Interaction of the Cytoplasmic Tail of CTLA-4 (CD152) with a Clathrin-Associated Protein Is Negatively Regulated by Tyrosine Phosphorylation

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ABSTRACT: CTLA-4 (CD152), high-avidity receptor for CD80 and CD86, is a powerful regulator of T cell activation. While CTLA-4 functions at the cell surface, it is primarily localized in intracellular vesicles and cycles to the cell surface. The CTLA-4 cytoplasmic domain contains sequences that direct its intracellular localization and regulate its signaling. Here we demonstrate that effector molecules involved in receptor trafficking and signaling interact with distinct, but overlapping, sequences in the CTLA-4 cytoplasmic domain. Using the yeast two-hybrid method, we demonstrate association of the μ2 subunit of AP-2, the clathrin-associated complex found in plasma membrane-associated coated pits, with the cytoplasmic tail of CTLA-4, but not CD28. The μ1 subunit of AP-1, found in Golgi-associated coated pits, associated with neither CTLA-4 nor CD28. Sequences required for interaction of μ2 and CTLA-4 were localized to residues, ^{161}TTGVY in CTLA-4; this sequence is N-terminal to, but overlaps with, a previously identified SH2 binding motif, ^{165}YVKM, involved in CTLA-4 signaling. μ2 interacted preferentially with CTLA-4 when residue ^{165}Y was nonphosphorylated, whereas a PI3 kinase SH2 domain interacted preferentially when ^{165}Y was phosphorylated. In co-transfection experiments, both tyrosine residues in the cytoplasmic tail of CTLA-4 (^{165}Y and ^{182}Y) were phosphorylated by the T lymphocyte-associated tyrosine kinase, p56lck. Thus, phosphorylation of CTLA-4 residue ^{165}Y may reciprocally regulate signaling and trafficking of CTLA-4 by determining which effector molecules bind to its cytoplasmic tail.

T lymphocyte receptors CTLA-4 and CD28 are structurally homologous members of the immunoglobulin superfamily which share common ligands, the CD80 and CD86 molecules on antigen-presenting cells (*1*). CTLA-4 and CD28 are closely linked on chromosome 2 in humans, suggesting that they evolved from a common ancestral gene (*2*). Despite similarities, these receptors also differ in several important ways (*3*). CD28 expression is constitutive, while CTLA-4 is expressed only after T cell activation (*4*). CTLA-4 also binds CD80 and CD86 with several-hundred-fold higher avidity than CD28 (*5*). Moreover, mice deficient in these receptors have greatly different phenotypes: while CD28-deficient mice show mild immunosuppression (*6*), CTLA-4-deficient mice exhibit profound lymphoproliferation (*7, 8*). Taken together, these findings suggest that CTLA-4 and CD28 are not redundant but have distinct roles in the development and function of the immune system.

CTLA-4 and CD28 also have different subcellular localization. CD28 is found primarily at the cell surface, while the majority of CTLA-4 molecules are contained in intracellular Golgi or post-Golgi vesicles (*9*). CTLA-4 is expressed on the cell surface at very low levels in activated T cells and traffics between intracellular stores and the cell surface (*10*). CTLA-4 also localizes intracellularly in numerous transfected cell types (*9*). In transfected COS cells, CTLA-4 intracellular localization was regulated (*9*) by an 11-amino acid tyrosine-containing motif in its cytoplasmic tail, ^{161}TTGVYVKMPPT. Contained within this motif is the sequence, ^{165}YVKM, which binds the p85 subunit of PI-3 kinase (*11*) and has been implicated in CTLA-4 signaling (*11, 12*).

Thus, overlapping sequences in the CTLA-4 cytoplasmic tail control its subcellular localization and signaling. While the functional relationship between these processes remains unclear, the close proximity of cytoplasmic sequences regulating CTLA-4 trafficking and function (i.e., signaling) suggests that they are interrelated. We therefore hypothesized that an analysis of CTLA-4 trafficking will lead to new insights into CTLA-4 function.

The trafficking of integral membrane proteins is controlled by sorting sequences in their cytoplasmic domains. Typically, many of these sorting sequences contain a YXXØ motif, where Y is tyrosine, X is any amino acid, and Ø is a bulky hydrophobic residue (13, 14). While the molecular details of how these sequences regulate trafficking are not well-understood, recent evidence suggests that certain tyrosine-containing sorting sequences interact with clathrin-associated coated protein complexes (15−18). Ohno et al. (19) used the yeast two-hybrid method to demonstrate the interaction between the medium chains (μ1 and μ2) of two clathrin-associated protein complexes and a tyrosine-containing sorting sequence (SDYQRL) in the cytoplasmic tail of the Golgi-integral membrane protein TGN38. This and other sorting motifs which interact with the clathrin-associated μ1 and μ2 chains (19) are reminiscent of the motif which regulates the intracellular localization of CTLA-4 (9). We therefore tested whether the CTLA-4 cytoplasmic tail would

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interact with the clathrin-associated μ1 and μ2 chains. Here, we demonstrate interaction of CTLA-4 and μ2 using both a yeast two-hybrid system and a biochemical technique. We find that this interaction is negatively regulated by phosphorylation of residue ^{165}Y.

METHODS

Plasmid Construction

Recombinant yeast and mammalian expression plasmids were constructed by PCR. All plasmid constructions were verified by DNA sequencing.

lexA Fusions. These were constructed in plasmid pBTM116, provided by P. Bartel and S. Fields. PCR products encoding the desired cytoplasmic regions were digested with *Sal*I restriction enzyme and cloned into pBTM116. Yeast were transformed with recombinant plasmids, and lysates were prepared and tested by immunoblotting with anti-lexA antiserum to confirm the expression of lexA fusion proteins.

The pBTM116 fusions of CTLA-4 and CTLA-4 mutants T161A, T162A, G162A, V164D, and Y165F, were constructed by PCR amplification using mutant plasmids (*9*) as templates and the oligonucleotide primers ATACGTGACTAATGCACGTGGCCCAGCCTGCTGTG (forward) and ACGCGTCGACTCAATTGATGGGAATAAAATAAGGC (reverse). The resulting fusions contained amino acid residues ^{155}K to ^{187}N of CTLA-4.

The pBTM116 fusions of CD28 and CD28/CTLA-4 chimeras R1-R4 were generated using previously described plasmids (*9*) as templates and primers CGTCGACCTAGTAAGAGGAGCAGGCTCCTG (forward) and AGGTCGACGTCAGGAGCGATAGGCTGCGAAGTC (reverse). The CD28 fusions contained residues ^{163}S to ^{202}S of CD28. Chimera R6 (see Figure 2) was generated by PCR from the pBTM116-CD28 plasmid using the forward primer GTCGACCTAGTAAGAGGAGCAGGCTCCTGCACGGGGTCTATGTG and the reverse primer described above for the pBTM116-CD28 fusion.

The pBTM116 fusion of TGN46 was constructed using a PCR product corresponding to amino acids 387−418 of TGN46 (accession number X94333), generated by reverse transcriptase-coupled PCR (RT-PCR) from human spleen RNA (Clontech, Palo Alto, CA), and primers GAATTCAAGCGGAAGATCATTGCTTTTGTC (forward) and GTCGACTTAGGACTTCTGGTCCAAACGTTG (reverse).

VP16 Fusions. These were constructed in plasmid pVP16 (*20*) which was modified to contain additional cloning sites, creating plasmid pVP16.1. PCR products encoding μ1 or μ2 were cloned into this plasmid such that they were fused in frame with VP16.

Human μ1 cDNA was generated by RT-PCR using fetal brain RNA (Clontech, Palo Alto, CA) as template and primers GAATTCATGTCCGCCAGCGCCGTCTACGT (forward) and CTCGAGCTACTGGGTCCGGAGCTGGTAATC (reverse). These primers correspond to the beginning and end of the murine μ1 cDNA sequence (*21*). The cDNA product amplified from human cDNA with these primers encoded a protein that differed from murine μ1 by three amino acids: ^{178}S in the human sequence replaced the corresponding murine residue ^{178}A, ^{295}I replaced ^{295}V, and ^{345}I replaced ^{345}V. This cDNA product was used as the human homologue of murine μ1.

A cDNA encoding human μ2 was generated by RT-PCR using human spleen RNA (Clontech, Palo Alto, CA) as template and primers GGTACCATGATTGGAGGCTTATTCATC (forward) and CTCGAGCTAGCAGCGAGTTTCATAAATGCC (reverse). The protein encoded by this PCR product showed two differences from the published human μ2 sequence: ^{116}L in the published sequence was converted to ^{116}V, which is also found at that position in murine μ2 (*19*), and residues 142 and 143 were deleted. We have observed this deletion in four of nine independent μ2 cDNA clones isolated from human spleen RNA, one of six from thymus RNA, and zero of six from human brain RNA. Human μ2 clones with or without this deletion showed no differences in their ability to interact with CTLA-4; thus, these two amino acids were not required for interaction of CTLA-4 and μ2. Deletion of these residues has also been described for murine μ2 (*19*).

pCDNA1 Expression Plasmids. Mammalian expression plasmids pCDNA1-CTLA-4 and pCDNA1-Y165F were described previously (*9*). pCDNA1-Y182F was amplified by PCR using pCDNA1-CTLA-4 as template and primers CACTGCTTACTGGCTTATCGA (forward) and TCTAGATCAATTGATGGGAATAAAGAAAGGCT (reverse). pCDNA1−Y165F/Y182F was amplified using the same primers and pCDNA1-Y165F as template. An SRα-based plasmid encoding full length p56lck was provided by Dr. Chris Rudd. The insert of this plasmid was subcloned in to the pCDNA1 vector.

Yeast Culture and Methods. Standard yeast media were used for cell growth (*22*). Strain L40 (*20*) was used for two-hybrid analysis. Transformants bearing pBTM116 and pVP16.1 plasmids were selected using standard genetic methods and maintained in synthetic medium lacking leucine and tryptophan. For quantitative β-galactosidase assays, transformants were grown to midlog phase in selective medium. β-Galactosidase activity was assayed on lysates prepared from three independent transformants for each strain and expressed in Miller units (*23*). Values represent the mean ± SD in mg/mL per min.

In Vitro CTLA-4/μ2 Binding Assay. ^{35}S-Labeled μ2 was prepared by in vitro translation of μ2 mRNA. A full-length μ2 cDNA was cloned into pCDNA1 (Invitrogen, San Diego, CA); the resulting plasmid was then linearized with *Xho*I. Capped μ2 RNA was generated using T7 Cap-Scribe (Boehringer Mannheim, Indianapolis, IN). Radiolabeled μ2 was generated from translation of 10 μg of capped μ2 RNA using a rabbit reticulocyte lysate kit (Boehringer Mannheim, Indianapolis, IN) and Trans S-35 Label (ICN Biochemicals, Irvine, CA). Binding of ^{35}S-labeled-μ2 to CTLA-4 was measured by precipitation analysis using CTLA-4 peptide-coupled beads. CTLA-4 peptides corresponding to amino acids 155−174 (containing an addition N-terminal cysteine residue) were synthesized using solid phase methods by Dr. James Blake of our institution. Two peptides were made, one having native ^{165}Y and the other having tyrosine-phosphorylated ^{165}Y. Peptides were coupled to SulfoLink beads (Pierce, Rockford, IL) via the N-terminal cysteine and used for precipitation analysis. ^{35}S-Labeled μ2 protein was incubated with peptide-coupled beads in binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin) for 16 h at 4 °C. Beads were then collected by centrifugation and washed, and bound material was fractionated by SDS−PAGE on

precast acrylamide gels (Novex, San Diego, CA). Gels were fixed, soaked in Amplify (Amersham, Arlington Heights, IL), dried, and exposed to X-ray film (Kodak XAR-5). Peptide-conjugated beads were also used to precipitate a GST fusion of the SH2 domains of the p85 subunit of PI3 kinase (GST-PI3K), kindly provided by James Esselstyn (*24*). Peptide-conjugated beads were incubated with GST-PI3K fusion protein, collected, washed, and subjected to SDS−PAGE analysis. Gels were blotted to PVDF membranes, which were then incubated with monoclonal anti-PI3 kinase mAb (Transduction Laboratories, Lexington, KY). Binding of the anti-PI3 kinase mAb was detected using horseradish peroxidase (HRP)-conjugated goat-anti-mouse immunoglobulin antibody (BioSource International, Camarillo, CA), followed by the ECL reaction (Amersham, Arlington Heights, IL) and exposure to X-ray film. In some experiments, beads conjugated with tyrosine-phosphorylated CTLA-4 peptide were incubated with a GST-CD45 fusion protein (*25*) prior to their use for precipitation analysis.

Transfection Assay

COS cells were transfected with pCDNA1 vectors encoding CD28, or wild type or mutant forms of CTLA-4. Forty eight hours post-transfection, cells were incubated with or without the tyrosine phosphatase inhibitor pV(phen) (25 μM) (*26*) for 2 h at 37 °C. Equal numbers of cells were then lysed in RIPA buffer (1% NP-40, 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Extracts were precleared with immobilized protein G-sepharose beads and incubated with anti-CTLA-4 mAb 11D4 (6 μg/mL) (*4*). Antigen−antibody complexes were collected by centrifugation and immunoprecipitates were analyzed by SDS−PAGE run under nonreducing conditions. Gels were blotted to PVDF membranes, which were then reacted with alkaline-phosphatase-conjugated anti-phosphotyrosine (p-Tyr) mAb, RC20AP (Transduction Labs, Lexington, KY). Anti-p-Tyr reactions were detected by chemiluminescence using the Tropix Western Light Kit (Tropix, Bedford, MA). The membrane was then stripped and reprobed with rabbit anti-CTLA-4 antiserum. This antiserum was raised by immunizing rabbits with recombinant extracellular domain of CTLA-4 (CTLA-4tp, (*5*). Binding of this antiserum was detected with HRP-conjugated goat-anti-rabbit immunoglobulin antibody (BioSource International, Camarillo, CA), followed by the ECL reaction (Amersham, Arlington Heights, IL). The anti-p56lck mAb (clone 28, Transduction Labs, Lexington, KY) was detected using HRP-conjugated goat-anti-mouse immunoglobulin antibody (BioSource International, Camarillo, CA).

YT Cell CTLA-4 mAb Uptake Assay

cDNAs encoding full-length CTLA-4 or mutant Y165F were cloned into the retroviral vector pLXSN (*27*). Constructs were introduced into the packaging cell line ψ2 using Lipofectin (Life Technologies, Gaithersburg, MD). Recombinant retroviruses were amplified in the packaging line PA317 (*28*). YT cells (*29*) were infected with recombinant retroviruses for 4 h in the presence of 4 μg/mL polybrene (Aldrich, St. Louis, MO). After 18 h, transduced cells were selected with G418 (1 mg/mL). The amount of CTLA-4 expressed by each cell line was measured using a double determinant immunossay for CTLA-4 standardized with recombinant CTLA4Ig (*4*). The uptake of BODIPY-labeled anti-CTLA-4 mAb 11D4 was measured as described (*10*).

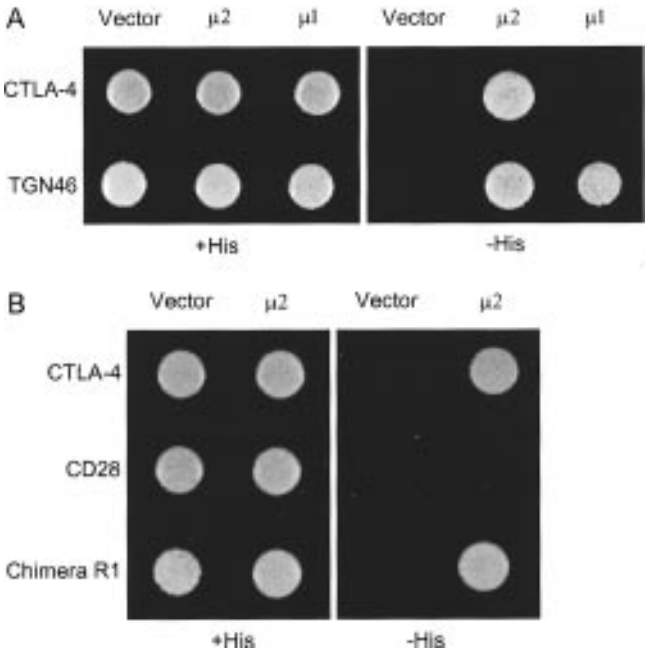

FIGURE 1: Interaction of CTLA-4 and the clathrin-associated protein μ2. Yeast strains containing pBTM116-derived (bait) plasmids and VP16.1-derived (prey) plasmids were assayed for activation of the lexA-dependent HIS3 reporter gene, by growth in histidine-deficient medium. (A) Interaction of CTLA-4 and μ2. Bait plasmids encoding the cytoplasmic regions of CTLA-4 or TGN46 fused to *lex*A were assayed for interaction with VP16.1-derived prey plasmids (empty vector, VP16.1−μ1, or VP16.1−μ2. For assay of *HIS3* expression, equal numbers of cells for each strain were spotted onto agar plates in the presence (+His) or absence (−His) of histidine. The histidine-deficient plates were supplemented with 3-aminotriazole (50 mM). Plates were maintained at 30 °C for 2−3 days before inspection for cell growth. (B) CD28 does not interact with μ2. Strains containing bait (pBTM116-derived) plasmids encoding the cytoplasmic regions of CTLA-4, CD28, or CTLA-4/CD28 chimera R1 fused to *lex*A were assayed for interaction with VP16.1-derived prey plasmids, as in A.

RESULTS

Two-Hybrid Approach Demonstrates the Interaction of CTLA-4 and μ2. To extend the studies of Ohno et al. to CTLA-4, we first needed to show that clathrin-associated protein μ1 and/or μ2 is expressed in human T cells. Since antibodies to these proteins are not readily available, we instead tested for transcripts for these proteins in primary human T cells and T cell lines. Both transcripts were detected in Jurkat cells using PCR analysis, and μ2 transcripts were detected by RNA blot analysis in activated human $CD4^+$ T cells. Thus, human T cells express μ1 and μ2.

We then tested whether CTLA-4 interacts with μ1 and μ2 clathrin-associated proteins using the yeast two-hybrid method (*30*). We constructed a bait plasmid encoding a genetic fusion of the CTLA-4 cytoplasmic tail and the lexA repressor, and prey plasmids encoding μ1 and μ2 fusions with the transcriptional transactivator, VP16. Plasmids were introduced into yeast, and strains bearing both plasmids were tested for activation of *HIS*3 and *LacZ* reporter genes. In strains containing plasmids encoding the CTLA-4 cytoplasmic tail and the clathrin-associated protein μ2, both reporter genes were activated, indicating interaction between these proteins (Figure 1A and Table 1). In contrast, no interaction

Table 1: Interaction of CTLA-4 and the Clathrin-Associated Protein μ2 As Detected by β-Galactosidase Activity[a]

	β-galactosidase activity (Miller units)		
	vector	μ2	μ1
CTLA-4	4.2 ± 0.1	300 ± 33	2.3 ± 0.5
CD28	4.0 ± 0.5	3.9 ± 0.1	nd[b]
chimera R1	4.6 ± 1.4	440 ± 30	nd
TGN46	3.5 ± 1.0	62 ± 34	3.6 ± 0.3

[a] Yeast strains containing pBTM116-derived (bait) plasmids and VP16.1-derived (prey) plasmids described in Figure 1 were assayed for activation of the lexA-dependent βGAL reporter gene as described in Materials and Methods. [b] Not detected.

between CTLA-4 and μ1 was detectable. In the experiment shown in Figure 1A, both μ1 and μ2 interacted with the Golgi protein, TGN46, as reported for its murine homologue, TGN38 (*19*). However, in contrast to this previous report (*19*), the interaction of μ1 with TGN46 was less robust than the interaction of μ2 and TGN46. Only a fraction of μ1-containing strains (9 of 25 independent isolates) showed any growth on histidine-deficient medium and those strains that grew showed little β-galactosidase activity (Table 1). This occasionally observed growth may be the consequence of leakiness of the *HIS*3 reporter gene, which would sustain growth in the absence of a meaningful two hybrid interaction.

Although the CD28 cytoplasmic tail also contains a YXXØ motif, it did not interact with μ2 (Figure 1B). However, the CTLA-4/CD28 chimera, R1, did interact with μ2 (Figure 1B). In this chimera, residues 161−171 of CTLA-4 (Figure 2A) were inserted into the corresponding region of CD28 (residues 169−179) (chimeric sequence shown in Figure 2B). This region was previously demonstrated to confer intracellular localization to CD28 chimeras (*9*). These data indicate that sequences responsible for the interaction of CTLA-4 and μ2 were contained within this 11-amino acid region from CTLA-4.

To further define the residues in CTLA-4 critical for interaction with μ2, plasmids containing different CTLA-4/CD28 chimeras and a series of site-specific mutants of CTLA-4 were prepared and tested in the two-hybrid assay. These results suggested that residues ^{161}T−^{165}Y were minimally essential for the interaction of CTLA-4 and μ2. As shown in Figure 2, chimeras R2 and R3 interacted with μ2, whereas R4 and R6 did not. The difference in β-galactosidase activity obtained with R2 and R3 (Figure 2C) indicated that CTLA-4 residues ^{166}V and ^{167}K were required for maximal interaction with μ2. Site-specific mutagenesis indicated that residues ^{163}G, ^{164}V, and ^{165}Y were also critical for this interaction. Mutation of residues ^{161}T and ^{162}T did not eliminate the interaction of CTLA-4 and μ2, although substitution at ^{162}T reduced the strength of the interaction, as judged by β-galactosidase activity. Thus, ^{162}T may enhance the interaction of CTLA-4 and μ2, although it is not required. This conclusion in supported by results with the R6 chimera, which contains both ^{163}G and ^{164}V, but does not interact with μ2. Taken together, these data suggest that the site in CTLA-4 which interacts with μ2 is defined by residues ^{161}TTGVY.

The relationship between cell surface expression of different mutant forms of CTLA-4 and the strength of their interaction with μ2, as judged by β-galactosidase activity, was examined in Figure 2D. COS cells were transfected with different mutant forms of CTLA-4, cell surface expression was measured by indirect immunofluorescence and flow cytometry, and mean fluorescence intensity (MFI) values were recorded. These values were then normalized for differences in expression levels by dividing by the total amount of mutant or wild type CTLA-4 expressed on a per cell basis, as determined by a capture immunoassay (*4*). Normalized cell surface expression values for each mutant protein (i.e., the ratio of cell surface MFI to total CTLA-4) were then plotted versus the levels of β-galactosidase activity that mutant protein produced in a two-hybrid assay. As shown in Figure 2D, this analysis showed that there was a negative correlation between cell surface expression of CTLA-4 mutants and their ability to bind μ2, suggesting a role for μ2 in retaining CTLA-4 in an intracellular compartment(s).

Biochemical Demonstration of CTLA-4 Interaction with μ2. To confirm biochemically the interaction of CTLA-4 and μ2, we tested whether μ2, which was synthesized and radiolabeled with ^{35}S by in vitro translation, could be precipitated with beads conjugated with a peptide corresponding to residues 155−174 from the CTLA-4 cytoplasmic tail. As shown in Figure 3A, beads conjugated with CTLA-4 peptide precipitated more μ2 than did control beads having no peptide attached (Figure 3A, compare lanes 1 and 2). We also used this technique to test whether phosphorylation of ^{165}Y in CTLA-4 was important for the interaction with μ2. Beads conjugated with peptide 155−174 having phosphorylated ^{165}Y were unable to precipitate μ2 (Figure 3A, lane 3). Treatment of phosphorylated peptide 155−174 with soluble CD45 phosphatase restored the capacity of the peptide to bind μ2 (lane 4), suggesting that the failure of phosphorylated 155−174 to bind was due to its phosphorylation. This conclusion was strengthened by control experiments which showed that phosphorylated CTLA-4 cytoplasmic tail peptide was an efficient substrate for soluble CD45 phosphatase (data not shown). Thus, these data support the conclusion from the two-hybrid data that CTLA-4 residues 155−174 contain a binding site for μ2.

Residues 155−174 in CTLA-4 have also been reported to contain a binding site(s) for SH2 domains of effector molecules involved in signaling (*11, 12*). To compare this binding with that of μ2, the same peptide-conjugated beads used in Figure 3A were tested for their ability to precipitate SH2 domains from the P85 subunit of PI3 kinase. In contrast to μ2, GST-PI3K fusion was precipitated only with beads conjugated with phosphorylated peptide 155−174 (Figure 3B, lane 3). This was in agreement with previous findings that binding of these SH2 domains requires tyrosine phosphorylation (*31*). Thus, phosphorylation of ^{165}Y enhanced binding of PI3 kinase SH2 domains, but prevented binding of μ2 to peptide 155−174.

Tyrosine Phosphorylation of ^{165}Y in Vivo. The results shown in Figure 3 suggested that phosphorylation of ^{165}Y may determine which of two different effector molecules binds to CTLA-4 peptide 155−174. For this to be an important regulatory mechanism, ^{165}Y must be phosphorylated *in vivo*. While this paper was in preparation, Shiratori et al reported similar findings to ours on the interaction of CTLA-4 and μ2 (*32*). These authors also showed that CTLA-4 was tyrosine phosphorylated in activated murine T cells and suggested that this event controlled internalization of CTLA-4 by regulating its interaction with μ2. However, these authors did not identify which of two tyrosine residues

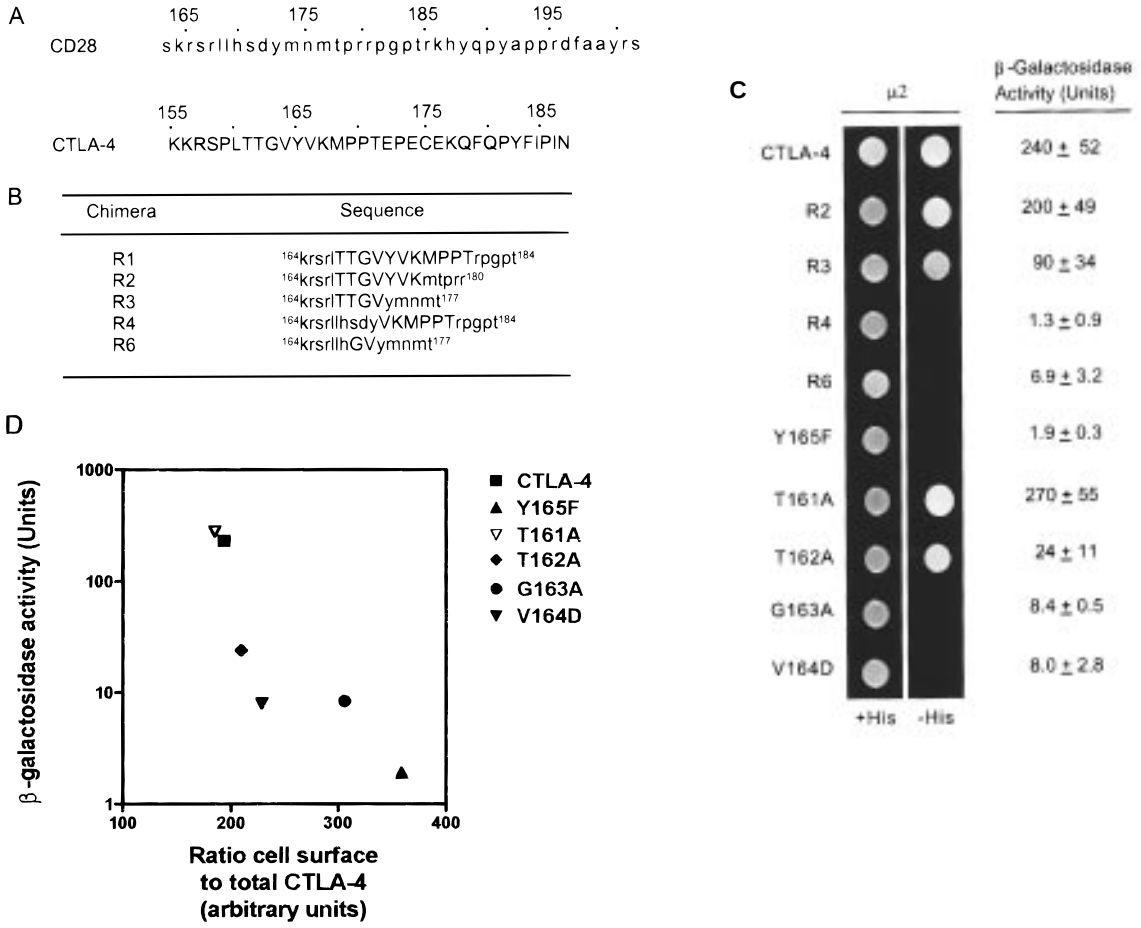

FIGURE 2: Determination of residues of CTLA-4 required for interaction with μ2. (A) Amino acid sequences of the cytoplasmic regions of CD28 and CTLA-4. Sequences shown are from human CTLA-4 (accession L15006) and CD28 (accession J02988). (B) Amino acid sequences of CTLA-4/CD28 chimeras. Shown are the sequences of CTLA-4/CD28 cytoplasmic tail chimeras (*9*). Upper case letters denote residues of CTLA-4 and lower case letters, CD28. (C) Definition of residues in CTLA-4 required for interaction with μ2. Bait plasmids encoding *lex*A fusions with the cytoplasmic regions of CTLA-4, the CTLA-4/CD28 chimeras, or various single amino acid substitutions in the cytoplasmic region of CTLA-4 were assayed for interaction with μ2 by growth in histidine-deficient medium, as described in Figure 1, or by β-galactosidase activity, as described in Table 1. (D) Correlation between CTLA-4 cell surface expression and μ2 binding. COS cells were transfected with different mutant forms of CTLA-4, and cell surface expression of CTLA-4 was determined by indirect immunofluorescence and flow cytometry following staining with the 11D4 mAb (*9*). Mean fluorescence intensity (MFI) values were recorded and corrected for nonspecific staining by subtracting values obtained with an isotype control mAb (MFI < 2% of maximal values). Corrected MFI values were then normalized by dividing by the amount of total CTLA-4 expressed per cell, as determined by a capture immunoassay (*4*). Normalized cell surface expression values (i.e., the ratio of cell surface MFI to total CTLA-4) were then plotted versus the β-galactosidase activity from Figure 2C.

in the cytoplasmic tail of CTLA-4 was phosphorylated, nor did they identify T-cell-associated kinase(s) which could mediate this phosphorylation. To further investigate these aspects of tyrosine phosphorylation of CTLA-4, we performed cotransfection experiments in COS cells (Figure 4).

COS cells were cotransfected with plasmids encoding wild type CTLA-4 or mutants containing tyrosine-to-phenylalanine substitutions, together with a plasmid encoding the T lymphocyte kinase, p56lck. Transfected cells were then lysed, and CTLA-4 was tested for phosphorylation on tyrosine residues by immunoprecipitation and immunoblotting. As shown in Figure 4, CTLA-4 was phosphorylated on tyrosine when co-transfected with p56lck. Moreover, mutant forms of CTLA-4 containing Y165F or Y182F substitutions were also phosphorylated. Mutant Y165F was phosphorylated more than Y182F, indicating that ^{182}Y was a favored substrate for p56lck in COS cells. Phosphorylation was not detected in the mutant containing both tyrosine substitutions. Thus, both cytoplasmic tyrosine residues in CTLA-4 may be phosphorylated *in vivo* by T-cell-associated kinase, p56lck.

Functional Role of ^{165}Y in Internalization of Anti-CTLA-4 mAbs. Residue ^{165}Y was required for binding of clathrin-associated protein μ2 (Figures 3 and 4), suggesting a role for this residue in intracellular trafficking of CTLA-4. To support this possibility, we next tested the role of ^{165}Y in CTLA-4 trafficking, by measuring the uptake of anti-CTLA-4 mAb. We previously showed that incubation of CTLA-4-positive cells with BODIPY-conjugated anti-CTLA-4 mAb resulted in the continuous and time-dependent uptake of labeled anti-CTLA-4 mAb; the amount of mAb which accumulated intracellularly was much greater than that bound to the cell surface (*10*). These observations suggested that CTLA-4 cycles continuously to and from the cell surface.

We therefore transduced YT cells, a CTLA-4 negative NK cell-like line (*29*), with retroviral vectors encoding either wild type CTLA-4 or the mutant Y165F. We then tested transduced cells for their ability to take up BODIPY-conjugated anti-CTLA-4 mAb (Figure 5). The amounts of CTLA-4 per cell were determined using a capture immunoassay (*4*), standardized with recombinant CTLA-4Ig. CTLA-4-transduced YT cells contained

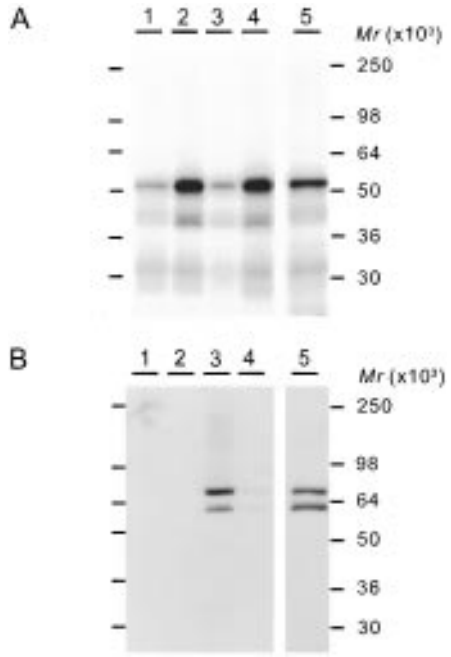

FIGURE 3: Phosphorylation of tyrosine 165 in CTLA-4 prevents the interaction with μ2. (A) Precipitation of in vitro translated μ2 by immobilized CTLA-4 cytoplasmic tail peptides. Peptides corresponding to amino acids 155−174 from CTLA-4 were conjugated to SulfoLink beads and incubated for 16 h with [^{35}S]-methionine-labeled, *in vitro* synthesized μ2. Beads were then collected by sedimentation, washed, and bound material was analyzed by SDS−PAGE (10% acrylamide): lane 1, precipitation with unconjugated beads; lane 2, beads conjugated with CTLA-4 peptide; lane 3, beads conjugated with CTLA-4 peptide phosphorylated at ^{165}Y; lane 4, beads as in lane 3, but treated with soluble CD45 phosphatase before incubation with μ2; lane 5, sample of *in vitro* synthesized μ2 (1% of that incubated with beads in lanes 1−4). (B) Precipitation of GST-PI3K-fusion protein by immobilized CTLA-4 cytoplasmic tail peptides. Peptide-conjugated beads from panel A were tested for their abilities to precipitate a GST-PI3K fusion protein. Beads were incubated for 16 h with GST-PI3K, collected by centrifugation, and washed, and bound material was analyzed by SDS−PAGE (10% acrylamide), followed by immunoblotting analysis. PI3 kinase SH2 domains were detected using an anti-PI3 kinase mAb. Lane 1, precipitation with unconjugated beads; lane 2, beads conjugated with CTLA-4 peptide; lane 3, beads conjugated with CTLA-4 peptide phosphorylated at ^{165}Y; lane 4, beads as in lane 3, but treated with soluble CD45 phosphatase before incubation with μ2; lane 5, sample of GST-PI3K (100 ng). Two bands reactive with anti-PI3K mAb were precipitated with beads conjugated with CTLA-4 peptide phosphorylated at ^{165}Y; the upper band corresponds to bona fide GST-PI3K, whereas the other probably represents a proteolytic cleavage product.

∼270 molecules per cell of CTLA-4, whereas Y165F-transduced YTcells contained ∼180 molecules per cell of mutant Y165F.

When these YT cells were incubated with BODIPY-conjugated anti-CTLA-4 mAb, Y165F-transduced cells showed less mAb uptake than CTLA-4-transduced cells. Plots of mean fluorescence intensity versus time for different transduced cell lines yielded linear rates of mAb uptake for CTLA-4- and Y165F-transduced cells (Figure 5). The rate of mAb uptake (determined from the slope of these plots) was ∼3-fold less for Y165F-transduced cells than for CTLA-4-transduced cells (0.0057 fluorescence units/min for Y165F-transduced cells versus 0.0165 fluorescence units/min for wild type CTLA-4−transduced cells). When normalized for differences in the number of CTLA-4 molecules per cell (i.e., Y165F-transduced cells contained ca. two-thirds of the

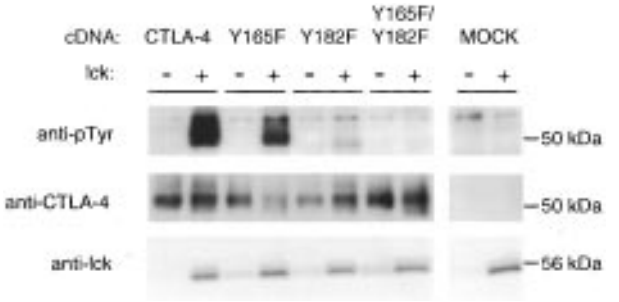

FIGURE 4: Phosphorylation of CTLA-4 on residues ^{165}Y and ^{182}Y. COS cells were cotransfected with expression plasmids encoding CTLA-4, tyrosine-to-phenylalanine mutants Y165F, Y182F, or Y165F/Y182F, and p56 lck. Detergent extracts of cells were then immunoprecitated with anti-CTLA-4 mAb. Immunoprecipitates were fractionated by SDS−PAGE on 6−18% acrylamide gradient gels run under nonreducing conditions and analyzed by immunoblotting analysis. The upper panel shows the membrane after reaction with anti p-Tyr antibody, the middle panel shows the same membrane after stripping and reprobing with a rabbit anti-CTLA-4 antiserum, and the lower panel shows the same membrane after probing with anti-p56lck mAb.

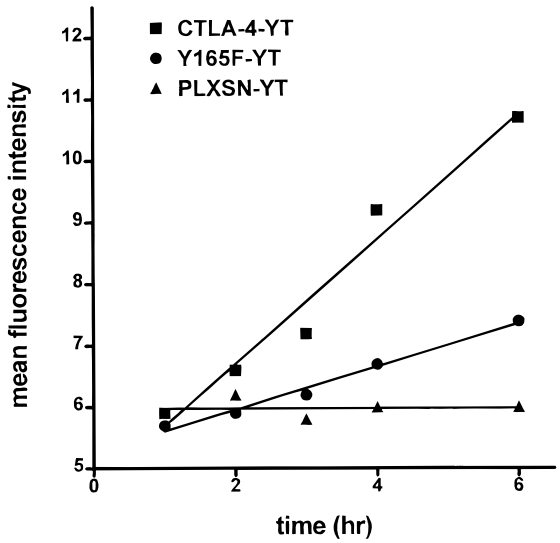

FIGURE 5: Tyrosine 165 is required for efficient uptake of anti-CTLA-4 mAb. YT cells were transduced with the retroviral vector PLXSN (triangles), the same vector encoding CTLA-4 (squares), or mutant Y165F (circles). Transduced YT cells were then incubated with BODIPY-conjugated anti-CTLA-4 mAb 11D4 at 37 °C for the indicated periods of time and washed, and cell-associated fluorescence was measured by flow cytometry. Shown are the mean fluorescence intensity values (arbitrary units) and linear regression lines calculated from these values. The mean fluorescence intensity of YT cells incubated with a BODIPY-conjugated control mAb (52.4) was 5.3 ± 0.7.

number of CTLA-4 molecules as wild-type CTLA-4 transduced cells), Y165F mediated (on a per molecule basis) ca. half of the rate of mAb uptake as wild-type CTLA-4. Thus, mutation of ^{165}Y reduced the rate of mAb uptake, indicating that this residue is important for intracellular trafficking of CTLA-4.

DISCUSSION

We have demonstrated an interaction between the cytoplasmic domain of CTLA-4 and the clathrin-associated protein, μ2. The crucial region for the interaction overlapped with a YXXØ motif, similar to the sequences shown by Ohno et al. (*19*) to interact with μ1 and μ2. Thus, our data support those of Ohno et al. in demonstrating interaction of tyrosine-

based sorting signals and clathrin-associated proteins. Also in agreement with these authors, we demonstrate the requirement of a tyrosine residue (i.e. ^{165}Y) in YXXØ sorting signals for association with μ2.

However, our data differ from those of Ohno et al (*19*) in two important ways. First, we showed that CTLA-4 interacted only with μ2 and not μ1. This was also shown by Shiratori et al. (*32*) in a paper published while ours was under preparation. In contrast, TGN46, interacted with both μ1 and μ2, in agreement with previous findings (*19*). Thus, CTLA-4 shows more specificity than TGN46 in which type of clathrin-associated molecule it interacted with. Second, we show that residues other than the YXXØ motif are required for interaction of CTLA-4 and μ2.

Ohno et al. (*19*) demonstrated interaction of μ1 and μ2 with YXXØ motifs presented either as triple repeats or singly as fusions with other sequences. This suggested direct interaction of μ1 or μ2 with the YXXØ motif. Here we showed that a YXXØ motif in CTLA-4 interacted with μ2, whereas the homologous motif in CD28 did not. Thus, a YXXØ motif was necessary but not sufficient for interaction with μ2. Transferring the sequence ^{161}TTGVYVKMPPT from CTLA-4 to the corresponding region of CD28 (R1 chimera) conferred the ability to interact with μ2. Furthermore, the R3 chimera (containing residues ^{161}TTGVY from CTLA-4) interacted with μ2 at a reduced level, relative to the R1 chimera. These data indicate that residues ^{161}TTGV, N-terminal to the YXXØ motif, are required for interaction with μ2 when these residues are presented in the context of the CD28 cytoplasmic tail.

Intracellular localization of CTLA-4 may in theory be achieved by either (a) intracellular retention; or (b) rapid internalization of cell surface-expressed molecules. The internalization and intracellular accumulation of anti-CTLA-4 mAbs support the latter possibility (see Figure 5). The binding of μ2 to CTLA-4 suggests a plausible mechanism to achieve such rapid internalization. We therefore propose that binding of μ2 to CTLA-4 triggers its rapid internalization from the cell surface and subsequent intracellular localization.

The interaction of CTLA-4 and μ2 is dependent upon whether ^{165}Y is phosphorylated. When ^{165}Y is not phosphorylated, μ2 can bind CTLA-4, whereas when ^{165}Y is phosphorylated, no binding was detected. This contrasts with the interaction of YXXØ motifs with SH2 domains, where tyrosine phosphorylation is required for interactions (*31*). Therefore, tyrosine phosphorylation of ^{165}Y motif may regulate whether the CTLA-4 cytoplasmic tail binds SH2 domain(s) or clathrin-associated molecules.

Does ^{165}Y become phosphorylated under physiological conditions? Signaling studies have suggested that phosphorylation of ^{165}Y does occur during T cell activation (*11, 12*), but this has not been directly demonstrated. It was also unknown which, if any, T-cell-associated tyrosine kinases has activity toward the CTLA-4 cytoplasmic tail. We showed here that ^{165}Y is phosphorylated in COS cells cotransfected with the T-cell-associated kinase p56lck. ^{182}Y was a preferred substrate for p56lck, but ^{165}Y was clearly also phosphorylated. Thus, T-cell-associated kinases can also phosphorylate CTLA-4 residue ^{165}Y, although the degree to which this occurs during T cell activation remains to be determined. Shiratori et al. (*32*) demonstrated phosphorylation of CTLA-4 in activated murine T cells, but did not distinguish which of these tyrosine residues is phosphorylated.

Our data suggest that tyrosine phosphorylation of a YXXØ motif in CTLA-4 reciprocally regulates binding of effector molecules involved in intracellular trafficking and signaling. This suggests that tyrosine phosphorylation regulates the balance between CTLA-4 trafficking and signaling. Thus, CTLA-4 cell surface expression may be reduced when it is not phosphorylated, because nonphosphorylated CTLA-4 would be internalized from the cell surface as a consequence of its association with clathrin-coated pits. When CTLA-4 is triggered by ligand binding (*11*), it may become phosphorylated and signal via binding of SH2-containing effectors. However, phosphorylated CTLA-4 would not bind μ2 and would, therefore, remain at the cell surface. Another factor which may regulate which type of effector molecule becomes bound to CTLA-4 is its reported association with tyrosine phosphatase SYP (*12*).

Others have also observed a close relationship between the processes of receptor trafficking and signaling (*33*). Most notably, the receptor for the epidermal growth factor (EGFR), a receptor tyrosine kinase, associates with the clathrin-associated complex AP-2 subsequent to ligand binding (*34*−*36*). This interaction requires the tyrosine kinase activity of EGFR, but receptor autophosphorylation is not required (*34*), suggesting the involvement of other factor(s) in the interaction (*35*).

CTLA-4 receptor trafficking and signaling appear different than those of EGFR. Association of AP-2 and EGFR is ligand-induced and requires tyrosine kinase activation (*34, 35*), whereas these events are not required for the CTLA-4/μ2 interaction. It should also be pointed out that while EGFR/AP-2 interactions are well-established, direct interaction of μ2 and EGFR has not been demonstrated. Nonetheless, the parallels between CTLA-4/μ2 interactions and EGFR binding to AP-2 may be indicative of a more general relationship between receptor trafficking and signaling. In this light, it is worthwhile considering a recent report (*37*) showing that endocytic trafficking of ligand-activated EGFR establishes and regulates specific EGFR signaling pathways. It is intriguing to speculate that trafficking of CTLA-4 may likewise regulate signaling in activated T cells, and thereby elicit at least some of the unique functional activities of this receptor.

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