-mediated suppression of human T cells. SHP-1 has been implicated in negatively regulating the signaling thresholds of the TCR (20–22). To test this hypothesis, PTPase assays were conducted on CTLA-4 tail precipitates originating from SHP-1-immunodepleted purified human CD4+ T cells as well as from SHP-1-deficient, motheaten (me/me) cell lysates. Lysates from CD4+ T cells were subjected to four rounds of SHP-1 immunodepletion or, instead, were exposed to protein G-Sepharose as a control. Samples were then used for PTPase assays and for blotting, displaying significant, although incomplete, depletion of SHP-1 (Fig. 7B, left panel). Similar amounts of proteins were subjected to the analysis, as confirmed by blotting for growth factor receptor binding protein 2 (Grb-2; Fig. 7B). SHP-1 binding was dependent on phosphorylation of the Y218 or Y201 and Y218 motifs within the CTLA-4 cytoplasmic tail. Interestingly, however, little SHP-1 coassociated with the pY201VKM motif. SHP-1 immunodepletion resulted in a reduction by 80% of SHP-1 binding to the peptides (Fig. 7B). This reduced SHP-1 binding correlated with a striking decrease in PTPase activity associated with the pY218 (75% reduction) and pY201 and pY218 (50% reduction) motifs compared with the nondepleted samples. These results strongly support the view that SHP-1 represents the major PTPase activity associated with the phosphorylated cytoplasmic tail of CTLA-4 (Fig. 7B).

To substantiate these findings, similar experiments were conducted on T cell lysates originating from motheaten (me/me) mice, which completely lack the expression of SHP-1, and from wild-type mice. We used the nonphosphorylated and the doubly phosphorylated version of the CTLA-4 peptide tail as the affinity reagents for these experiments. Blotting the lysates for Grb-2 revealed that protein concentrations were similar in wild-type and me/me samples (Fig. 7C). Consistent with the immunodepletion experiments, the level of PTPase activity associated with the phosphorylated CTLA-4 cytoplasmic tail was reduced by 50–75% (n = 2) compared with that in wild-type controls (Fig. 7C). Taken together, these findings suggest that SHP-1 coassociates with CTLA-4 and constitutes an important PTPase activity in the CTLA-4 complex. However, SHP-1 is not solely responsible for the observed PTPase activity, because 25–50% PTPase activity was still detectable even in SHP-1-deficient cells.

In summary, this study shows for the first time that coengagement of CTLA-4 on resting human CD4+ T cells inhibits TCR induced p56\(^{Lck}\)-ZAP-70 complex formation, which correlates with reduced ZAP-70 Y319 phosphorylation, causing inhibition of downstream signaling events and of IL-2 secretion. The major active PTPase recruited to the phosphorylated CTLA-4 cytoplasmic tail appears to be SHP-1.

**Discussion**

While CTLA-4 is well documented to be a negative regulator of cytokine production and T cell proliferation (31), the molecular mechanisms of CTLA-4 signaling are largely unknown. This study was designed to investigate CTLA-4-mediated inhibition of TCR signaling in primary human CD4+ T cells. To validate our stimulation protocol using beads coated with Abs, IL-2 cytokine assays were performed. Cross-linking of CTLA-4 in conjunction with CD3/CD28 resulted in down-regulation of IL-2 cytokine produc-
tion, confirming an inhibitory role of CTLA-4 in our experimental model (Fig. 1). We noticed that CTLA-4 is constitutively expressed, both intracellularly and on the surface of resting CD4⁺ T cells (Fig. 2). However, there was variation of CTLA-4 surface expression levels among the different blood donors. There is still some controversy regarding the level and kinetics of CTLA-4 expression. CTLA-4 is thought to be undetectable in naive T cells, but is rapidly up-regulated upon T cell activation. CTLA-4 mRNA can be readily detected within 1 h of TCR engagement and peaks at ~24–36 h (32). It is likely in the present work that the donors had encountered Ag in vivo that may have mobilized existing intracellular stores of CTLA-4 to the membrane. Thus, the variation observed among the donors may reflect heterogeneity in the immune histories of the different donors. Our observations suggest that the low CTLA-4 expression level could be functionally significant in inhibiting early T cell activation events. Interestingly, recent studies have shown that CTLA-4 is detectable and constitutively expressed on murine CD25⁺CD45RBlowCD4⁺ regulatory cells and that CTLA-4 is likely to be involved in their immune-suppressive function (33). It remains to be established.

FIGURE 7. SHP-1 represents the major PTPase activity associated with the cytoplasmic tail of CTLA-4. A. Wild-type and SHP-2−/− DT40 cell lysates were incubated with CTLA-4 cytoplasmic tail peptides covalently bound to Sepharose beads. Precipitates were washed in lysis buffer and PTPase assay buffer and used in an ELISA-based in vitro PTPase assay as described in Materials and Methods. CTLA-4 peptide precipitates were subjected to SDS-PAGE and anti-SHP-2 Western blotting. B. Postnuclear lysates originating from human purified CD4⁺ T cells were subjected to four consecutive rounds of SHP-1 immunoprecipitations or exposed to protein G-Sepharose only (control). SHP-1-depleted and control samples were then incubated with immobilized CTLA-4 peptides, washed, and used for in vitro PTPase assay, SDS-PAGE, and SHP-1 and Grb-2 blotting. C. Lysates from wild-type and me/me mice were incubated with CTLA-4 peptide beads, used for in vitro PTPase assay, and blotted for SHP-1 and Grb-2. Results are representative of two independent experiments performed in duplicate.
whether expression of CTLA-4 is restricted predominantly to human T regulatory cells.

Analysis of CTLA-4-mediated signaling events in resting human CD4+ T cells showed that CD3-evoked protein tyrosine phosphorylation events were decreased upon CTLA-4 ligation. This was paralleled with reduced ERK activation (Fig. 3). These data suggest that CTLA-4 interferes with proximal TCR signaling. However, CD3-induced tyrosine phosphorylation of the TCR ζ-chains and subsequent ZAP-70 recruitment were not affected by CTLA-4 (Fig. 4), indicating that CTLA-4 acts downstream of these molecules. Our results are consistent with a previous study using preactivated, murine T cells, which also found that these phosphorylation events and ZAP-70 recruitment remain unaltered upon CTLA-4 engagement (4). In contrast, another report demonstrated reduced TCR ζ-chain and linker for activation of T cells phosphorylation following CTLA-4 cross-linking (16). However, these latter results were obtained using preactivated murine T cells and different stimulation conditions, and it is possible that T blasts may display different signaling characteristics due to their altered activation state.

Because ZAP-70 recruitment to the TCR ζ-chain was not affected by CTLA-4 and because ZAP-70 has been implicated as a critical intermediary between TCR stimulation and Ras activation (34), we investigated whether recruitment and/or activation of kinases in the ZAP-70 immunocomplex were altered by CTLA-4. Phosphorylation of multiple ZAP-70 tyrosine residues, mediated by autophosphorylation, Src kinases, or other cellular kinases, is thought to result in the association of downstream signaling components by SH2 domain-phosphotyrosine interactions (35). Importantly, phosphorylation of Y319 was shown to be a binding site for the SH2 domain of p56Lck, and this interaction is crucial for ZAP-70 activation and subsequent amplification of downstream signaling (19, 34). In this study we demonstrate for the first time that CTLA-4 interferes with the regulation of ZAP-70. This was evidenced by reduced phosphorylation of ZAP-70 (Fig. 5A) and diminished coassociation or reduced phosphorylation of ZAP-70 with unknown proteins of 25 and 28 kDa (Fig. 5A). Moreover, the TCR-ζ-associated ZAP-70 pool was found to be hypophosphorylated, as assessed by in vitro kinase assays of TCR-ζ immunoprecipitates (Fig. 5C). Current experiments are in progress to identify pp25/pp28. One possible candidate could be the p23 isoform of TCR-ζ; however, immunoblotting for TCR-ζ was inconsistent with this idea (data not shown). More importantly, CTLA-4 ligation resulted in perturbed TCR-induced complex formation of p56Lck with ZAP-70 following ZAP-70 blotting of p56Lck immunoprecipitates (Fig. 5B). It is this reduction in p56Lck-ZAP-70 association rather than any reduction in ZAP-70 activity that most likely explains the reduced phosphorylation of ZAP-70 shown in Fig. 5A. When phospespecific ZAP-70 Y319 Abs were used to immunoprecipitate and to blot for phosphorylated ZAP-70, we were able to demonstrate that CTLA-4 caused a striking inhibition of ZAP-70 Y319 phosphorylation. Because phosphorylation at this site has been shown to be important for the induction of the NFAT transcription factor complex (34), a reduction in Y319 phosphorylation could readily explain the inhibition of IL-2 secretion (Fig. 1) that we observed upon CTLA-4 engagement. The reduced phosphorylation could be explained by inhibition of a kinase or increased actions of a phosphatase. We were unable to detect any reduction in p56Lck kinase activity following CTLA-4 engagement (Fig. 6A). Because p56Lck is most likely the kinase that phosphorylates Y319, this points to increased phosphatase action as the possible explanation for the reduction in Y319 phosphorylation. A CTLA-4-associated PTPase could dephosphorylate ZAP-70 Y319, leading to diminished interaction with the p56Lck SH2 domain, resulting in inhibited TCR signaling.

To identify potential PTPases that associate with CTLA-4 in an active form, we took an approach using the CTLA-4 cytoplasmic tail in nonphosphorylated and phosphorylated forms as affinity reagents to precipitate PTPase activities. One candidate PTPase that has been shown to interact with and negatively regulate ZAP-70 is SHP-1 (36, 37). By immunodepletion of SHP-1 from human CD4+ T cell lysates and by using SHP-1-deficient me/me cell lysates, we show that SHP-1 accounts for the majority of the PTPase activity associated with the CTLA-4 cytoplasmic tail phosphorylated at Y218 or at Y201 and Y218 (Fig. 7, B and C). The preferential binding motif for SHP-1 appears to be the pY218FIP motif, and we detected neither significant PTPase activity nor SHP-1 binding to the pY201VKM motif (Fig. 7B and data not shown). The CTLA-4 cytosolic domains do not possess immunoreceptor tyrosine-based inhibition motifs, which could mediate direct binding to SHP-1, indicating an indirect association. The absence of direct SHP-1 binding to CTLA-4 does not preclude a functional role, because the interaction may be mediated by one or more adaptor molecules. We were unable to detect interaction between endogenous CTLA-4 and SHP-1 in pervanadate treated primary T cell blasts (data not shown), which may be due to the extremely small amounts of CTLA-4 expressed in these cells.

From our results it is evident that SHP-1 does not constitute the sole CTLA-4-associated PTPase, because appreciable activities (25–50%) were detected in the me/me mouse cell lysates. Using the SHP-2-deficient DT40 cell line we could exclude the contribution of SHP-2 to the PTPase activity associated with CTLA-4, although SHP-2, presumably catalytically inactive, binds to the phosphorylated cytoplasmic tail (Fig. 7A). It is possible that the N-SH2 domain of SHP-2 interacts with its PTPase domain, and SHP-2 adopts a closed conformation, thereby rendering the enzyme catalytically inactive when bound to the CTLA-4 cytoplasmic tail.

Although it was impossible to directly prove the involvement of SHP-1 using primary human cells, we speculate that SHP-1 may be critical for the observed CTLA-4-induced inhibition of TCR signaling. There is evidence that CTLA-4 interacts with the TCR ζ-chain (16), and SHP-1 is normally excluded from lipid rafts for initiation of TCR signaling to occur (38, 39). It is possible that CTLA-4 targets SHP-1 to the TCR complex, thereby interfering with the phosphorylation of signal effectors, e.g., ZAP-70, and with its association state with p56Lck (40). A study using lymph node T cells from motheaten viable mice has shown that proliferation and IL-2 production could still be inhibited by CTLA-4 engagement (40), suggesting that SHP-1 may not be essential for mediating the inhibitory effects of CTLA-4 in vivo. Clearly, the availability of conditional knockout SHP-1−/− mice would help to elucidate the possible role of SHP-1 in vivo in mediating the negative effects of CTLA-4 in the T cell lineage. The phenotype of the T cells from such mice might provide a more complete understanding of SHP-1 function, because the development of hemopoietic cell lineages are not expected to be as severely affected as in motheaten viable mice. Furthermore, our findings do not exclude other potential mechanisms for CTLA-4-mediated inhibition, such as the possibility that CTLA-4 competes with CD28 for binding to CD80 and CD86 ligands.

In summary, our results using primary resting CD4+ T cells point to a highly selective molecular mechanism of action of CTLA-4 in down-regulating TCR signal transduction coupling.
Acknowledgments

We are indebted to the following for their provision of reagents: Prof. P. Beverley, Dr. J. Matthews, Prof. C. Terhorst, Dr. M. Glennie, Prof. T. Kurosaki, and Dr. L. Samelson.

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