

Tyrosine Phosphorylation Controls Internalization of CTLA-4 by Regulating Its Interaction with Clathrin-Associated Adaptor Complex AP-2

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Summary

CTLA-4 is a costimulation receptor that binds to the same ligands, CD80 and CD86, as CD28 with high affinity and is transiently expressed on the cell surface of activated T cells. CTLA-4 delivers an inhibitory signal through association of a phosphotyrosine-containing motif in the cytoplasmic domain with Syp tyrosine phosphatase. We now demonstrate that CTLA-4 interacts with the μ_2 subunit of the plasma membrane-associated adaptor complex, AP-2, through the same motif involved in the interaction with Syp, except that the interaction with μ_2 requires unphosphorylated tyrosine. The interaction with μ_2 likely induces rapid internalization of CTLA-4 from the cell surface. Our results suggest that the phosphorylation state of a single tyrosine residue determines whether CTLA-4 delivers a negative signal or is internalized.

Introduction

The T cell surface molecules CTLA-4 and CD28 are homologous, and both function as receptors for CD80 and CD86 expressed on antigen-presenting cells (Brunet et al., 1987; Harper et al., 1991; Linsley and Ledbetter, 1993; Linsley et al., 1991; June et al., 1994; Jenkins, 1994; Leung et al., 1995). Whereas the signal through CD28 is required for the full T cell activation as the costimulatory signal, CTLA-4 delivers an inhibitory signal (Walrus et al., 1994; Tivol et al., 1995; Waterhouse et al., 1995; Marengere et al., 1996). The most striking evidence is massive proliferation of activated T cells in CTLA-4-deficient mice (Tivol et al., 1995; Waterhouse et al., 1995). It has been suggested that the negative signal is mediated through the association of a tyrosine-containing motif in the cytoplasmic tail of CTLA-4 with Syp tyrosine phosphatase upon phosphorylation (Marengere et al., 1996).

While CD28 is constitutively expressed on the cell surface, CTLA-4 is transiently expressed during T cell

activation (Walrus et al., 1994), indicating that the expression of CTLA-4 is strictly regulated. Only a fraction of synthesized CTLA-4 is expressed on the cell surface upon T cell receptor (TCR) stimulation, and the rest localizes intracellularly (Leung et al., 1995; Linsley et al., 1996). It has been suggested that the same tyrosine-containing motif that has been shown to interact with the SH2 domain of Syp also controls subcellular localization of the molecule (Leung et al., 1995). However, the mechanism controlling the intracellular trafficking of CTLA-4 remains to be elucidated.

Clathrin-coated vesicles are required for the transport of specific proteins from one compartment to the other in cells (Pearse and Robinson, 1990). Clathrin-coated vesicles bud from two membrane components: plasma membrane and trans-Golgi network (TGN). The former is required for the internalization of cell surface molecules especially receptors to endosomes, while the latter is necessary to transport lysosomal proteins from TGN to lysosomes. Most of the proteins transported by the clathrin-coated vesicles bear a specific amino acid sequence motif recognized by adaptor complexes. One of the motifs is a tyrosine containing sequence, YXX \emptyset , in which tyrosine (Y) is separated by two seemingly random amino acids (X) from an amino acid with a bulky hydrophobic side chain (\emptyset) (Davis et al., 1987; Trowbridge et al., 1993). This motif is thought to be recognized by adaptor complexes. One adaptor complex is associated with plasma membrane, and the other is associated with TGN. Recently we have shown that the medium chains, μ_1 and μ_2 of adaptor complexes, recognize and associate with the sorting motif YXX \emptyset of TGN38, LAMP-1, CD68, and H2-Mb (Ohno et al., 1995). The mechanism that regulates the internalization of various receptors upon ligand binding has not been elucidated.

Here we identify μ_2 as a molecule that recognizes the cytoplasmic region of CTLA-4 by yeast two-hybrid system. This interaction is also demonstrated in vitro. Y-165 in the cytoplasmic region of CTLA-4 is critical for the association with μ_2 . The medium chain of TGN adaptor complex (μ_1) did not bind to the CTLA-4 cytoplasmic region. The cytoplasmic region of CD28 harboring a similar motif YNM did not interact with μ_2 . Phosphorylation of Y-165 reduced the affinity of the interaction, suggesting that the internalization of CTLA-4 from the plasma membrane is mediated by the medium chain of the adaptor complex μ_2 and regulated through phosphorylation and dephosphorylation of Y-165.

Results

Cytoplasmic Tail of CTLA-4 Interacts with the Medium Chain of the Clathrin-Associated Adaptor Complex

To analyze the signal transduction and cell surface expression of CTLA-4, we searched for proteins binding to the cytoplasmic region of CTLA-4 by utilizing a yeast two-hybrid system. The entire cytoplasmic region (K-152–N-187) of mouse CTLA-4 was used as bait. We isolated six positive clones, all of which encoded the

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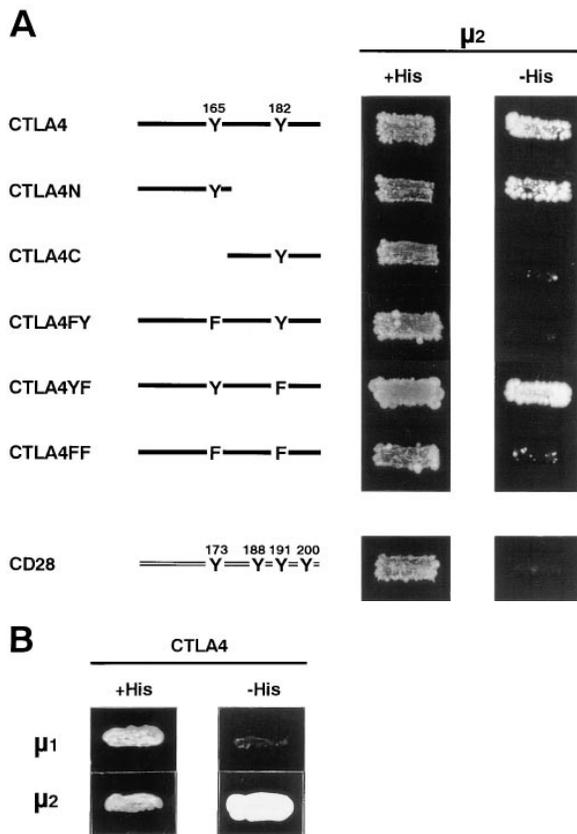


Figure 1. Specific Interaction of the Cytoplasmic Tail of CTLA-4 with the Medium Chain (μ_2) of Clathrin-Associated Adaptor Complex in Yeast

(A) Interaction of μ_2 with the cytoplasmic tails of CTLA-4 and CD28 and with tyrosine mutants of the CTLA-4 tail in yeast cells. Yeast cells were cotransformed with plasmids encoding GAL4bd fused to the sequences indicated at left, and a plasmid encoding the GAL4ad- μ_2 . Transformed cells were streaked on histidine-containing (+His) or histidine-deficient (-His) plates. CTLA-4, CTLA-4N, and CTLA-4C: full-length, N-terminal half, and C-terminal half of the cytoplasmic tail of CTLA-4, respectively; CTLA-4FY, CTLA-4YF, and CTLA-4FF: phenylalanine substitution mutants of two tyrosines (Y-165 and Y-182) (see Experimental Procedures).

(B) Interaction of CTLA-4 with μ_1 and μ_2 . CTLA-4 binds to μ_2 but not to μ_1 in yeast.

medium chain (μ_2) of the clathrin-associated adaptor complex of the plasma membrane, AP-2. We had previously shown that μ_2 interacts with a subset of the tyrosine-containing motif, YXX \emptyset , where Y is tyrosine, X is any amino acid, and \emptyset is an amino acid with a bulky hydrophobic side chain (Ohno et al., 1995). Since the cytoplasmic region of CTLA-4 harbors two tyrosine residues (Y-165 and Y-182), the N-terminal half carrying Y-165 (CTLA-4N) and the C-terminal half carrying Y-182 (CTLA-4C) were tested for the interaction with μ_2 in the two-hybrid system. As shown in Figure 1A, yeast carrying CTLA-4N but not CTLA-4C grew on plates lacking histidine and was positive on a β -galactosidase assay (data not shown), indicating that the cytoplasmic region containing Y-165 is important for interaction with μ_2 .

Next, we examined the effect of substituting phenylalanine for either or both of the tyrosine residues. Substitution of Y-165 but not Y-182 abolished the interaction

with μ_2 , suggesting that Y-165 within the motif YVKM is involved in the interaction with μ_2 . Although CD28 possesses a similar tyrosine-containing motif with which PI3 kinase has been shown to associate upon phosphorylation, the cytoplasmic tail of CD28 failed to interact with μ_2 (Figure 1A). Furthermore, μ_1 , the medium chain of the TGN-associated adaptor complex AP-1, which is also able to interact with various tyrosine-containing motifs (Ohno et al., 1995), did not interact with the cytoplasmic tail of CTLA-4. This suggests that the YVKM motif is specifically involved in the internalization of cell surface CTLA-4 from the plasma membrane to endosomes (Figure 1B). The specific interaction between the cytoplasmic region of CTLA-4 and μ_2 as well as the importance of Y-165 for this association was also examined in vitro by using a GST-CTLA-4 fusion protein and 35 S-labeled in vitro translated μ_2 . As shown in Figure 2, the wild-type CTLA-4 tail bound μ_2 , whereas CTLA-4 carrying a phenylalanine or glycine substitution at Y-165 did not.

Functional Association In Vivo between the Tyrosine-Based Motif of CTLA-4 and μ_2

To analyze the functional association between the YVKM motif of CTLA-4 and μ_2 within cells, we used an assay system in which overexpression of a YXX \emptyset -containing motif appended to CD25 (Tac antigen) saturates the endogenous pools of μ_2 and μ_1 , resulting in cell surface accumulation of CD63, an endogenous lysosomal protein with a cytoplasmic YXX \emptyset -targeting motif (Marks et al., 1995, 1996). Tac fusion constructs containing GVVVKM (from wild-type CTLA-4), GVGVKM (from the tyrosine mutant of CTLA-4), or LSYTRF (from the transferrin receptor; positive control) were transiently transfected into HeLa cells, and the cell surface expression of the endogenous CD63 was analyzed. Introduction of three Tac constructs induced similar levels, though a broad range, of surface Tac expression (Figure 3A). Analysis of transfectants expressing a high level of surface CD25 (Figure 3A) revealed that overexpression of Tac-GVVVKM but not Tac-GVGVKM augmented the cell surface expression of CD63 (Figure 3B), demonstrating that the YVKM motif of CTLA-4 functionally interacts with the sorting machinery in intact cells.

A Tyrosine-Based Motif of CTLA-4 Is Responsible for Internalization from the Cell Surface

Since it has been suggested that the association of YXX \emptyset -targeting motifs with μ_2 mediates endocytosis (Marks et al., 1995), we next examined whether the YVKM motif of CTLA-4 is responsible for the internalization of the surface CTLA-4. We measured the internalization of radioiodinated anti-Tac antibody bound to the cell surface of HeLa cells transiently transfected with Tac-GVVVKM, Tac-GVGVKM, or Tac alone (Marks et al., 1995, 1996). As shown in Figure 4, the GVVVKM motif induced internalization of almost half of the cell surface Tac-chimeric molecule (43%), whereas the GVGVKM motif and Tac alone failed to mediate internalization (17% and 12%, respectively), suggesting that the interaction of the YVKM motif of CTLA-4 with μ_2 is responsible for the internalization of the surface CTLA-4.

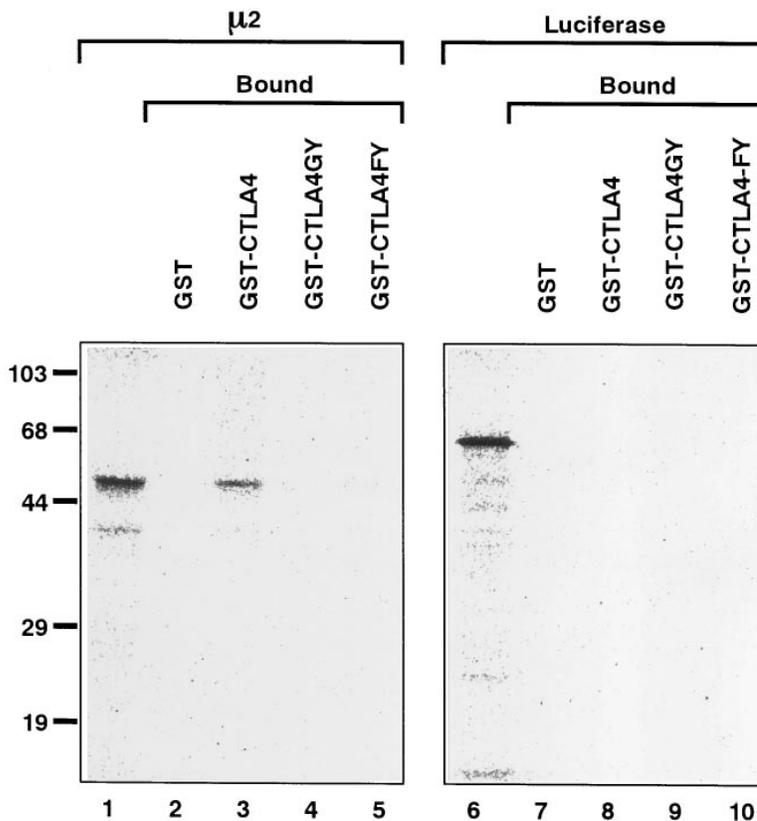


Figure 2. Specific Binding of In Vitro Translated μ_2 to the Cytoplasmic Tail of CTLA-4 through a Tyrosine Residue

In vitro-translated, ^{35}S -labeled μ_2 (lanes 1–5) and luciferase as a control (lanes 6–10) were allowed to interact with GST fusion proteins with various forms of CTLA-4 tail, CTLA-4GY, and CTLA-4FY (glycine and phenylalanine substitutions of the Y-165 of CTLA-4) (see Experimental Procedures) and analyzed on SDS-PAGE. One hundredth of the total amount of each in vitro-translated product was applied to the lanes 1 and 6 for comparison. Molecular size markers are indicated at left.

Phosphorylation of CTLA-4 Inhibits the Interaction with μ_2

Previous studies have suggested that the phosphorylated Y-165 of CTLA-4 interacts with signal transducers such as tyrosine phosphatase Syp and the p85 subunit of PI3 kinase through their SH2 domains by using phosphopeptide (Schneider et al., 1995; Marengere et al., 1996). However, since phosphorylation of CTLA-4 upon T cell activation has not yet been demonstrated in vivo, we examined the tyrosine phosphorylation of CTLA-4 in activated murine spleen cells. Splenic T cells were stimulated with anti-CD3 ϵ monoclonal antibody (MAb) cross-linking for 2 days, and a portion of the stimulated cells were surface-biotinylated to detect surface CTLA-4 biochemically. Two-dimensional gel analysis revealed an off-diagonal spot for biotinylated CTLA-4 (Figure 5A). We observed a low level of tyrosine phosphorylation of CTLA-4 upon TCR stimulation (data not shown) and detected a more significant level of phosphorylated CTLA-4 on activated T cells by a 10 min treatment with pervanadate, known as an inhibitor of tyrosine-phosphatases (Figure 5B).

To analyze the effect of tyrosine phosphorylation of CTLA-4 on the interaction with μ_2 , peptides carrying the phosphorylated (GVpYVKM) and nonphosphorylated (GVYVKM) forms of the tyrosine motif were analyzed for their ability to act as competitors of the interaction between CTLA-4 and μ_2 . As shown in Figure 6, the non-phosphorylated peptide efficiently competed with the binding of GST-CTLA-4 to ^{35}S -labeled in vitro-translated μ_2 , while the phosphorylated peptide failed to do so. These data demonstrate that phosphorylation of Y-165

in the YVKM motif of CTLA-4 inhibits the interaction with the μ_2 subunit of AP-2.

To demonstrate the correlation between tyrosine phosphorylation and internalization, the surface expression of CTLA-4 was analyzed upon pervanadate treatment of activated T cells. While T cells without TCR stimulation did not express any CTLA-4 on the cell surface (Figures 7a and 7b), T cells stimulated with anti-CD3 antibody for 1 day expressed a significant level of the surface CTLA-4 (Figure 7c). When these T cells were treated with pervanadate for 10 min, the surface expression of CTLA-4 was significantly increased on TCR-stimulated T cells but not on unstimulated T cells (Figure 7d). This result supports the conclusion that tyrosine phosphorylation of CTLA-4 influences the internalization and then the cell surface expression of CTLA-4.

Discussion

Here we have shown that the tyrosine-containing motif YVKM in the cytoplasmic region of CTLA-4 interacts specifically with the medium chain (μ_2) of plasma membrane-associated adaptor complexes (AP-2) both in vivo and in vitro. Although CD28 is homologous to CTLA-4 and harbors several tyrosines in its cytoplasmic region, μ_2 does not interact with CD28. The unique binding of CTLA-4 to μ_2 probably explains the transient expression of CTLA-4 on the cell surface and its intracellular localization as compared with the constitutive surface expression of CD28. When phosphorylated, the YVKM motif of CTLA-4 cannot bind to μ_2 . This finding suggests that phosphorylation of Y-165 inhibits the interaction

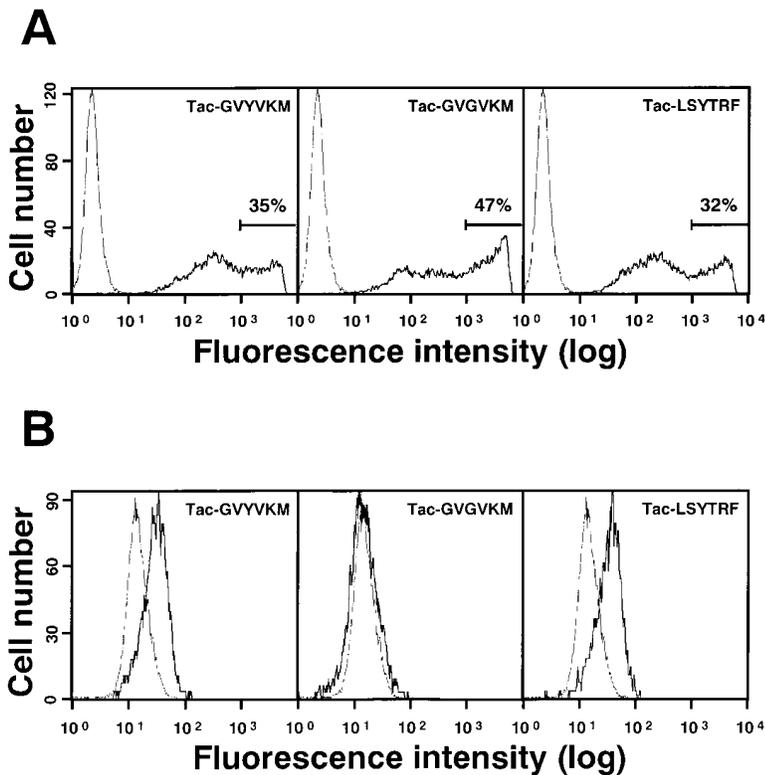


Figure 3. In Vivo Sorting Function of the YVKM Motif from CTLA-4: Overexpression of Tac-CTLA-4 Motif Results in Missorting of CD63 to the Cell Surface

HeLa cells were transiently transfected with either mock vector (light lines) or Tac-chimeric constructs (bold lines) with the tyrosine motifs of CTLA-4 (GVYVKM), its Y→G mutant (GVGVKM), and of the transferrin receptor (LSYTRF). At 48 hr posttransfection, cells were stained with FITC-anti-CD63 MAb and PE-anti-Tac MAb. (A) Expression of Tac on the cell surface of transfected HeLa cells. The numbers represent the population expressing a high level of Tac and used for the analysis of CD63 expression in (B). (B) FACS profiles of CD63 expression on the cell surface of the population expressing a high level of Tac in HeLa transfectants.

with μ_2 and thus regulates both signal transduction and surface expression.

CTLA-4 disappears from the plasma membrane within 4–5 days after stimulation. Our findings that μ_2 binds to the YVKM motif in the cytoplasmic region of CTLA-4 and that the substitution of Y-165 inhibits the internalization of CTLA-4 suggest that the down-regulation of CTLA-4 from the cell surface of T cells involves clathrin-dependent endocytosis of the molecule. Most of the CTLA-4 protein is localized intracellularly in activated T cells. Previous studies had narrowed down the region required for intracellular localization to amino acid sequence 161–171, TTGVYVKMPPT, in which Y-165 was found to be especially important (Leung et al., 1995). However, the machinery and the mechanism for the intracellular sorting has been totally unknown. Our observation that the medium chain (μ_1) of the TGN-associated adaptor complex does not interact with CTLA-4 despite its high homology to μ_2 suggests that CTLA-4 is not targeted to lysosomes from the TGN but is first transported to the plasma membrane and then internalized by virtue of its association with μ_2 . Generation of a negative signal during T cell stimulation requires a means of keeping CTLA-4 at the cell surface long enough for interaction with signal transduction molecules. We think that phosphorylation may play such a role, and indeed we detected tyrosine phosphorylation of CTLA-4 in vivo. The fact that tyrosine phosphorylated CTLA-4 was clearly observed in pervanadate-treated TCR-stimulated cells but weakly upon TCR stimulation alone may suggest that phosphorylated CTLA-4 is rapidly dephosphorylated. In addition to successful detection of phosphorylated CTLA-4, pervanadate treatment of TCR-stimulated T cells induced high expression of surface CTLA-4 within

a short period, demonstrating that phosphorylation regulates the surface expression and endocytosis of CTLA-4. This was also supported by our recent observation that a T cell clone that was deficient in expression of endogenous CTLA-4 and that constitutively expressed transfected CTLA-4 only in the cytoplasm was able to express CTLA-4 on the cell surface upon stimulation with either anti-CD3 antibody cross-linking or pervanadate treatment (S. M. et al., unpublished data).

It was reported that the YVKM motif is recognized

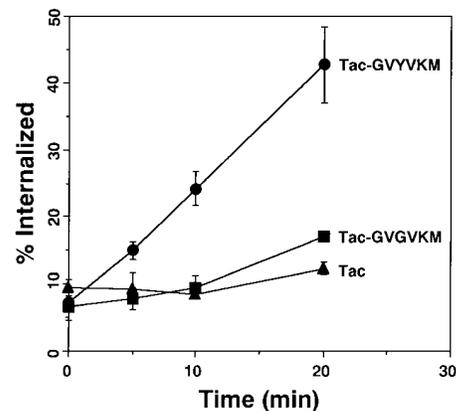


Figure 4. The YVKM Motif of CTLA-4 Is Responsible for the Internalization

HeLa cells were transiently transfected as described in Figure 3, with plasmids coding Tac (triangles), Tac-GVYVKM (circles), and Tac-GVGVKM (squares). At 40 hr after transfection, cells were reacted with 125 I-labeled anti-Tac MAb, and internalization of surface-bound anti-Tac MAb was measured.

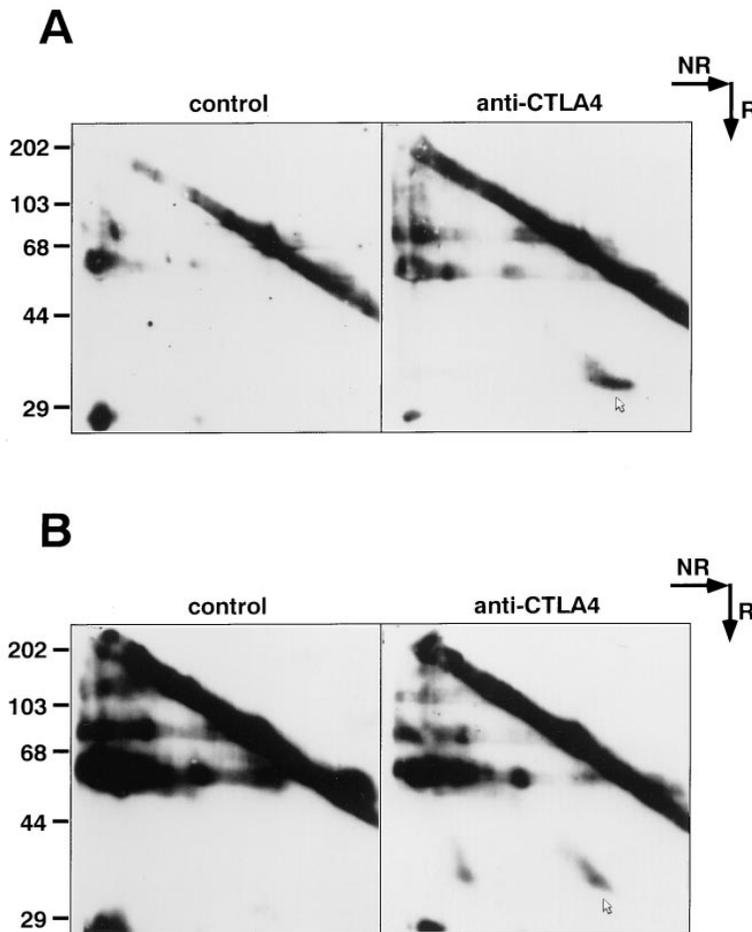


Figure 5. Tyrosine Phosphorylation of CTLA-4 Occurs In Vivo upon Stimulation

(A) Detection of CTLA-4 on the cell surface of activated T cells. Splenic T cells were stimulated with anti-CD3 ϵ MAb for 2 days, followed by 10 min treatment with pervanadate and then surface biotinylation. Immunoprecipitates with a control hamster immunoglobulin G and anti-CTLA-4 MAb were analyzed on nonreducing (NR) (10%)–reducing (R) (12%) two-dimensional SDS-PAGE.

(B) Tyrosine phosphorylation of CTLA-4 in splenic T cells upon TCR stimulation. Another gel with the same samples as in (A) was transferred onto a membrane that was immunoblotted with anti-phosphotyrosine MAb (4G10). The specific spots of CTLA-4 in (A) and tyrosine-phosphorylated CTLA-4 in (B) are indicated by open arrows. Molecular size markers are indicated at left.

by the SH2 domains of signal transducers such as the tyrosine phosphatase Syp and p85 subunit of PI3 kinase upon phosphorylation of CTLA-4. The association of CTLA-4 with Syp appears to be responsible for delivering the negative signal in T cell activation (Marengere et al., 1996). We have shown that the phosphorylation of Y-165 inhibits the binding of CTLA-4 to μ_2 , probably leading to the transient retention of CTLA-4 on the cell surface upon T cell activation. Therefore, it is likely that dephosphorylation of the Y-165 makes CTLA-4 unable to associate with Syp and to transduce a negative signal and causes it to become endocytosed by interaction with AP-2. This is the first demonstration that a single tyrosine residue in the cytoplasmic tail of a receptor molecule can participate in both signal transduction and intracellular protein trafficking, depending on its phosphorylation state. Considering that the phosphotyrosine-containing motifs recognized by various SH2 domains are very similar to tyrosine-containing sorting signals, it is likely that there will be other receptor systems in which the same tyrosine motif mediates both signal transduction and protein trafficking, depending on its phosphorylation state.

Experimental Procedures

Plasmid Construction

The cytoplasmic regions of mouse CTLA-4 and CD28 were generated by polymerase chain reaction (PCR) and subcloned into pGBT9

(Clontech) and pAS1-CYH2 to yield GAL4 DNA-binding domain (GAL4bd) fusion plasmids. DNA fragments carrying deletions and point mutations of the cytoplasmic region of CTLA-4, N (K-152–P-169), C (P-170–N-187), FY (Y-165→F), YF (Y-182→F), FF (Y-165→F, Y-182→F), and GY (Y-165→G) were constructed by PCR and cloned into pGBT9 and the pGEX-4T-1 vectors (Pharmacia). The μ_1 cDNA was isolated from mouse spleen cDNA by PCR and cloned into the vector pACT11 to yield GAL4 transcription activation domain (GAL4ad) fusion plasmid. The plasmid encoding GAL4ad fused to μ_2 was isolated from the mouse spleen cDNA library by the interaction with the (SDYQRL)₃ bait sequence using a yeast two-hybrid system.

Yeast Two-Hybrid System

A GAL4ad cDNA fusion library in pACT11 was constructed from BALB/c mouse spleen mRNAs. The library was introduced into the HF7c strain of *Saccharomyces cerevisiae* (Clontech) carrying the plasmid encoding GAL4bd fused to the cytoplasmic region of mouse CTLA-4. Screening was performed on 1.4×10^7 transformants.

In Vitro Binding Assay

Expression of GST fusion protein was induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside. Cells were collected; resuspended in a buffer consisting of 0.5% NP-40, 20 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM EDTA; and disrupted using a sonicator. The supernatant was incubated with glutathione-Sepharose beads (Pharmacia) at 4°C for 30 min. GST fusion protein was eluted with elution buffer (5 mM glutathione, 50 mM Tris [pH 8.0]), and dialyzed against phosphate-buffered saline (PBS). In vitro translation was done using an in vitro translation kit (Promega) in the presence of ³⁵S-labeled methionine with a full-length mouse μ_2 generated by PCR and subcloned into pcDNA3 (Invitrogen). ³⁵S-labeled, in vitro-translated μ_2 was precleared with glutathione-Sepharose beads and

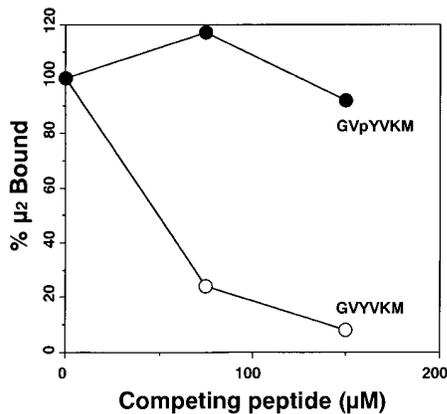


Figure 6. Phosphorylation of Y-165 of CTLA-4 Inhibits the Interaction of CTLA-4 with μ_2

In vitro-translated ^{35}S -labeled μ_2 was incubated with GST-CTLA-4 in the presence or absence of graded concentrations of the competing peptides GVVYKM (open circles) or GVPYVKM (closed circles), which contains the phosphotyrosine residue. μ_2 bound to GST-CTLA-4 was isolated and resolved by 12% SDS-PAGE, and the radioactive bands on a membrane were quantitated by an image analyzer. Data are given as the percentages of μ_2 bound in the absence of the competing peptide.

incubated with 10 μg of GST fusion proteins in 600 μl of binding buffer 2 (0.05% Triton-X, 50 mM HEPES, 10% glycerol, 0.1% bovine serum albumin, 100 mM KCl, 2 mM MgCl_2 , 0.1 mM CaCl_2 , 0.05 mM dithiothreitol [pH 7.3]) for 2 hr at room temperature. The mixture was adsorbed to glutathione-Sepharose beads.

In Vivo μ_2 Saturation Assay

A chimeric insert encoding the full-length Tac cDNA fused at the C-terminus to the tyrosine-based signal from the human transferrin receptor, LSYTRF, was constructed by PCR and ligated into pCDM8.1 to yield Tac-LSYTRF. Tac-GVVYKM and Tac-GVGVKM were similarly constructed. HeLa cells were transfected with Tac fusion constructs by the calcium phosphate method. After 48 hr, transfected cells were stained with fluorescein isothiocyanate (FITC)-anti-CD63 MAb (CLB-gran/12, Immunotech S. A.) and phycoerythrin (PE)-anti-Tac MAb (1HT44H3, Coulter Immunol.) and were analyzed using a FACScan and the CellQuest 1.2 analysis program.

Cell Surface Internalization Assay

This assay was performed as previously described (Marks et al., 1995). Transfected cells were incubated with ^{125}I -labeled anti-Tac MAb (7G7. B6, American Type Culture Collection), on ice for 30–60 min. Cells were washed and resuspended in Dulbecco's modified Eagle's medium/10% fetal bovine serum and then warmed to 37°C. At the indicated times, cells were diluted into ice-cold PBS and pelleted. Half of the cells were resuspended in PBS alone and the other half in PBS containing proteinase K, and all cells were incubated on ice for 30 min. Cells were pelleted through a cushion of fetal bovine serum to remove degraded label, and radioactivity in the cell pellets were counted with a Packard γ counter. Counts of untransfected cells at each time point were subtracted from those of transfectants to give corrected counts. The final percentage internalization was then calculated as the corrected counts obtained with proteinase K treatment divided by those obtained without treatment at each time point.

Analysis of Phosphorylation and Surface Biotinylation

BALB/c spleen cells were stimulated in the presence of 10 $\mu\text{g}/\text{ml}$ anti-CD3 ϵ MAb (145–2C11) and murine interleukin-2. Two days later, 2.5×10^6 cells were treated with pervanadate and lysed with 0.5% NP-40 lysis buffer (0.5% NP-40, 50 mM Tris, 50 mM NaCl, 5 mM

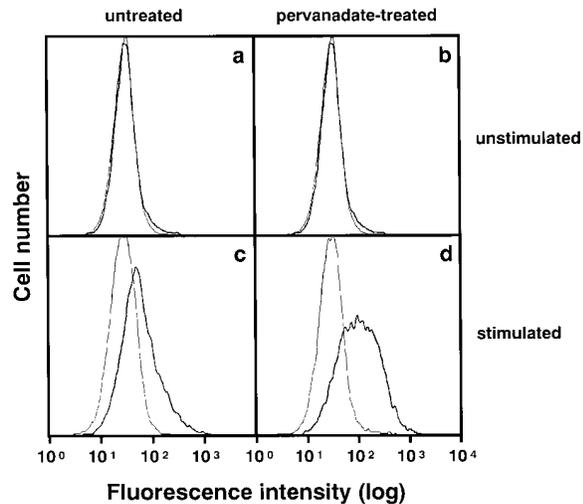


Figure 7. Pervanadate Treatment of Activated T Cells Augments the Surface Expression of CTLA-4

Splenic T cells were unstimulated (a and b) or stimulated (c and d) with anti-CD3 ϵ MAb for 1 day and treated (b and d) or untreated (a and c) with pervanadate for 10 min. The cells were then stained with (bold lines) or without (light lines) anti-CTLA-4 MAb and analyzed on a FACScan.

EDTA, 7.7 U/l aprotinin, 2.5 $\mu\text{g}/\text{ml}$ antipain, 2.5 $\mu\text{g}/\text{ml}$ chymostatin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 5 $\mu\text{g}/\text{ml}$ pepstatin A, 1 mM PMSF, 5 mM iodoacetamide, 2 mM Na_3VO_4 , 10 mM NaF). The lysate was immunoprecipitated with anti-mouse CTLA-4 MAb (UC10.4F10.11) or hamster immunoglobulin G. The immunoprecipitates were subjected to two-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel under nonreducing conditions for the first dimension and 12% gel under reducing conditions for the second dimension). Proteins were transferred to a polyvinylidene-difluoride membrane, immunoblotted with anti-phosphotyrosine MAb (4G10), and developed using the ECL system (Amersham).

For surface biotinylation, 1.5×10^8 spleen cells were biotinylated for 1 hr at 4°C as previously described (Ono et al., 1995) and lysed with 0.5% NP-40 lysis buffer. The cell lysate was immunoprecipitated as described above, and the blotted membrane was developed by the ABC kit (Vector Laboratories).

In Vitro Competition of CTLA-4- μ_2 Association

In vitro-translated μ_2 was incubated with 10 μg of GST-fusion proteins in 600 μl of binding buffer (0.05% Triton X-100, 50 mM HEPES, 10% glycerol, 0.1% bovine serum albumin [pH 7.3]) in the presence or absence of the competing peptides GVVYKM or GVPYVKM (SAWADY Technology) for 2 hr at room temperature, adsorbed to glutathione-Sepharose beads for 30 min, washed, and resolved by SDS-PAGE on 12% gels. Radiolabeled products were detected by fluorography. Quantitation was done with a Phosphorimager (Molecular Dynamics).

Pervanadate Treatment

The pervanadate stock solution (50 \times) was made fresh by mixing 0.45 volumes of H_2O , 0.5 volumes of 1 M H_2O_2 (final concentration 10 mM), and 0.05 volumes of 100 mM Na_3VO_4 (final concentration 100 μM), and left for 15 min at room temperature. Cells were added 1/50 volume of the pervanadate stock solution and incubated for 10 min at 37°C.

Analysis of Surface Expression of CTLA-4

BALB/c spleen cells were stimulated by anti-CD3 ϵ MAb cross-linking in the presence of murine interleukin-2. One day later, 5×10^6 cells were treated with pervanadate as described above, washed in ice-cold PBS, incubated with anti-FcR MAb (2.4G2) for 30 min at

4°C, stained with FITC-anti-Thy1.2 MAb (53-2.1) and biotin-anti-CTLA-4 MAb, and incubated with PE-streptavidin. Flow cytometry was performed using a FACScan and analyzed using the CellQuest 1.2 program.

Acknowledgments

Correspondence should be addressed to T. Saito (e-mail: saito@med.m.chiba-u.ac.jp). We thank S. J. Elledge for the gift of pAS1-CYH2 and pACTII vectors; P. Golstein for CTLA-4 cDNA; J. A. Bluestone for anti-CTLA-4 MAb; M. Muramatsu, K. Matsumoto, T. Yamazaki, and K. Wakizaka for discussion and help with experiments; M. Hayashi, H. Watanabe, H. Shoji, T. Iida, M. Ohta, K. Takase, M. Otsuji, Y. Kimura, and M. Sakuma for technical help; and H. Yamaguchi for secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, and from the Agency for Science and Technology.

Received November 18, 1996; revised February 20, 1997.

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