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Inhibition of CTLA-4 Function by the Regulatory Subunit of Serine/Threonine Phosphatase 2A

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The catalytic subunit of the serine/threonine phosphatase 2A (PP2A) can interact with the cytoplasmic tail of CTLA-4. However, the molecular basis and the biological significance of this interaction are unknown. In this study, we report that the regulatory subunit of PP2A (PP2AA) also interacts with the cytoplasmic tail of CTLA-4. Interestingly, TCR ligation induces tyrosine phosphorylation of PP2AA and its dissociation from CTLA-4 when coligated. The association between PP2AA and CTLA-4 involves a conserved three-lysine motif in the juxtamembrane portion of the cytoplasmic tail of CTLA-4. Mutations of these lysine residues prevent the binding of PP2AA and enhance the inhibition of IL-2 gene transcription by CTLA-4, indicating that PP2A represses CTLA-4 function. Our data imply that the lysine-rich motif in CTLA-4 may be used to identify small molecules that block its binding to PP2A and act as agonists for CTLA-4 function. The Journal of Immunology, 2002, 168: 5070–5078.

Materials and Methods

Cells

The panel of Jurkat T cells transfected with a regulatable, doxycycline-sensitive CTLA-4 cDNA has been previously reported (6, 13). The K-less mutant was generated using the PFU polymerase-based QuikChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to manufacturer’s instructions using the oligonucleotides: CCTCACAGCTGTTGTAAGAGGGCTTCTTCCCGCTAGCCATTGCGCTCAAAGAAACAGCTGTGAGG as mutagenic primers and its complement, CCCCTGTTGTAAGAGGGCTTCTTCCCGCTAGCCATTGCGCTCAAAGAAACAGCTGTGAGG and its complement. CCCCTGTTGTAAGAGGGCTTCTTCCCGCTAGCCATTGCGCTCAAAGAAACAGCTGTGAGG as mutagenic primers and human CTLA-4/pBIG2i as template. Plasmid isolates were recovered and human CTLA-4, K-less mutant clones were confirmed by sequencing. A
luciferase reporter CDNA under the control of the IL-2 promoter and enhancer elements (kindly provided by Dr. A. Weiss, University of California, San Francisco, CA) was transfected into these cells, and clones isolated after limiting dilution were used for these experiments. The 0.45 lymphoblastoid B cell line that expresses HLA-DR1 and B7.1 was kindly provided by Dr. E. Long (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Both cell lines were cultured under standard conditions.

**Plasmid construction**

The cytoplasmic regions of mouse CTLA-4, CD28, and ICOS were generated by PCR. The cDNA encoding murine CTLA-4 (cytoplasmic domain) was inserted into the EcoRI site of bait vector PEG202 (Origene Technologies, Rockville, MD) to yield a Lex-A DNA binding fusion plasmid. Similarly, cytoplasmic regions of murine CD28 and ICOS were subcloned into the EcoRI site of bait vector PEG202.

The 1-kb SalI fragment of clone 54 or the 1.7-kb SalI fragment of full-length murine PP2A was inserted into the SalI site of vector pCMV-myc (Clontech Laboratories, Palo Alto, CA), which contains an oligonucleotide encoding the myc peptide inserted into the 5′ end of the multiple cloning site of the mammalian expression vector pCMV. Full-length CTLA-4 was expressed as a hemagglutinin (HA)-tagged fusion protein by inserting the CTLA-4 cDNA into the EcoRI/XhoI sites of pCMV-HA (Clontech Laboratories). Similarly, full-length CD28 and ICOS were cloned into the EcoRI/SalI sites of pCMV-HA.

**Yeast two-hybrid system**

A yeast two-hybrid screen of a murine Th1 cell library was performed by cotransfecting the bait CTLA-4-PEG202 into the yeast strain EGY4-8 (Origin Technologies) along with a murine Th1 cell line library constructed in the B42 activation domain of pJG4-5 (Origene Technologies), using the Duplex A yeast two-hybrid system (Origene Technologies). The Th1 library was obtained from Drs. S. Szabo and L. Glimcher (Harvard School of Public Health, Boston, MA). Screening was performed on 2 million transformants.

**Protein interactions**

The plasmid vectors encoding the myc-tagged PP2A (392–589) or full-length PP2A and HA-tagged CD28, CTLA-4, or vector alone were cotransfected into human 293 cells using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and following the manufacturer’s protocol. Transfected cells were harvested and lysed at 4°C in 1% Nonidet P-40 lysis buffer. Cell lysates were preclarified and immunoprecipitated using protein G beads coated with anti-HA Abs. After an overnight incubation at 4°C, the immunoprecipitates were washed with 1% Nonidet P-40 lysis buffer. Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/15% PAGE, and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 3% BSA in PBS and then incubated with anti-myc Ab or anti-myc Abs for detecting PP2A (392–589). For the detection of CTLA-4 and CD28, polyvinylidene difluoride membranes were blotted with anti-CD28 and anti-CTLA-4 Abs. Subsequently, the membranes were incubated with HRP-conjugated secondary Abs, before visualization using chemiluminescence reagents.

**Biochemistry**

Jurkat T cells (cell number normalized for protein content) were cultured overnight in the absence or presence of doxycycline (5 μg/ml), and were stimulated for 10 min with 0.45 cells (5:1 ratio) preincubated with Staphylococcal enterotoxin E (SEE) (Toxin Technology, Sarasota, FL) for 40 min at 37°C. Cells were subsequently lysed in standard lysis buffer containing Triton X-100 (1%). Lysates were preclarified with protein G agarose beads (Roche, Laval, Canada), followed by immunoprecipitation with diethio bis(succinimidyl) proprionate)–cross-linked Abs on protein G agarose beads. mAb-coated beads were prepared with anti-CD3 mAb (1 μg/ml), followed by immunoprecipitation with diethio bis(succinimidyl) proprionate)–cross-linked Abs on protein G agarose beads, and Western blotting as previously described (13, 18, 19).

**Cell surface biotinylation**

Doxycycline-induced wild-type (WT)–CTLA-4–transfected cells were incubated with biotin (Pierce, Rockford, IL) (0.5 μg/ml) for 30 min at room temperature. Cells were washed with PBS and incubated with 0.45 cells for 10 min at 37°C. Cell lysates were prepared and used to immunoprecipitate with anti-biotin Abs (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoprecipitates were either eluted with sample buffer under reducing conditions or with lysis buffer (200 μl) for 10 min at 95°C to perform a second immunoprecipitation with a mAb anti-MHC class I molecule (HC10) or a mixture of anti-CTLA-4 Abs (CTLA-4-11 and CTLA-4-24), followed by immunoblotting for CTLA-4 or PP2AA.

**Reagents**

Abs used for these experiments were: a goat polyclonal antiserum against PP2AA regulatory subunit, and a goat polyclonal antiserum against Dok-1 (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse mAb against PP2A catalytic subunit (from Santa Cruz Biotechnology and from Upstate Biotechnology, Lake Placid, NY), a mouse mAb against phosphotyrosine (kindly provided by Dr. B. Drukker, Oregon Health Sciences University, Portland, OR), a mouse mAb against human CTLA-4-11, the chimeric B7.2-human IgG1 molecule (B7.2 IgG1) was obtained from Genetics Institute (Cambridge, MA), and a goat polyclonal antiserum against a peptide from the extracellular portion of human CD28 was purchased from Santa Cruz Biotechnology, PMA and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO).

**Luciferase assay**

Doxycycline-treated Jurkat T cells (0.25 × 10^6 cells/group) were stimulated for 4 h with 0.45 cells (ratio 2:1) preincubated overnight with different concentrations of SEE or with anti-CD3/CTLA-4 mAb-coated tosyl beads. mAb-coated beads were prepared with anti-CD3 mAb (1 μg/ml) and anti-CTLA-4 mAb (4 μg/ml) as previously described (6, 13). Beads were added to untreated or doxycycline-induced cells (ratio 1:1) in the presence of soluble anti-CD28 mAb (20 μg/ml; CD28.2; BD Pharmingen, Mississauga, Canada). Luciferase assay was performed using the Promega Luciferase Assay System (Promega, Madison, WI).

**Flow cytometry**

Doxycycline-treated Jurkat T cells (1 × 10^6 cells/group) were washed and stained for CTLA-4 expression using a PE-labeled mAb against human CTLA-4 (BD Pharmingen). Cells were examined by flow cytometry using a FACScan Flow Cytometer (BD Biosciences, Mountain View, CA). Statistical analyses were performed with CellQuest computer software (BD Biosciences).

**Confocal microscopy**

Doxycycline-treated Jurkat T cells (1 × 10^6 cells/group) were incubated at 37°C for 2 h on 35-mm polylysine-coated glass bottom microwell dishes (MatTek, Ashland, MA). APC (0.5 × 10^6) were stained for CTLA-4 expression using a PE-labeled mAb against human CTLA-4 (BD Pharmingen) and FITC-CD3 (BD Pharmingen) for 30 min each. Analysis was performed on a confocal microscope (Zeiss, Oberkochen, Germany) and analyzed by LSM 510 software (Zeiss and Microsoft, Redmond, WA).

**Results and Discussion**

To gain an understanding of the molecular mechanisms involved in CTLA-4–mediated T cell down-regulation, we undertook a yeast two-hybrid screen to identify putative proteins interacting with the cytoplasmic domain of CTLA-4. Because it was more likely that such proteins would be expressed in an activated T cell, we screened an activated Th1 T cell library with the cytoplasmic domain of mouse CTLA-4 fused to the DNA-binding domain of Lex-A as bait. Of the 2 million transformants screened, two clones interacted specifically when tested for nutritional selection and β-galactosidase activity (Fig. 1A). Both clones were identified as containing a cDNA insert spanning aa 392–589 of the C-terminal end of PP2AA.

To verify the specificity of this interaction, we determined the ability of the mouse 392–589 domain of PP2AA (mPP2AA [392–589]) to interact with CD28, a closely related molecule which shares extensive structural and sequence homologies with CTLA-4. As an additional control, we used the cytoplasmic tail of the newly discovered CD28 family member ICOS. cDNAs encoding the cytoplasmic domains of CD28 and ICOS were cloned in the bait vector, and used to retransform yeast cells along with mPP2AA (392–589). When assessed for growth on nutritionally selective media and β-galactosidase activity, we found that mPP2AA (392–589) did not interact with CD28 and ICOS (Fig. 1B), demonstrating that the mPP2AA (392–589) contained an interacting motif specific for the cytoplasmic domain of mouse
Inhibited. Positive interactions were assigned a score of 1/1001 control (lower panel on X-Gal containing plates. Plasmid vector with no insert was included as main of either CTLA-4, CD28, or ICOS. Transformed cells were streaked 392 – 589, and a plasmid encoding the GAL4bd fused to cytoplasmic do-

This observation in a mammalian cell system, verified the findings in the yeast system. Additionally, it suggested that the domain of mPP2AA encompassing aa 392–589 probably contains anchor residues that mediate binding to CTLA-4, but not its close homolog CD28.

To extend this observation to full-length PP2AA, we amplified this ubiquitously expressed protein from activated murine spleen cells. Recombinant mouse PP2AA migrated as a 61-kDa protein when expressed in H293K cells. To test for interaction of full-length PP2AA with CTLA-4, PP2AA was expressed as a myc-tagged protein and cotransfected with either HA-CTLA-4, HA-CD28, or HA-vector alone. Surprisingly, upon immunoprecipitating the lysates of H293K cells with anti-HA Ab, both PP2AA-CTLA-4 and PP2AA-CD28 immune complexes could be detected by Western blotting. (Fig. 2B). This was in marked contrast to the results observed with mPP2AA (392–589) domain in both yeast and H293K cells. Together, the yeast two-hybrid screen and the coimmunoprecipitation data in the H293K system indicated that the full-length mPP2AA can interact with both CD28 and CTLA-4. However, these molecules associate with PP2AA using distinct domains for interaction. Specifically, the domain containing residues 392–589 binds exclusively to CTLA-4, but residues 1–392 either alone or together with other residues associate with CD28.

Transient cell surface expression of CTLA-4 coupled with low levels of surface expression have been a major impediment in elucidating the molecular mechanisms involved in CTLA-4-mediated

FIGURE 1. Genetic evidence for the association of PP2AA with CTLA-4. A, The cytoplasmic domain of murine CTLA-4 was used to screen a cDNA library generated from a Th1 clone, using the Duplex A yeast two-hybrid system. Positive interaction was confirmed by expression of both the Leu2 and lacZ genes, thereby conferring ability of positive clones to grow on media lacking Leu2 and turn blue on media containing X-Gal. Of the 15 CTLA-4-specific clones identified, 2 encoded the regulatory subunit of murine PP2A. B, To establish the specificity of interaction, plasmids containing clones 54 and 48, identified as molecules interacting with cytoplasmic domain of CTLA-4 in yeast, were resolated from the library plasmid, pJG4-5. This was then retransformed into EGY4-8 along with the cytoplasmic domain of CTLA-4, CD28, and ICOS. Positive interaction was scored by the expression of Leu2 or lacZ on media containing Leu/Gal or Gal/X-Gal. The relative β-galactosidase activity is indicated. Positive interactions were assigned a score of 1+ (upper panels). Specific interaction of the C-terminal of PP2AA (aa 392–589) with the cytoplasmic tail of CTLA-4; yeast cells were cotransfected with plasmids encoding the GAL4bd fused to the library-derived fragment of PP2AA (aa 392–589), and a plasmid encoding the GAL4bd fused to cytoplasmic domain of either CTLA-4, CD28, or ICOS. Transformed cells were streaked on X-Gal containing plates. Plasmid vector with no insert was included as control (lower panel).

CTLA-4, only CTLA-4, but not CD28, was found to associate with mPP2AA (392–589) (Fig. 2A). The absence of interaction between mPP2AA (392–589) and CD28, even in a H293K expression system, verified the findings in the yeast system. Additionally, it suggested that the domain of mPP2AA encompassing aa 392–589 probably contains anchor residues that mediate binding to CTLA-4, but not its close homolog CD28.

To extend this observation to full-length PP2AA, we amplified this ubiquitously expressed protein from activated murine spleen cells. Recombinant mouse PP2AA migrated as a 61-kDa protein when expressed in H293K cells. To test for interaction of full-length PP2AA with CTLA-4, PP2AA was expressed as a myc-tagged protein and cotransfected with either HA-CTLA-4, HA-CD28, or HA-vector alone. Surprisingly, upon immunoprecipitating the lysates of H293K cells with anti-HA Ab, both PP2AA-CTLA-4 and PP2AA-CD28 immune complexes could be detected by Western blotting. (Fig. 2B). This was in marked contrast to the results observed with mPP2AA (392–589) domain in both yeast and H293K cells. Together, the yeast two-hybrid screen and the coimmunoprecipitation data in the H293K system indicated that the full-length mPP2AA can interact with both CD28 and CTLA-4. However, these molecules associate with PP2AA using distinct domains for interaction. Specifically, the domain containing residues 392–589 binds exclusively to CTLA-4, but residues 1–392 either alone or together with other residues associate with CD28.

Transient cell surface expression of CTLA-4 coupled with low levels of surface expression have been a major impediment in elucidating the molecular mechanisms involved in CTLA-4-mediated

FIGURE 2. Distinct domains on murine PP2AA mediate interaction with CD28 and CTLA-4 in H293K cells. A, Association of mPP2AA (392–589) with CTLA-4. H293K cells were transiently transfected with myc-tagged mPP2AA (392–589) and either HA-CTLA-4, HA-CD28, or HA-vector alone. HA-tagged proteins were immunoprecipitated and immunoblotting. (Fig. 2B). This was in marked contrast to the results observed with mPP2AA (392–589) domain in both yeast and H293K cells. Together, the yeast two-hybrid screen and the coimmunoprecipitation data in the H293K system indicated that the full-length mPP2AA can interact with both CD28 and CTLA-4. However, these molecules associate with PP2AA using distinct domains for interaction. Specifically, the domain containing residues 392–589 binds exclusively to CTLA-4, but residues 1–392 either alone or together with other residues associate with CD28.

Transient cell surface expression of CTLA-4 coupled with low levels of surface expression have been a major impediment in elucidating the molecular mechanisms involved in CTLA-4-mediated
T cell function. We circumvented this problem using a well-characterized system in which Jurkat cells are induced to express transfected CTLA-4 upon exposure to doxycycline (6, 13). We have used this system previously to elucidate the structure-function relationship of CTLA-4 and its role on T cell down-regulation, and thus it offered us an excellent tool to determine the functional relevance of the CTLA-4-PP2AA association. We first investigated whether Jurkat cells expressed PP2AA. We observed that resting, noninduced Jurkat cells expressed abundant PP2AA that migrated as a 65-kDa band on Western blotting (data not shown). Furthermore, the level of endogenous PP2AA expression remained constant even after doxycycline mediated induction of CTLA-4. Hence, we concluded that the levels of endogenous PP2AA are not affected by doxycycline induction of Jurkat cells.

Next, we sought to establish the association between PP2AA and CTLA-4 in transfected Jurkat cells. We found that immunoprecipitation of the 65-kDa regulatory subunit of PP2A coprecipitated a band with a size and blotting reactivity comparable to that of CTLA-4 (Fig. 3A). This band was absent in H293K cells, and in a HLA-DR-1⁺, B7⁺ lymphoblastoid B cell line used as APCs, as well as in immunoprecipitates of CTLA-4-expressing cells with a control goat antiserum against Dok-1 (Fig. 3A, lane labeled as IP: Ctrl Ab). To test whether PP2AA was associated with surface CTLA-4, we looked at the levels of CTLA-4 and PP2AA in biotinylated CTLA-4-expressing cells after immunoprecipitation with anti-biotin alone (Fig. 3B, 1st ip) or a sequential immunoprecipitation with anti-biotin and anti-MHC class I Ab as control Ab, or with anti-biotin and anti-CTLA-4 Abs. As shown in Fig. 3B, CTLA-4 was only detectable in cells induced with doxycycline either after immunoprecipitation with anti-biotin or after sequential immunoprecipitation with anti-biotin and anti-CTLA-4 Abs. A 36-kDa band of unknown identity was occasionally seen upon CTLA-4 blotting of these immunoprecipitates. Furthermore, as shown in Fig. 3C, PP2AA was detected after immunoprecipitation with anti-biotin alone, suggesting that PP2AA binds to different T cell surface molecules (Fig. 3C). Most importantly, we detected a small fraction of PP2AA specifically associated to CTLA-4, as implied by its presence after reprecipitation with mAb against CTLA-4 but not after reprecipitation with a mAb against MHC class I molecules (Fig. 3C).

**FIGURE 3.** The regulatory subunit of PP2A (PP2AA) associates with surface CTLA-4 in T cells. A, CTLA-4-transfected Jurkat T cells (40 x 10⁶ cells/group) plus APCs (30 x 10⁶ cells/group) were incubated with or without doxycycline (5 μg/ml) for induction of CTLA-4 expression. Subsequently, cell lysates were prepared and used for immunoprecipitation of PP2AA, followed by immunoblotting for CTLA-4 (upper panel) and PP2AA (bottom panel). A nonlymphoid cell line (H293K, 30 x 10⁶ cells/group), APC (0.45 cells; 30 x 10⁶ cells/group), and CTLA-4-transfected cell lysates immunoprecipitated with a goat antiserum against the cytosolic protein Dok-1 (IP: Ctrl Ab) were added as controls. Beads: immunoprecipitating Ab without cell lysate. Whole-cell lysates from the same samples were used for direct immunoblotting for CTLA-4 to confirm induction of CTLA-4 expression and for expression of PP2AA. B, Doxycycline-induced or noninduced CTLA-4-transfected cells (100 x 10⁶ cells/group) were biotinylated as indicated in Materials and Methods. Cell lysates were immunoprecipitated with anti-biotin alone (1st ip) or with anti-biotin followed by a second immunoprecipitation with anti-CTLA-4 Abs (2nd ip) and immunoblotted for CTLA-4. A control mAb against MHC class I molecules (Ctrl. lanes) was used for the second immunoprecipitation following anti-biotin ip. C, Doxycycline-induced or noninduced CTLA-4-transfected cells (100 x 10⁶ cells/group) were biotinylated as indicated in Materials and Methods. Cell lysates were immunoprecipitated with anti-biotin alone (1st ip) or with anti-biotin followed by a second immunoprecipitation with either a control Ab against MHC class I molecules or with anti-CTLA-4 Abs (2nd ip). These immunoprecipitates were then immunoblotted for PP2AA. Beads, immunoprecipitating Ab without cell lysate; CL, cell lysate.
Because parental Jurkat cells only express CD28 but not CTLA-4, we used these cells to establish the association of PP2AA with CD28 using B7.2 IgG fusion protein. Western blotting with anti-PP2AA Ab after immunoprecipitation with B7.2 IgG revealed that PP2A also bound to CD28. These data confirmed our findings obtained in the H293K system in that PP2AA associated with CD28 as well as CTLA-4 (Fig. 4A). The association between PP2A and CTLA-4 also involved the catalytic subunit of this phosphatase (PP2AC), consistent with reports indicating that free catalytic subunit of PP2A cannot be found in intact cells (20). As shown in Fig. 4B, we found that PP2AC coprecipitated with CTLA-4, indicating that both the regulatory and catalytic subunits of PP2A can interact with CTLA-4.

Next, we examined the effect of TCR-CTLA-4 coligation on the association between CTLA-4 and PP2AA. Because Jurkat cells express a TCR Vα1/Vβ8.1 Ag receptor complex, we tested the effects of TCR or TCR-CTLA-4 coligation using a system in which the superantigen SEE is presented by HLA-DR1-expressing, B7.1+ APC (21). As shown in Fig. 5A, coligation of the TCR and CTLA-4 with SEE and APC resulted in a time-dependent decrease in the levels of PP2AA associated with CTLA-4, while the total levels of PP2AA and PP2AC remained constant. Previous reports have implicated phosphorylation of the catalytic subunit of PP2A by several kinases including p56lck in the inactivation of PP2A (22). This prompted us to investigate whether TCR ligation resulted in the tyrosine phosphorylation of PP2A as well. We found that TCR ligation by SEE and APC caused an increase in tyrosine phosphorylation of the regulatory subunit of PP2A in a time-dependent fashion (Fig. 5B). The decrease in PP2AA-CTLA-4 association was not due to a loss of CTLA-4, because the levels of surface CTLA-4 increased with stimulation as predicted from previous characterization of the system (Fig. 5C). The precise kinetics and stoichiometry of the association between PP2AA and CTLA-4 is currently under investigation. It will be interesting to examine whether TCR internalization and/or decreased TCR-mediated signaling might be involved in the specific turnover of this association.

Our data are consistent with the possibility that TCR-dependent tyrosine phosphorylation of PP2AA may regulate the association of PP2A with CTLA-4 and subsequent effects of TCR-CTLA-4 coligation. Various studies have documented the importance of the PP2A holoenzyme as both a negative and positive regulator of cell growth and cell cycle progression proteins (23–25). Considering the fact that both PP2AA and CTLA-4 can exist in resting T cells, the association of PP2AA and CTLA-4 may be a mechanism by which the phosphatase prevents the inhibitory function of CTLA-4 before TCR-CTL-4 coligation. According to this model, activation and subsequent coligation of CTLA-4 and TCR could result in the tyrosine phosphorylation of both CTLA-4 and PP2AA, resulting in retention of CTLA-4 in the cell surface and the dissociation of CTLA-4 from PP2AA, respectively. Reversible phosphorylation of PP2A and its association with various intracellular molecules that regulate cell cycle progression have been previously reported (23, 26). Dissociation from PP2AA could then result in the restoration of CTLA-4 functional activity.

If the above model is correct, then a mutant CTLA-4 incapable of binding to PP2AA should be a better inhibitor of T cell function than the WT molecule. It has been reported that the sequence HKXXX in SV40 small T Ag and in the kinase domain of Casein kinase 2α is the sequence required for binding of these proteins to the PP2A core enzyme (23, 24). Based on this evidence, we looked for the presence of a similar sequence in the cytoplasmic tail of CTLA-4 and in those proteins known to form stable complexes with PP2A (25). We found that the cytoplasmic tail of CTLA-4 contained a K-rich motif, SKMLKKRSP, in the juxtamembrane portion of its cytoplasmatic tail. Such sequence meets a consensus also found in PP2A-binding proteins (23–25), XK/R/HXXK/R/HKXXX, and located within regions identified as important for binding to the regulatory subunit of PP2A (Ref. 27; Fig. 6A). The consensus sequence in CTLA-4 is located immediately upstream of tyrosine residue 165, which was shown to be critical for the binding between CTLA-4 and the catalytic subunit of PP2A in the yeast two-hybrid study reported by Thompson and colleagues (17). The consensus sequence requirements for binding of proteins to the PP2AA subunit or to the PP2AC subunit remain to be determined. However, the linear arrangement suggested by Chuang et al. (17) and our data may secure the association of both the catalytic and the regulatory subunits of PP2A with CTLA-4 (20). Of interest, we could not find any similar sequence in the cytoplasmatic tail of CD28, and this could explain the differential binding observed for PP2AA and PP2AC between CD28 and CTLA-4 (Ref. 17 and our data).

Based on the previous analysis, we generated a Jurkat T cell clone that expressed a mutant K-less CTLA-4 molecule lacking the three lysine residues in the juxtamembrane region (K152A/K155A/K156A). Upon induction with doxycycline, the K-less CTLA-4 mutant was found to be expressed at significantly lower levels than WT CTLA-4 (Fig. 6B). The cause for the lower levels of cell surface expression of this mutant molecule is currently under investigation. However, the K-less CTLA-4 molecules were able to colocalize with the TCR in a manner similar to that seen with WT CTLA-4 (Fig. 6C), suggesting that these molecules do not disturb the gross arrangement of the immunological synapse.

Next, we investigated whether the mutant K-less CTLA-4, which lacks the potential anchor residues that may mediate interaction with PP2AA, is still capable of forming CTLA-4-PP2A complexes. Because the level of CTLA-4 surface expression in the
K-less-transfected T cells is lower than in the WT CTLA-4-expressing cells, we sorted these cells by FACS to achieve similar levels of surface CTLA-4 expression. Jurkat cell lysates from these cells were used to immunoprecipitate PP2AA (Fig. 7). Unlike the WT CTLA-4, mutant K-less CTLA-4 failed to coimmunoprecipitate with PP2AA in significant amounts. This was not due to the inability or decreased reactivity of anti-CTLA-4 Abs used to detect mutant K-less CTLA-4. This finding confirmed our assumption that the lysine residues are indeed critical for binding of CTLA-4 to PP2AA. Furthermore, it offered us an opportunity to delineate the functional relevance of the CTLA-4-PP2A interaction in T cells.

To assess the functional effects of K-less CTLA-4 on T cell responses, Jurkat cells expressing WT CTLA-4 or mutant CTLA-4 were cotransfected with a luciferase reporter gene under the control of the IL-2 promoter and enhancer elements. Surprisingly, upon stimulation of doxycycline-induced Jurkat cells with SEE and APC, we observed that K-less CTLA-4 was far more efficient than WT CTLA-4 at inhibiting IL-2 gene transcription (Fig. 8, A and B). The enhanced inhibition of the K-less mutant was verified by comparing the percentages of inhibition at maximal relative luciferase unit response, to rule out intrinsic differences between the mutants ability to transcribe IL-2. In addition, stimulation with ionomycin and PMA showed similar responsiveness in both WT CTLA-4 and K-less CTLA-4 T cells (Fig. 8C). Specifically, luciferase activity was inhibited by 70–80% in K-less mutants compared with 35–55% by the WT CTLA-4 upon TCR-CTLA-4 coligation. This enhanced inhibition is particularly significant in the context of a much lower surface expression of K-less CTLA-4. Therefore, the lack of association between PP2AA and K-less CTLA-4 correlated with an enhanced inhibition of IL-2 gene transcription by CTLA-4, implying that PP2A could operate as a negative regulator of CTLA-4 function. This required the lysine residues at the juxtamembrane region of CTLA-4.

To compare the functional implications of PP2A interactions with CTLA-4 and CD28, we examined the response of WT CTLA-4-expressing T cells and K-less CTLA-4-expressing T cells after stimulation with anti-CD3 or anti-CD3/anti-CTLA-4-coated beads in the presence of soluble, nonlimiting concentrations of anti-CD28 mAb (6, 13). Jurkat T cells do not produce IL-2 in response to TCR ligation with anti-CD3-coated beads in the absence of anti-CD28 soluble (data not shown). Importantly, IL-2 response after TCR/CD28 ligation is not significantly different between WT
and K-less CTLA-4-expressing T cells in the presence or absence of doxycycline (Fig. 8D), ruling out any intrinsic difference in IL-2 responses due to CTLA-4 expression in the absence of its ligation. The effects of the association of PP2A with CTLA-4 were also validated in this system (Fig. 8D), although the enhanced function of K-less CTLA-4 over that of WT CTLA-4 using Ab-coated beads was less pronounced than with SEE/APC stimulation. This could be due to better inhibition of WT CTLA-4 upon forced coligation with the TCR. Furthermore, the inhibition of T cell activation by K-less CTLA-4 is due to negative signaling, because IL-2 gene transcription decreased after direct coligation of CD3 and CTLA-4 using mAb-coated beads (Fig. 8D).

**FIGURE 6.** A, Sequence conservation of a putative PP2A binding site on CTLA-4. The sequences of proteins reported to form complexes with PP2A were examined for the presence of K-based charged stretches. Bold residues are those conserved in different molecules arranged from more to less conservation of the three main residues of the motif. B, Expression of WT and K-less CTLA-4 on the cell surface upon doxycycline induction. WT ( ● ) or K-less ( ○ ) CTLA-4-transfected T cells were stained with an Ab against CTLA-4 in the presence of increasing concentrations of doxycycline for 18 h, and examined by flow cytometry (MFU, mean fluorescence units). C, WT and K-less CTLA-4 colocalize into the immunological synapse upon coligation with TCR. WT CTLA-4-expressing and K-less CTLA-4-expressing Jurkat T cell transfectants (1 × 10⁶) were induced with doxycycline and stimulated with 100 ng/ml of SEE and DR.1/B7-expressing APC for 30 min. Cells were double immunostained for CTLA-4 (red, top left panel) and CD3 (green, lower left panel) and analyzed by confocal microscopy. The two color panels were overlayed on the light field panel ( upper right panel ) to reveal points of overlap (yellow, lower right panel).

**FIGURE 7.** Loss of PP2AA binding by K-less CTLA-4 mutant. Doxycycline-treated WT CTLA-4 (0.1 μg/ml) and K-less CTLA-4 (5 μg/ml) transfected T cells were sorted by FACS to obtain similar levels of surface CTLA-4 expression ( left panel ). Whole-cell lysates from these cells (30 × 10⁶/8 cells/group) were prepared and used for immunoprecipitation of PP2A, followed by immunoblotting for CTLA-4 ( right panel ) and PP2AA ( bottom right panel ). Beads, immunoprecipitating Ab-coated beads without cell lysate.
Because PP2A plays a critical role in regulating diverse functions including cell proliferation, differentiation, and survival in different cell types (23–25), it is not difficult to envision a role for this molecule in regulating T cell-dependent immune responses. The recent identification of the catalytic subunit of PP2A as a potential modulator of the function of T cells by association with CD28 supports the concept that PP2A may be a major mediator in controlling T cell responsiveness, expansion, and homeostasis (17). Our study demonstrates an association of the regulatory subunit of PP2A with the cytoplasmic domain of both CTLA-4 and CD28. Sharing a 30% sequence within their cytoplasmic domains, both molecules have a consensus phosphatidylinositol 3-kinase src homology 2 domain-binding motif and proline-rich sequences (28), and can be targets for src kinases (17, 29, 30). Hence, the association of CTLA-4 and CD28 with PP2A positions these receptors as major regulators in kinase cascades modulating signaling events in T cell activation. Both PP2A and CTLA-4 have been shown to regulate the activity of extracellular signal-related kinase (ERK) in the mitogen-activated protein kinase pathway (13, 20, 31). Interestingly, whereas CTLA-4 coligation with the TCR results in the inhibition of ERK activation, TCR ligation induces ERK activation that can be prolonged by okadaic acid, an inhibitor of PP2A, suggesting a role for PP2A in the dephosphorylation of ERK. A plausible explanation could be that TCR-CTLA-4 coligation could result in the release of PP2A from the CTLA-4-PP2A complex, with the subsequent dephosphorylation of ERK by PP2A and down-regulation of mitogen-activated protein kinase-dependent T cell responses.

The ability of CTLA-4 to cocap with the TCR at the immunological synapse on receptor coligation suggests that a more elaborate mechanism might be operational. The cytoplasmic domain of CTLA-4 has been demonstrated to associate with SHP-2 (10) and the TCRζ chain (11). Thus, one may argue that SHP-2-mediated dephosphorylation of TCRζ may involve a CTLA-4-initiated translocation of signaling components, including PP2A, to the center of the immunological synapse that will prevent activation-induced serine-threonine phosphorylation of CD28. However, the fact that the K-less mutant can cocap with TCR just as well as the WT, but is more effective at inhibiting IL-2 in the face of lower association with PP2A, seems to suggest that this may not be the case. On the contrary, these findings imply that PP2A is indeed a negative regulator of CTLA-4 function. In light of the recent definition of the crystal structure of CTLA-4:B7 complex (32–34), it is tempting to speculate that the formation of supramolecular arrays of CTLA-4 may be favored upon dissociation of PP2A from CTLA-4, and thus facilitate the inhibition of TCR-mediated activation.

The precise nature of the interaction between PP2AA and the K-rich region of the CTLA-4 cytoplasmic tail remains to be defined. One could propose that electrostatic interactions maintain these two molecules together, as it has been reported for the association between Ly-49 with DAP-12 in NK cells (35–38). Co-ligation of TCR and CTLA-4 may favor electrostatic interactions between TCR and CTLA-4, following phosphorylation and dissociation of PP2A from CTLA-4. This hypothesis is further supported by recent data that also identify the first 7 aa of the CTLA-4 tail (and particularly the two distal lysine residues) as important for the proper interaction between CTLA-4 and TCRζ and subsequent inhibition of TCRζ-mediated signaling (K. Lee, M. Griffin, and J. A. Bluestone, unpublished observations). However, our data indicate that this region is important for the negative regulation of CTLA-4 function before TCR ligation and T cell activation. Because the occupancy of this domain by either PP2AA or TCR is dictated by the activation status of the cell, it is possible that the juxtamembrane region functions as an anchor for PP2A in resting cells “containing” T cell activation. In contrast, after T cell activation, the lysine-rich motif becomes available for TCR association and T cell down-regulation mediated by CTLA-4. This may explain why the lack of the three juxtamembrane lysine residues on the CTLA-4 tail enhance the function of CTLA-4 by causing a net increase in PP2AA-free CTLA-4 that can then interact with TCRζ, albeit in less kinetically favorable conditions.

The multilayered complexity of CTLA-4 function likely carries the advantage of a very precise regulation of its function, something not surprising to those studying the biology of this molecule. However, the definition of the lysine-rich motif in CTLA-4 as the binding site for PP2A provides an important tool for the search of small molecules that can interfere with the association of CTLA-4...
with PP2AA, and thus allows to target CTLA-4 for down-modulation of T cell-mediated immune responses.

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References