Cytolytic T Lymphocyte-Associated Antigen-4 and the TCRζ/CD3 Complex, But Not CD28, Interact with Clathrin Adaptor Complexes AP-1 and AP-2

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The negative signaling receptor cytolytic T lymphocyte-associated Ag-4 (CTLA-4) resides primarily in intracellular compartments such as the Golgi apparatus of T cells. However, little is known regarding the molecular mechanisms that influence this accumulation. In this study, we demonstrate binding of the clathrin adaptor complex AP-1 with the GVYVKM motif of the cytoplasmic domain of CTLA-4. Binding occurred primarily in the Golgi compartment of T cells, unlike with AP-2 binding that occurs mostly with cell surface CTLA-4. Although evidence was not found to implicate AP-1 binding in the retention of CTLA-4 in the Golgi, AP-1 appears to play a role in shuffling of excess receptor from the Golgi to the lysosomal compartments for degradation. In support of this, increased CTLA-4 synthesis resulted in an increase in CTLA-4/AP-1 binding and a concomitant increase in the appearance of CTLA-4 in the lysosomal compartment. At the same time, the level of intracellular receptor was maintained at a constant level, suggesting that CTLA-4/AP-1 binding represents one mechanism to ensure steady state levels of intracellular CTLA-4 in T cells. Finally, we demonstrate that the TCR/CD3 complex (but not CD28) also binds to AP-1 and AP-2 complexes, thus providing a possible link between these two receptors in the regulation of T cell function. The Journal of Immunology, 1999, 163: 1868–1879.

D28 and cytolytic T lymphocyte-associated Ag-4 (CTLA-4) are differentially expressed on T cells, bind to common ligands CD80 and CD86 (albeit with different avidities), and are required for optimal T cell activation (1–3). CD28 is expressed on the surface of resting and activated T cells, while structurally related CTLA-4 is expressed only on activated T cells (4, 5). CD28 and CTLA-4 have different functions on the activation of T cells. Although CD28 augments lymphokine production and the T cell response (1, 6–8), Ab-blocking studies have implicated the tyrosine phosphatase SHP-2 in binding from CTLA-4-deficient mice are hyperresponsive to anti-CD3 ligand (9, 10). Consistent with this negative function, T cells from CTLA-4-deficient mice are hyperresponsive to anti-CD3 ligation, and show extensive lymphadenopathy (11, 12). Several studies have implicated the tyrosine phosphatase SHP-2 in binding to CTLA-4 and causing the dephosphorylation of the TCRζ chain and the adaptor protein LAT (13, 14). CTLA-4 may therefore disrupt TCRζ/CD3 signaling (15, 16), as well as down-regulating CD28-mediated potentiation of activation (17). The blockage of CD28-CD80 binding has also been reported to reverse the hyperresponsive phenotype of CTLA-4-negative mice (18).

In addition to negative signaling, CTLA-4 can be distinguished from CD28 by the fact that it is primarily an intracellular Ag (19). Surface expression is low and tightly regulated, occurring as early as 6 h postactivation and reaching its highest levels by some 36 h (5). Even at its highest levels, however, CTLA-4 is expressed at only some 3% relative to CD28 (5). This may be due to a high level of compartmentalization in the trans-Golgi network (TGN) and secretory vesicles (19, 20), and rapid internalization from the surface of T cells (20, 21). The molecular basis for this intracellular accumulation is unclear, although it has been reported to be linked to a tyrosine-containing motif in the CTLA-4 cytoplasmic tail (19). This intracellular localization is unusual for a surface receptor, and has led to speculation regarding its function, possibly allowing intracellular signaling, or serving as a mechanism to control the polarized release of CTLA-4 at sites of contact between T cells and APC (21).

Little is known regarding the processes that control the intracellular accumulation of CTLA-4 in T cells. Clathrin-coated vesicles are involved in selective intracellular trafficking of membrane proteins in eukaryotes (22). At least three distinct tetrameric adaptor complexes, AP-1, AP-2, and AP-3, associate with clathrin (23). AP-1 complexes are localized in the TGN and are involved in lysosomal and cell surface targeting, while AP-2 complexes are found at the plasma membrane and regulate endocytosis (24–26). AP-3 complexes play a role in cargo-selective transport from the Golgi to intracellular vacuoles (27–29). AP-1 and AP-2 complexes contain two large subunits (γ and β-1 vs α and β-2), one medium chain (μ-1 (AP-47) vs μ-2 (AP-50), and a small chain (σ1 (AP-19) vs σ-2 (AP-17) (30, 31–33). The β-1 and β-2 chains are most closely related to each other, and bind to clathrin (34). AP-1 localization with the Golgi membrane requires the core complex and a small GTP-binding protein ADP-ribosylation factor (35, 36). Subsequent recruitment of cytosolic clathrin is dependent on the presence of intact AP-1 (33, 37, 38).

Although somewhat poorly defined, the μ-1 and μ-2 chains bind to nonphosphorylated tyrosine-based sorting motifs with...
the sequence YXXO, where X can be any amino acid and Ø is a large hydrophobic amino acid (39–42). Using the two-hybrid screening assay, several laboratories found that the CTLA-4 cytoplasmic tail can bind to the µ-2 chain of the AP-2 complex (43–46). A similar tyrosine-based motif may function in the case of the TGN to the endosomal/lysosomal pathway (39). By contrast, a similar motif YMNK in the cytoplasmic tail of CD28 did not interact with the µ-2 chain. AP-2 binding to CTLA-4 provides a possible mechanism by which CTLA-4 becomes endocytosed; however, it leaves unresolved the mechanism responsible for the possible mechanism by which CTLA-4 becomes endocytosed; however, it leaves unresolved the mechanism responsible for the accumulation of CTLA-4 in the TGN (19, 20).

In this study, we report the identification of an interaction between the clathrin adaptor complex AP-1 and intracellular forms of CTLA-4, found primarily in the Golgi compartment. AP-1 appears to play a role in regulating the shuttling of excess receptor from the Golgi to the lysosomal compartments for degradation thereby maintaining a constant level of intracellular receptor. CTLA-4/AP-1 binding serves as one mechanism to regulate intracellular levels of CTLA-4 in T cells. Finally, we demonstrate that the TCR/CD3 complex (but not CD28) also binds to AP-1 and AP-2 complexes, thus providing a potential link between these two receptors in the regulation of T cell function.

Materials and Methods

**Cells, reagents, and Abs**

The murine T cell hybridoma DC27.10 (gift of Dr. R. Zamoyska, Medical Research Council, London, U.K.) was cultured in RPMI 1640 medium supplemented with 5% (v/v) FBS (Intergen, Purchase, NY), 100 U/ml penicillin, 100 mg/ml streptomycin (Life Technologies, Grand Island, NY), 2 mM L-glutamine (Life Technologies), and 50 µM 2-ME. PBL were isolated from the buffy coat by lymphocyte separation medium (Ficoll-Paque) density-gradient centrifugation. Adherent cells were depleted from the PBL by plastic absorption. The nonadherent cells were cultured for 2 days in RPMI 1640 supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, PHA (1 µg/ml), and human rIL-2 (50 U/ml).

Anti-CTLA-4 (11D4) has been previously described (47), while anti-CTLA-4 (BNI3.1) was purchased from Immunotech (Marseille, France). Anti-CD28 (4B10) was a kind gift from Coulter (Hialeah, FL). Anti-murine CD3 (145-2C11) was purchased from American Type Culture Collection (Manassas, VA). Anti-phospho PI-3 kinase was provided by Dr. M. White (Joslin Diabetes Center, Boston, MA). Anti-γ-subunit Ab specific for AP-1 was kindly provided by Dr. L. Traub (Washington University, St. Louis, MO); the specific anti-β-chain antisera by Dr. T. Kirchhausen (Center for Blood Research, Boston, MA). Unlabeled and PE-labeled mouse IgG2a (specific for 2,4,6-trinitrophenol; TNP) and PE-labeled anti-CTLA-4 were purchased from PharMingen (San Diego, CA). Monoclonal anti-Golgi 58K protein was obtained from Sigma (St. Louis, MO). mAb lamp-2 was purchased from Developmental Studies Hybridoma Bank (Iowa). Ionomin and PMA were obtained from Calbiochem (San Diego, CA).

**Transfections of human CTLA-4 and chimera**

Human CTLA-4 was obtained by PCR using pCDM8-CTLA-4 (kindly provided by G. Freeman, Dana-Farber Cancer Institute, Boston, MA) as a template. Primers for PCR were: GAA TTC ATG GTG TGC GTG CTC GGT TTT. The PCR product was digested with EcoRI and subsequently cloned into the EcoRI site of the SRE expression vector (48). Amino acid residue at position Y-201 as well as CD28GVYVK and CTLA-4SYMMNN were expected to direct mutated on the protocol provided by Promega (Madison, WI). Mutation of the designated tyrosine motif and the generation of the chimera were verified by dideoxynucleotide sequence analysis. Stable transfections were generated using the T cell hybridoma DC27.10 transfected with human CTLA-4 WT, mutant Y201F, CTLA-4CD28(SYMNN), or CD28CTLA-4(GVYVKM) plasmid together with pSinneo containing a neomycin resistance gene. Electroporation was conducted at 260 V and 1600 µF. Cells were selected with 1.5 mg/ml of G418 for 2 wk, and cells from different populations were assayed for Ag expression by FACS, as described (49).

**Subcellular fractionation**

DC27.10 cells were harvested, resuspended in 0.25 M sucrose, pH 7.4, homogenized, and centrifuged at 3000 rpm for 15 min to pellet the nuclei and unbroken cells. Cytoplasmic organelles from the postnuclear supernatant were separated by a Percoll/metrizamide discontinuous density gradient, as described (50). Lysosomal and lysosomal/mitochondrial membranes were obtained as described (51). The supernatants contained matrix proteins. The membranes were washed twice with 0.5 M NaCl and 0.1 M DTT, and then resuspended in PBS. A mixture of protease inhibitors was added to the lysosomal fractions before lysis (1 mM EDTA, 0.1 mM peptatin A, 0.1 mM leupeptin, and 0.1 mM AEBSF). The purity of the isolated fractions was tested by immunoblotting using Abs for Golgi and lysosomes. In addition, the purity of lysosomes was verified by the lysosomal marker enzyme β-hexosaminidase, as described (50). Protein determinations were performed using the Lowry assay (52). For precipitations, the amount of protein was about 900 µg, and for whole cell lysate generally 30 µg. In some experiments, cell fractionations were prepared following treatment of cells for 48 h with 20 mM ammonium chloride to prevent lysosomal degradation.

**Peptide competition assay**

Peptides were synthesized and HPLC purified by the Molecular Biology Core Facility (Dana-Farber Cancer Institute). The sequence of the peptides used was as follows: TTGVYVKMPTE (unphosphorylated peptide), TTGVpYVKMPTE (phosphorylated peptide). Purification of AP-1 and peptide photocross-linking analysis were conducted as described (42, 53).

**Immunoprecipitation and immunoblotting**

For immunoprecipitations, T cells were lysed in ice-cold lysis buffer containing 1% Triton X-100 (v/v) in 20 mM Tris-HCl, pH 8.3, 150 mM NaCl. The lysis buffer contained 1 mM PMSF, 1 mM Na3VO4, 10 mM NaF, and 1 mM Na3P04. For precipitations, 100 × 106 cells/ml were generally used, while whole lysates are comprised of 1 × 108 cells/lane. Lysates were incubated for 20 min on ice before centrifugation at 15,000 × g for 15 min at 4°C. Postnuclear lysates were incubated for 1 h with agitation at 4°C with a tyrosine-mutated mAb A or a phospho A-Sepharose beads (30 µl). Phosphorylated proteins were washed and washed in lysis buffer, were added and incubated for 1 h at 4°C. The beads were washed three times in cold lysis buffer, and proteins were eluted by boiling for 5 min in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting. The membranes were blocked with 5% milk in TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) and incubated with the indicated Ab. Bound Ab was revealed by enhanced chemiluminescence using HRP-conjugated protein A/G using enhanced chemoluminescence (ECL; Amersham, Arlington Heights, IL).

**Metabolic labeling**

To monitor the synthesis of newly synthesized CTLA-4, CTLA-4 wild-type cells were treated with ionomycin (1 µM) or PMA (50 ng/ml). After metabolic labeling for 30 min with 1 mCi/ml [35 S]methionine, cells were lysed, immunoprecipitated with CTLA-4 mAb or isotype-specific mAb, and analyzed by 10% SDS-PAGE.

**Results**

**Binding of CTLA-4 to the AP-1 complex**

Stable CTLA-4 transfectants were generated from the mouse T cell hybridoma DC27.10 using previously described approaches (54). As seen in Fig. 1A, human CTLA-4 WT (b) and CTLA-4 mutant Y201F (tyrosine at residue 201 was mutated to phenylalanine) (c) were expressed at moderate levels on cell surface of the T cells. Generally, the CTLA-4 Y210F mutant was expressed at slightly higher levels on the surface of cells, consistent with the importance of the YVKM site in regulating surface expression of the receptor (19).

Previous studies have shown that the majority of CTLA-4 accumulates in the intracellular compartments in T cells (19, 20). One likely candidate to influence this intracellular accumulation of protein is the AP-1 complex. AP-1 is a complex that recognizes tyrosine-based motifs and is involved in the shuttling of various proteins to the lysosomal compartment of cells for degradation (33, 55, 56). To assess whether CTLA-4 was capable of interacting...
with the AP-1 complex, anti-CTLA-4 precipitates from the
DC27.10 transfectants were subjected to immunoblotting with an
Ab against the γ-chain of the AP-1 complex. As seen in Fig. 1B
(upper left panel), anti-CTLA-4 precipitated a polypeptide at 95–
100 kDa that was recognized by the anti-γ-chain antiserum (lane
3). A band of the same size was also recognized in the cell lysate
(lane 4). By contrast, anti-CD28 failed to precipitate the protein
(lane 2). As a negative control, anti-CTLA-4 failed to precipitate the
γ-chain from nontransfected CTLA-4-negative DC27.10 control
cells (lane 1). As a positive control, both CD28 and CTLA-4
precipitated PI 3-kinase as detected by anti-p85 immunoblotting
(Fig. 1B, middle panel, lanes 2 and 3, respectively). PI 3-kinase
binding to CD28 and CTLA-4 has been described (45, 57, 58).
Recent studies using the two-hybrid system found that CTLA-4
binds the μ-chain of the AP-2 complex (37, 43, 44, 45). Using our
transfectants and a specific anti-β-chain antiserum, anti-CTLA-4
was found to precipitate β-subunits of both the AP-1 and AP-2
(Fig. 1B, lower panel, lane 3). The migration of the protein bands
corresponding to the AP-1 and AP-2 β-chains is identical to what
has been previously documented (37, 66). The interaction was spec-
fic in that no association between CD28 and AP-1/AP-2 was
observed (lane 2) and anti-CTLA-4 did not precipitate Ag from
vector transfectants (lane 1).

To verify that the CTLA-4/AP-1 interaction could occur in nor-
mal T cells, peripheral T cells that had been activated for 48 h with
PHA and rhIL-2 were subjected to anti-CTLA-4 precipitation and
anti-γ-chain immunoblotting (Fig. 1B, upper right panel, lanes
5–7). Again, an AP-1 γ-chain was readily detected in anti-CTLA-4
precipitates (lane 6), and in cell lysates (lane 7). These data dem-
onstrate that CTLA-4 associates in a specific manner with the
AP-1 complex in T cells, a feature that distinguishes CTLA-4 from
CD28. In addition, coprecipitation of CTLA-4 with AP-1 is un-
usual, being the first example of an AP-1 interaction with a protein
of sufficient avidity to withstand detergent and immunoprecipita-
tion conditions.

**Binding of AP-1 to the GVVVM motif of CTLA-4 in T cells**

Tyrosine-based endocytic motifs (tyrosine-polar-polar-large
hydrophobic) have been shown to interact with the μ-1 chain of the
AP-1 complex (39). CTLA-4 possesses a YVVK motif that could
meet this requirement (4). To assess this further, a peptide
competition assay was conducted using phosphorylated and nonphos-
phorylated peptides encoding TTGYYKMPPET and a peptide
containing the UV-induced activation of cross-linker benzoylphen-
ylalanine (42, 53). Peptide YQTI corresponds to the sequence of
the complete 11-amino-acid cytoplasmic tail of lamp-1 containing
the Y-motif YQTI. Under this regime, nonphosphorylated peptide
competition was observed at 1000 μM peptide (lanes 5–7). Densitometric readings of the exposed film showed a clear
dose-dependent competition between 1–100 μM peptide (right
panel). The absence of CTLA-4 peptide served as a positive con-
trast (lanes 1 and 6). By contrast, phosphorylated peptide did not
dissociate the complex between 1–100 μM (lanes 3–5), while a
competition was observed at 1000 μM (lane 2). Whether the effect
of this high peptide concentration is related to a small contamina-
tion of nonphosphorylated peptide is unclear. The concentration

**FIGURE 1.** CTLA-4 binds to AP-1 (and AP-2) complexes in T cells. A, Wild-type and mutant Y201F CTLA-4 expression on transfected T cell
hybridoma DC27.10. The T cell hybridoma DC27.10 was transfected with wild-type and mutant human Y201F CTLA-4, as outlined in Materials and
Methods. Expression levels of CTLA-4 were analyzed by flow cytometry using mAb 11D4. The control corresponds to cell staining with secondary Ab
alone (FITC-conjugated goat anti-mouse mAb). B, CTLA-4 binding to AP-1 complexes. *Upper left panel,* Vector-transfected, human CD28-, and CTLA-
4-expressing DC27.10 were subjected to immunoblotting with anti-CTLA-4 or anti-CD28 mAb and were subjected to immunoprecipitation with
anti-γ-chain antisera of AP-1. Lane 1, Anti-CTLA-4 precipitation from vector-transfected cells; lane 2, anti-CD28 from CD28 transfectants; lane 3,
anti-CTLA-4 from CTLA-4 transfectants; lane 4, cell lysate. *Upper right panel,* PHA-stimulated PBLs were lysed; immunoprecipitated with CD28 mAb
(lane 5) or CTLA-4 mAb (lane 6), and subjected to immunoblotting with anti-γ-chain antisera of AP-1. The corresponding band recognized in cell lysate
served as a positive control (lane 7). *Middle panel,* DC27.10 cells were subjected to immunoprecipitation as described in upper panel and subjected to
immunoblotting with anti-p85 of PI 3-kinase. Lane 1, Anti-CTLA-4 precipitation from vector-transfected cells; lane 2, anti-CD28 from CD28 transfectants;
lane 3, anti-CTLA-4 from CTLA-4 transfectants; lane 4, cell lysate. *Lower panel,* DC27.10 cells were subjected to immunoprecipitation as described in
B and subjected to immunoblotting with anti-β-chain antisera cross-reactive with AP-1 and AP-2. Lane 1, Anti-CTLA-4 precipitation from vector-
transfected cells; lane 2, anti-CD28 from CD28 transfectants; lane 3, anti-CTLA-4 from CTLA-4 transfectants; lane 4, cell lysate.
range of peptide needed for effective displacement of *YQTI/AP-1 binding corresponds to previously documented examples (42).

To complement the in vitro findings with in vivo data, chimeric receptors of CD28 and CTLA-4 were generated in which the tyrosine-based motifs of CD28(SDYMNM) and CTLA-4(GVYVKM) were exchanged (Fig. 2B, upper panel). DC27.10 cells were then stably transfected with either human CD28, the CD28-CTLA-4 (GVYVKM) chimera, hCTLA-4, or the CTLA-4-CD28(SDYMNM) chimera. Immunoprecipitation was conducted using anti-CD28 or anti-CTLA-4 Abs, followed by immunoblotting with an antiserum against AP-1 (γ-chain specific). As shown in Fig. 2B (lower panel), anti-CD28 and anti-CTLA-4 Abs precipitated the γ-chain of AP-1 from the CD28-CTLA-4(GVYVKM)- and CTLA-4-CD28(SDYMNM) transfected cells, respectively (lanes 2 and 3). By contrast, CD28WT and the CTLA-4-CD28(SDYMNM) chimera failed to precipitate the protein (lanes 1 and 4). Therefore, the GVYVKM motif alone provided minimal conditions for binding, albeit less efficiently than WTCTLA-4. The ability of the GVYVKM motif alone to confer AP-1 binding to the CD28 receptor provided further in vivo evidence implicating this motif in the binding to AP-1.

Finally, to define the role of the tyrosine residue itself within CTLA-4, Y-201 was mutated to phenylalanine within WTCTLA-4 and investigated for AP-1 binding in T cells (Fig. 2C). In this case, the loss of the tyrosine abrogated AP-1 binding (lane 3 vs lane 4). Together, these data demonstrate that the tyrosine-based YVKM motif serves as a binding site for the AP-1 complex.

Intracellular localization of CTLA-4/AP-1 complexes

The majority of the AP-1 complexes have been shown in well-documented studies to reside in the TGN (25, 30, 33). This suggested that unlike CTLA-4 binding to intracellular Ags such as the SHP-2 phosphatase and PI 3-kinase, CTLA-4 binding to AP-1 may occur primarily in the Golgi compartment, the site of accumulated intracellular CTLA-4. To address this further, intracellular CTLA-4 was separated from surface CTLA-4 and compared for the presence of AP-1 binding (Fig. 3). Surface CTLA-4 was distinguished from intracellular CTLA-4 by preincubating intact cells with anti-CTLA-4 Ab at 4°C, followed by washing to remove excess Ab, detergent lysis, and immunoprecipitation. Following this, lysates were subjected to a round of preclearing with protein A-Sepharose, followed by a second precipitation to purify intracellular CTLA-4. As seen in Fig. 3 (upper panel), more than 90% of CTLA-4/AP-1 complexes were found in the intracellular lysate (lane 4), while anti-CTLA-4 precipitates from the cell surface contained only a minor fraction (lane 3). Given the limitations of the Ab-prebinding assay with some possible redistribution of Ab in cell lysates, the significance of some 5–10% AP-1 binding to cell surface-expressed CTLA-4 is unclear. As a control, surface and intracellular CTLA-4/AP-1 was also precipitated from untreated

FIGURE 2. The AP-1 complex binds to a tyrosine-based motif in CTLA-4. A, Peptide competition of AP-1 binding. Peptide corresponding to the YVKM motif competes with the photoreactive *YQTI peptide for AP-1 binding. Phosphorylated YVKM peptide in the range of 1–100 μM did not dissociate the complex between AP-1 and *YQTI (lanes 3–5). At 1000 μM, a competition was noted (lane 2). Nonphosphorylated peptide could compete in a range of 1–1000 μM for the association between AP-1 and *YQTI (lanes 7–10). *YQTI/AP-1 complexes served as positive controls (lanes 1 and 6). B, Chimeric CD28 receptor with CTLA-4GVYVKM motif binds AP-1. DC27.10 cells transfected with human CD28 (hCD28), human CD28 with the cytoplasmic SDYMNM motif exchanged for the CTLA-4 GVKVKM motif (CD28-CTLA-4(GVYVKM) chimera), or human CTLA-4 in which the cytoplasmic GVKVKM motif has been exchanged for the CD28 SDYMNM motif (CTLA-4-CD28(SDYMNM)) chimera) were lysed and immunoprecipitated with either anti-CD28 (lanes 1 and 2) or anti-CTLA-4 mAbs (lanes 3 and 4). The precipitates were subjected to immunoblotting with anti-γ-chain antiserum. The corresponding band recognized in cell lysate served as a positive control (lane 5). C, The Y201F mutant of CTLA-4 fails to bind AP-1. DC27.10 cells transfected with vector, human CD28, CTLA-4-Y201F mutant, or CTLA-4-WT were lysed and immunoprecipitated with anti-CD28 (lane 2) or anti-CTLA-4 (lanes 1, 3, and 4) mAbs. The precipitates were subjected to immunoblotting with anti-γ-chain antiserum. The corresponding band recognized in cell lysate served as a positive control (lane 5).
cells (lane 2). As expected, the combination of surface and intracellular CTLA-4 precipitated AP-1, which equaled the total precipitated by anti-CTLA-4 against untreated cells (Fig. 3, lower panel). Anti-CD28 precipitates served as a negative control (lane 1). These data show that the majority of CTLA-4/AP-1 complexes are located in intracellular compartments of T cells.

**Mutation within the AP-1 binding site alters levels of intracellular CTLA-4**

CTLA-4 accumulates as an intracellular Ag in T cells (19). AP-1 binding to CTLA-4 could play a role in this event by regulating the shuttling of CTLA-4 to the lysosomal compartments for degradation. AP-1 has been shown in several other systems to regulate the trafficking of proteins such as the mannose 6-phosphate receptor to lysosomes (55, 59–62). Initially, wild-type and a form of CTLA-4 with a mutation at tyrosine 201 (which disrupts the AP-1 binding site (Y201F)) were compared for localization in various intracellular compartments. Intracellular membranes were subfractionated on a Percoll/metrizamide discontinuous density gradient to purify Golgi-enriched, mitochondrial, and lysosomal vesicles, as described in Materials and Methods (63). In addition to the Golgi-enriched fraction, two populations of lysosomes were defined according to their density in the Percoll/metrizamide gradients, one light and another heavier lysosomal fraction that cosediments with the mitochondria. Immunoblotting with antisera against a Golgi-specific 58-kDa protein confirmed the identity of the Golgi fraction (Fig. 4A, upper left panel, lane 1), and a marker for lysosomes lamp-2 was found in the two lysosomal fractions (lower panel, lanes 2 and 3). In addition, the purity of lysosomes was verified by the lysosomal marker enzyme β-hexosaminidase. As shown in Fig. 4A (upper right panel), significant β-hexosaminidase activity could be demonstrated in both the light and heavy lysosomal fractions, with no activity found in the cytosolic and Golgi-enriched fractions. We were therefore successful in separating Golgi and lysosomes from each other.

To assess the subcellular localization of the CTLA-4/AP-1 complexes, CTLA-4 and AP-1 were analyzed for the presence in the different cytoplasmic compartments. CTLA-4 was found in both Golgi-enriched and lysosomal fractions (Fig. 4B, middle left panel, lanes 2 and 3). Within the lysosomal fraction, wild-type CTLA-4 was found primarily in the light lysosomal fraction (Fig. 4B, middle panel, lane 3). By contrast, AP-1 was found in the Golgi-enriched fraction and the cytosol (Fig. 4B, lower left panel, lanes 1 and 2). Anti-CTLA-4 precipitation followed by anti-γ-chain immunoblotting showed that CTLA-4/AP-1 complexes were detected exclusively in the Golgi-enriched fraction (Fig. 4B, upper left panel, lane 2).

To address whether the intracellular binding of CTLA-4 and AP-1 influenced the intracellular accumulation and processing of CTLA-4, the steady state expression of wild-type CTLA-4 was compared to the mutant Y201F in the Golgi membranes. Significantly, Y201F transfectant showed a marked loss of CTLA-4 in the Golgi and lysosomal compartments (Fig. 4B, middle right panel, lanes 2 and 3). With the overexposure of the gel, it was possible to detect only small amounts of intracellular material (lane 6 vs 5). Furthermore, as expected with the loss of CTLA-4, anti-CTLA-4 coprecipitated no detectable AP-1 (Fig. 4B, upper right panel, lane 2). This occurred despite the presence of normal levels of AP-1 in the Golgi-enriched and cytosolic fractions of the Y201F-expressing cells (Fig. 4B, lower panel, lanes 1 and 2). These findings indicate that the disruption of the AP-1 binding site in CTLA-4 resulted in the loss of CTLA-4 from the Golgi and the lysosomes.

A difference in shuttling between the WTCTLA-4 vs Y201F mutant to the lysosomes was also apparent. In this case, cells were treated with ammonium chloride to neutralize lysosomal pH and block degradation (64). Only under these conditions could one begin to visualize the Y201F mutant in the lysosomal compartment (Fig. 4C, lanes 5 and 6). Although untreated cells showed no protein (Fig. 4C, lanes 1–3), ammonium chloride treatment allowed for the detection of small amounts of the mutant Y201F in both light and heavy lysosomal fractions (lanes 5 and 6). Further enrichment and separation of the matrix and membranes of the lysosomes showed receptor binding primarily to the lysosomal membranes (lanes 7 and 9 vs 8 and 10). These data demonstrate that the GVYYKM motif influences the accumulation of CTLA-4 in the Golgi and shuttling CTLA-4 from the Golgi to the lysosomes. Pulse-chase experiments showed that the difference in the levels of WTCTLA-4 vs Y201F mutant in the Golgi fraction was not due to differences in rates of protein biosynthesis (data not shown). AP-1 is therefore necessary for the efficient transport and degradation of CTLA-4 to the lysosomal compartment.

**Ca2+-dependent increase in cell surface expression**

Accumulated intracellular CTLA-4 can be released from intracellular stores to the cell surface by exposure to ionomycin, and to a lesser extent, by phorbol ester and TCR ligation (19–21). Our data combined with the data of others (21) on the effect of the Y201F

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**FIGURE 3.** Intracellular localization of CTLA-4/AP-1 complexes. CTLA-4/AP-1 complexes are found primarily in intracellular compartments. Surface CTLA-4 from wild-type CTLA-4 transfectants was precipitated by preincubating intact cells with anti-CTLA-4 Ab at 4°C, followed by washing and immunoprecipitation. Lysates were subjected to a round of preclearing with protein A-Sepharose, followed by a second precipitation to purify intracellular CTLA-4. Precipitates were blotted with anti-AP-1 antiserum. Lane 1, Anti-CD28 precipitation; lane 2, anti-CTLA-4 from whole cell lysates; lane 3, surface CTLA-4; lane 4, intracellular CTLA-4; lane 5, cell lysate.
FIGURE 4. Subcellular localization of CTLA-4/AP-1 complexes. A, Golgi 58-kDa protein and lamp-2 blotting of membrane fractions. Left panel, Lysates of subcellular fractions were immunoblotted with antiserum against a Golgi-specific 58-kDa protein (lanes 1–3, upper panel) or with antiserum (lamp-2) against lysosomes (lanes 1–3, lower panel). Right panel, Subcellular fractions were prepared as described in Materials and Methods, and each fraction was assayed for specific β-hexosaminidase activity. B (left), CTLA-4/AP-1 complexes are restricted to Golgi-enriched membranes. Subcellular fractionation of intracellular membranes was performed according to Materials and Methods. Each fraction was precipitated with anti-CTLA-4 mAb and immunoblotted with anti-γ-chain antiserum (lanes 1–4, upper panel) or anti-CTLA-4 mAb (lanes 1–4, middle panel). Lysates of subcellular fractions were immunoblotted with anti-γ-chain antiserum (lower panel). B (right), Disruption of AP-1 binding site results in a loss of CTLA-4 from Golgi and lysosomal membranes. Subcellular fractionation of intracellular membranes of DC27.10 cells transfected with CTLA-4Y201F mutant was performed as described. Each fraction was precipitated with anti-CTLA-4 mAb and immunoblotted with anti-γ-chain antiserum (lanes 1–4, upper panel) or anti-CTLA-4 mAb (lanes 1–4, middle panel). Lanes 5 and 6 represent overexposed version of lanes 1 and 2. Lysates of subcellular fractions were immunoblotted with anti-γ-chain antiserum (lower panel). C, Lysosomal and lysosomal/mitochondrial fractions from untreated (lanes 1–3) and ammonium chloride-treated (lanes 4–10) DC27.10 cells stably transfected with Y201F mutant were immunoblotted with anti-CTLA-4 mAb.
mutation on the accumulation of CTLA-4 in the Golgi compartment support the idea that the YVKM motif is needed for this retention. One possibility is that AP-1 binding could also influence the release of CTLA-4 from the Golgi to the cell surface. Unfortunately, dominant-negative forms of the AP-1 complex are not available to test this question directly. Instead, it was possible to assess whether the release of intracellular CTLA-4 was accompanied by a loss of CTLA-4/AP-1 binding in cells. As seen in Fig. 5A, the Ca^{2+} ionophore ionomycin caused a time-dependent increase in surface expression of CTLA-4 (left panel). Exposure to extracellular EGTA completely inhibited the increase in surface expression, indicating a requirement for a Ca^{2+} influx (Fig. 5A). The effect of ionomycin in increasing surface expression of CTLA-4 was not the result of a disruption of receptor endocytosis, as shown by internalization and uptake studies (data not shown). Significantly, we found that this increase in surface expression occurred in the absence of de novo transcription or protein synthesis. Actinomycin D, a potent inhibitor of transcription (65), had no effect on the increased expression (left panel). Furthermore, pulse labeling for 30 min with [35S]methionine at various times following ionomycin treatment showed no change in the synthesis of the protein (right panel, lanes 2–5). The ability of ionomycin to induce the release of receptor without inducing an increase in protein synthesis allowed an examination of changes in AP-1 binding upon the release of receptor to the cell surface.

To assess whether these events led to a diminished binding of AP-1, cells treated with ionomycin were subjected to precipitation with anti-CTLA-4 mAb, followed by immunoblotting with an anti-γ-chain antiserum (upper panel) or CTLA-4 mAb (middle panel). Equal amount of cell lysates was immunoblotted with anti-γ-chain antiserum for the presence of AP-1 complexes (lanes 2–4, lower panel).
Increased CTLA-4 synthesis is accompanied by increased AP-1 binding and accumulation in the lysosomes

Because AP-1/CTLA-4 binding is not influenced by the release of CTLA-4 to the cell surface, another issue was whether the interaction could regulate steady state levels of intracellular receptor. Under conditions in which there is an increase in CTLA-4 synthesis, but no release of receptor to the cell surface, the cell would need a mechanism to maintain steady state levels of the receptor in the Golgi. Otherwise, the Golgi would become overburdened with newly synthesized accumulated CTLA-4. From this analysis, we found that treatment of cells with phorbol ester (PMA) caused a 2–3-fold increase in protein synthesis, as detected by a pulse labeling with [35S]methionine (Fig. 6A, right panel). This occurred with only a moderate effect on surface expression (Fig. 6A, left panel). Significantly, the increased synthesis was accompanied by a 2–3-fold increase in AP-1 binding (Fig. 6B, middle panel; see histogram). The 2–3-fold increase in binding matched the increase in protein synthesis. At the same time, despite the increase in biosynthesis, as shown in two experiments (Fig. 6B), the overall level of intracellular CTLA-4 remained more or less the same, as detected by anti-CTLA-4 immunoblotting (upper panel). This maintenance of intracellular levels of receptor may be accounted for by an increase in AP-1 binding and its ability to facilitate trafficking to lysosomes for degradation. PMA also increased the expression of the Y201F mutant, although its ultimate fate remains to be established.

To assess whether the increased synthesis and AP-1 binding were accompanied by an increase in the appearance of intracellular CTLA-4 in the lysosomes, cells were labeled with a 30-min pulse and chased in the absence and presence of ammonium chloride (Fig. 6C). In the absence of ammonium chloride, both untreated and phorbol ester-treated cells showed degradation of protein by 2–4 h (lanes 1–3 and 7–9). As expected from increased synthesis, labeling of cells during the initial pulse was higher in phorbol ester-treated cells than untreated cells (lane 7 vs 1). Exposure to ammonium chloride caused a moderate rescue from degradation in untreated cells (lanes 4–6). By contrast, the combination of phorbol ester and ammonium chloride caused an increased accumulation of CTLA-4 in the lysosomes, which was significantly higher than in nonstimulated cells (lanes 10–12 vs 4–6). These data are therefore consistent with the hypothesis that the increase in CTLA-4 synthesis is accompanied by a preferential increase in shuttling of newly synthesized material to the lysosomal compartment. These observations support a model in which increased synthesis of receptor is accompanied by increased binding to AP-1 and an increase in its appearance in the lysosomal compartment without an increase in the accumulated receptor in the intracellular compartment. Overall, these observations are consistent with a role for CTLA-4/AP-1 binding as a homeostatic regulator of steady state levels of intracellular CTLA-4.

TCR/CD3 complex shares an ability to interact with AP-1 and AP-2 complexes

AP-1 and AP-2 failed to bind to the CD28 Ag, as assessed by immunoblotting of anti-CD28 precipitates (Fig. 1), and two-hybrid analysis (43–46). However, one possibility is that the adaptor complexes might bind to the TCR/CD3 complex in addition to CTLA-4. Such an interaction could hypothetically act to bridge the CTLA-4 with TCR in a manner that is not available to CD28. To assess a possible interaction, anti-CD3 was used to precipitate protein followed by blotting with the anti-γ-chain antiserum (Fig. 7A, upper panel, lane 2). From this, it is evident that a well-represented γ-chain was present that comigrated with the same band precipitated by anti-CTLA-4 (lane 4). A band of the same size was also recognized in the cell lysate (lane 5). By contrast, anti-CD28 failed to precipitate the protein (lane 3). As an additional negative control, anti-CTLA-4 also failed to precipitate the γ-chain from nontransfected CTLA-4-negative DC27.10 control cells (lane 1). Similarly, using a specific anti-β-chain antiserum, anti-CD3 was found to precipitate β-subunits of both the AP-1 and AP-2 (Fig. 7B, lower panel, lane 2). As discussed in Fig. 1, the position of the AP-1 and AP-2 β-chains has been previously well documented with the upper band corresponding to AP-1 and the lower band to AP-2 β-chains (37, 66). For specificity, we were also interested in whether AP-1 specifically bound to the TCR/CD3 complex in the Golgi compartment. As shown in Fig. 4 and described in Materials and Methods, intracellular membranes were subfractionated on a Percoll/metrizamide discontinuous density gradient to purify Golgi-enriched, mitochondrial, and lysosomal vesicles (63). As in the case of CTLA-4, anti-CD3-precipitated γ-chain was found restricted to the Golgi-enriched fraction (Fig. 7B, lane 2). As positive controls, AP-1 was found in the Golgi-enriched fraction, cytosol, and to a smaller extent in the lysosomal fraction (Fig. 7B, lanes 5–7). These data indicate that CTLA-4 and the TCR/CD3 complex share the property of being associated with the AP-1 and AP-2 complexes in T cells.

Discussion

CTLA-4 can be distinguished from CD28 due to its negative regulation of T cell proliferation and due to its unusual accumulation in the cytoplasm of T cells (19, 20). Little information exists regarding the mechanisms that influence the intracellular accumulation of CTLA-4 in T cells. This issue is of importance because factors that influence the level of accumulated intracellular receptor will control the amount of receptor available for release to the cell surface. In this study, we define an interaction between intracellular CTLA-4 and the clathrin adaptor complex AP-1 in the Golgi-enriched compartment of T cells (Fig. 4). Peptide competition and in vivo expression of chimeras further showed that AP-1 binds to the nonphosphorylated GVYVKM motif within the CTLA-4 cytoplasmic tail. AP-1 binding appears linked to its classical role in regulating the shuttling of proteins from the Golgi compartment to lysosomes for degradation (24–26). Consistent with this function, phorbol ester-induced increases in CTLA-4 synthesis were accompanied by an increase in AP-1 binding, and a concomitant increase in the appearance of [35S]methionine pulse-labeled CTLA-4 in neutralized lysosomes. At the same time, the overall level of intracellular CTLA-4 remained the same despite the increased rate of receptor synthesis. Together, these observations support a role for AP-1/CTLA-4 binding in the maintenance of steady state levels of CTLA-4 in the intracellular compartments of T cells. Finally, we demonstrate that the TCR/CD3 complex also binds to AP-1 and AP-2 complexes, providing a possible link between these two receptors in the regulation of T cell function.

The interpretation of the data was complicated by the fact that the AP-1 binding site (i.e., the GVYVKM site) also plays a role in regulating the release of the receptor from the Golgi to the cell surface (19, 20, 67). This latter observation was clearly supported by the observation of an almost complete loss of the Y201F mutant in purified Golgi-enriched membranes of T cells (Fig. 4). One possibility was that the AP-1 complex binding is responsible for this retention, a possibility that is difficult to establish without a dominant-negative form of AP-1 for testing in transfection studies. However, with the limited tools presently available, we could find no evidence to implicate AP-1 in retaining CTLA-4 in the Golgi.
Instead, an ionomycin-induced Ca\(^{2+}\) influx was observed to induce surface expression of the receptor without having an effect on the binding of AP-1 to the receptor. If AP-1 controlled the release of the receptor to the cell surface, one would have expected a decreased association between AP-1 and the receptor. Future studies will be needed to determine the nature of the YVKM-recognizing protein that regulates the surface expression of the CTLA-4 molecule. Candidate proteins include PI 3-kinase, SHP-2, and...
another adaptor complex, AP-3. Instead, our data are most compatible with the well-documented role of the AP-1 complex in sorting proteins in the TGN to the endosomal/lysosomal pathway for degradation. Consistent with this, AP-1/CTLA-4 complexes were found primarily in the Golgi-enriched membrane fraction, the site of shuttling to the lysosomes. Moreover, increases in CTLA-4 synthesis induced by phorbol ester and monitored by [35S]-methionine labeling were reproducibly accompanied by a 2–3-fold increase in CTLA-4 binding to AP-1 (Fig. 6). This in turn was followed by a marked increase in the accumulation of CTLA-4 in the purified lysosomal compartment, as monitored by [35S]-methionine labeling under conditions in which the degradation was blocked.

At the same time, although there was an increase in synthesis, there was no increase in the overall level of intracellular receptor, thus supporting the view that AP-1 serves to maintain intracellular CTLA-4 in defined level. Pulse-chase comparisons between WTCTLA-4 and Y201F mutant also showed a more rapid degradation of the WT receptor (data not shown). Without a mechanism to maintain steady state levels of receptor, the Golgi would become overburdened with newly synthesized CTLA-4. This might prevent leakiness in the expression of CTLA-4 at the cell surface. AP-1 binding to CTLA-4 would therefore act to maintain intracellular levels of CTLA-4 in the Golgi for its eventual release to the cell surface upon TCR/CD3 ligation. Small alterations in the levels of CTLA-4 expression could have major consequences on T cell function. For example, anti-CTLA-4 Abs can block proliferation as early as 6 h postactivation, where the levels of CTLA-4 expression are too low to be detected by FACS analysis (17).

The exact nature of the AP-1 subunit responsible for binding the CTLA-4GVYVKM motif remains to be clarified. Our in vitro and in vivo analysis showed binding to intact AP-1 tetrameric complexes. Previous studies using the two-hybrid screen failed to detect μ-1 binding to the CTLA-4 cytoplasmic domain, suggesting that other subunits are involved (43–46). Other chains in the complex are capable of binding proteins, such as in the case of the β-chain that was recently reported to bind CD4 (53). Future studies will be needed to identify the binding subunit.

Another feature of interest is the ability of AP-1 to form a stable biochemical complex in detergent lysates. This complex is the first example of binding between the AP-1 complex and a protein or receptor that can withstand exposure to detergent. In this context, a comparison can be made with AP-2 complex binding to the epidermal growth factor receptor (40). In both cases, some 1.5–3% of AP complexes remain stably associated with the receptor in detergent. In the case of the epidermal growth factor receptor, despite the low stoichiometry of binding as estimated in the formation of detergent-resistant complexes, AP-2 is widely accepted as the major mechanism by which the receptor is removed from the cell surface (40). A similar stoichiometry of binding was noted in our transfectants in the case of AP-2/CTLA-4 binding (data not shown). Detergent sensitive AP complexes are known to regulate the trafficking of other proteins. For example, recognition motifs have been identified in the two-hybrid system, but the integrity of these complexes is lost during the biochemical purification (39–42).

Finally, we demonstrate that CTLA-4 and the TCR/CD3 complex share an ability to bind to the AP-1 and AP-2 complexes, thus providing a potential link between these two receptors (Fig. 7). AP-1 binding to the TCR/CD3 complex was also found localized to the Golgi apparatus. The binding mechanism is unknown, but may involve dileucine-based motifs that are present in CD3γ subunits and bind to the β-chain of AP-1 (53). The shared use of AP-1 and AP-2 with binding to potentially distinct binding sites could facilitate an interaction between these receptors, both in the Golgi and the cell surface. AP complexes in turn have been noted to bind to each other (68). This could provide a mechanism to favor a CTLA-4 association with the TCR relative to CD28. This in turn could help facilitate the association between CTLA-4 and the TCR/CD3 complexes described by others (14). In addition, the shared AP-2 binding suggests a mechanism by which CTLA-4 might alter the redistribution of the TCR/CD3 complex needed for appropriate signaling. We are presently exploring the possible function of AP-1/AP-2 complex binding to the two receptors in T cell signaling.

FIGURE 7. TCR/CD3 complex binds to AP-1 and AP-2 complexes in T cells. A. TCR/CD3 complex binding to AP-1 complexes. Upper panel. Vector-transfected, human CD28- and CTLA-4-expressing DC27.10 cells were subjected to immunoprecipitation with anti-CD3, anti-CD28, or anti-CTLA-4 mAbs, and were subjected to immunoblotting with anti-γ-chain antisera of AP-1. Lane 1, Control, anti-CTLA-4 precipitation from vector-transfected cells; lane 2, anti-CD3 from CTLA-4 transfectants; lane 3, anti-CD28 from human CD28 transfectants; lane 4, anti-CTLA-4 from CTLA-4 transfectants; lane 5, cell lysate. Lower panel. Immunoprecipitations, as described in A, were subjected to immunoblotting with anti-β-chain antiserum cross-reactive with AP-1 and AP-2. Lane 1, Control (anti-CTLA-4 precipitation from vector-transfected cells); lane 2, anti-CD3 from CTLA-4 transfectants; lane 3, anti-CD28 from human CD28 transfectants; lane 4, anti-CTLA-4 from CTLA-4 transfectants; lane 5, cell lysate. B, TCR/CD3-AP-1 complexes are restricted to Golgi-enriched membranes. Subcellular fractionation of intracellular membranes was performed according to Materials and Methods. Each fraction was precipitated with anti-CD3 mAb and immunoblotted with anti-γ-chain antiserum (lanes 1–4). Lysates of subcellular fractions were immunoblotted with anti-γ-chain antiserum (lanes 5–8).
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