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Regulation of Cell Surface Expression of CTLA-4 by Secretion of CTLA-4-Containing Lysosomes Upon Activation of CD4+ T Cells

Tomohiko Iida,*† Hiroshi Ohno,§ Chiaki Nakaseko,* Machie Sakuma,* Mitsue Takeda-Ezaki,‡ Hisashi Arase,* Eiki Kominami,‡ Takehiko Fujisawa,‡ and Takashi Saito2,*

CTLA-4 is expressed on the surface of activated T cells and negatively regulates T cell activation. Because a low-level expression of CTLA-4 on the cell surface is sufficient to induce negative signals in T cells, the surface expression of CTLA-4 is strictly regulated. We previously demonstrated that the association of CTLA-4 with the clathrin-associated adaptor complex AP-2 induces internalization of CTLA-4 and keeps the surface expression low. However, the mechanism to induce high expression on the cell surface upon stimulation has not yet been clarified. To address this, we investigated the intracellular dynamics of CTLA-4 by analyzing its localization and trafficking in wild-type and mutant CTLA-4-transfected Th1 clones. CTLA-4 is accumulated in intracellular granules, which we identified as lysosomes. CTLA-4 is degraded in lysosomes in a short period, and the degradation process may serve as one of the mechanisms to regulate CTLA-4 expression. Upon TCR stimulation, CTLA-4-containing lysosomes are secreted as proven by the secretion of cathepsin D and β-hexosaminidase in parallel with the increase of surface expression of CTLA-4 and lysosomal glycoprotein 85, a lysosomal marker. These results suggest that the cell surface expression of CTLA-4 is up-regulated upon stimulation by utilizing a mechanism of secretory lysosomes in CD4+ T cells. The Journal of Immunology, 2000, 165: 5062–5068.

Cytotoxic T lymphocyte Ag-4 (CTLA-4) is a glycoprotein belonging to the Ig superfamily, and it possesses a similar structure to CD28 (1, 2). It functions as a coreceptor by binding to the common ligands with CD28 and CD80/CD86 on APCs or target cells mostly through the MYPPP motif in the transmembrane proximal region of the extracellular domains (3). However, CD28 and CTLA-4 are different mainly in three aspects. First, CD28 transduces an activation signal for T cell proliferation and cytokine secretion (4–8), whereas CTLA-4 down-regulates T cell activation (9, 10). Second, CTLA-4 has ~10-fold higher affinity for the ligands than CD28 (11), and, thus, a low level of CTLA-4 expression on the cell surface can readily compete with CD28 for ligand binding, resulting in inhibition of T cell activation. Third, the surface expression of CTLA-4 is strictly regulated: CTLA-4 is expressed transiently upon T cell activation, whereas CD28 is expressed constitutively (1, 2).

Recent studies have revealed that CTLA-4 is localized mainly in intracellular granules, and such granules containing CTLA-4 were partially overlapped with perforin-containing secretory granules and also with endocytic compartments in CD8+ T cells. It has been further revealed that trafficking of CTLA-4 is dynamically regulated between intracellular stores and the cell surface (12). However, because CD4+ T cells do not possess any typical secretory granule, the intracellular localization and trafficking of CTLA-4 in CD4+ Th cells remain unclear. Although CTLA-4 was originally identified as a CTL-specific molecule, CTLA-4 is also expressed in CD4+ T cells and is suggested to play essential roles in Th1/Th2 development, cytokine production, and tolerance induction (13–17). Indeed, CTLA-4-deficient mice revealed strong selection toward Th2 development, indicating a crucial role of CTLA-4 in Th1 development and function (18).

We have previously demonstrated that CTLA-4 binds to the μ2 subunit of the clathrin-associated adaptor protein complex AP-2 through the tyrosine-based motif containing GVVVKM within the cytoplasmic domain of CTLA-4, and that tyrosine phosphorylation of the motif prevents the binding to AP-2 and probably keeps CTLA-4 on the cell surface (19–22). We have suggested from these data that AP-2-mediated endocytosis of CTLA-4 plays an important role in the regulation of the surface expression of CTLA-4. Indeed, tyrosine mutants of CTLA-4, which failed to bind to μ2, exhibited a higher expression of cell surface CTLA-4 than wild-type (WT)3 CTLA-4 on resting T cells (23). However, we observed that a mutant CTLA-4 lacking most of the cytoplasmic tail exhibited much higher expression of the cell surface CTLA-4, and, in addition, the surface expression of the mutant CTLA-4 was further up-regulated upon T cell activation. These observations suggest that mechanism(s) other than AP-2-mediated endocytosis may be involved in the regulation of the surface expression of CTLA-4. More importantly, the mechanism to induce high expression of CTLA-4 from the intracellular pool to the cell surface has not yet been clarified.

3 Abbreviations used in this paper: WT, wild type; β-hex, β-hexosaminidase; CHX, cycloheximide; lgp, lysosomal glycoprotein; FasL, Fas ligand.
Here, we have studied the intracellular localization and trafficking of CTLA-4 in CD4⁺ T cells transfected with various mutants of CTLA-4. We found that intracellular CTLA-4 is mainly localized in lysosomes, and these lysosomes are secreted upon T cell activation. Therefore, our results demonstrate that CD4⁺ T cells enhance the surface expression of CTLA-4 upon activation by the mechanism of the activation-induced secretion of lysosomes. From these aspects, we will discuss the dynamic regulation of CTLA-4 expression.

Materials and Methods

Cells
A murine Th1-type T cell clone, 23-1-8 (24, 25), was cultured as previously described (23). 23-1-8 cells were transfected by retrovirus-mediated gene transfer with WT CTLA-4 and its mutants, and infected T cells were used as bulk population and not as a clone as previously described (23).

Antibodies
Anti-CTLA-4 mAb, UC10, was kindly provided by Dr. J. Bluestone (University of Chicago, Chicago, IL), and biotinylated UC10 was prepared by standard technique. Alexa488 (green fluorescence-conjugated UC10 was prepared using an Alexa488 Protein Labeling Kit (Molecular Probes, Eugene, OR). Rabbit anti-lyosomal glycoprotein (Igp) 110 Ab is directed to the peptide of the C region of the rat Igp110, and rabbit anti-cathepsin D Ab is prepared against rat cathepsin D. Rabbit anti-rat Igp85 was provided by Dr. M. Himeno (Kyushu University, Fukuoka, Japan). Biotinylated anti-mouse Fas ligand (Fasl) mAb, K10, was provided by Dr. K. Okumura (Juntendo University, Tokyo, Japan). Rabbit anti-human Rab5a Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated goat anti-aramenic hamster IgG Ab and Cy3-conjugated goat anti-rabbit IgG Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-goat anti-rabbit IgG Ab, streptavidin-PE, and rabbit IgG Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa488 (green fluorescence)-conjugated UC10 was prepared using an Alexa488 Protein Labeling Kit (Molecular Probes, Eugene, OR). Anti-CTLA-4 mAb, UC10, was kindly provided by Dr. J. Bluestone (University of Chicago, Chicago, IL)

Immunostaining and confocal laser scanning microscopy

Cells were cultured on coverslips overnight, and fixed in 4% (w/v) paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 15 min at room temperature. The coverslips were then washed and mounted onto slides with PermaFluor Aqueous Mounting Medium (Shandon, Pittsburg, PA) and examined with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).FITC-conjugated goat anti-aramenic hamster IgG Ab and Cy3-conjugated goat anti-rabbit IgG Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).FITC-goat anti-rabbit IgG Ab, streptavidin-PE, and rabbit IgG Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Flow cytometry

T cells were stained with first Ab for 45 min followed by appropriate second Ab for 45 min. All steps were performed at 4°C in a buffer containing 0.1% BSA and 0.05% sodium azide. Cells were analyzed by FACScan (Becton Dickinson, Mountain View, CA) with the CellQuest software.

T cell stimulation

For plate coating, 1 µg/ml anti-TCRβ mAb (H57-597), anti-CD3ε mAb (145-2C11), and anti-CD3ζ mAb (H146-968) as control were incubated on 24-well plates at 4°C overnight. Wells were washed with PBS four times. WT CTLA-4-transfected T cells were added at 2 × 10⁶/well in 600 µl to complete RPMI 1640 in the presence of 5 µg/ml murine IL-2 and incubated at 37°C with 5% CO₂ for 24 h. For detection of protease activity, the culture medium was collected and the supernatants after centrifugation at 11,000 × g for 5 min were used. For normal T cell stimulation, CD4⁺ T cells were isolated from spleen cells of C57BL/6 mice by depleting CD8⁺ cells and surface Ig⁺ cells using anti-CD8 mAb and anti-mouse IgAb and magnetic beads. After CD4⁺ T cells were stimulated with Con A with irradiated C57BL/6 splenocytes for 2 days, T cells were separated by lymphohight M and subjected for restimulation.

Assay for β-hexosaminidase (β-hex) activity

Fifty microliters of supernatants were preincubated for 5 min at 37°C with 150 µl of 0.2 M sodium acetate buffer (pH 4), and further incubated with 100 µl of 3 mM 4-methylumbelliferyl-β-D-glucosaminide. The reaction was stopped by addition of 2 ml of 0.1 M glycine (pH 10.5). Fluorescence was measured with an F-2000 spectrophotometer (Hitachi, Tokyo, Japan) at excitation 360 nm/emission 450 nm. The culture medium of nonstimulated T cells was also measured and subtracted as background from each sample.

Assay for cathepsin D activity

Fifty microliters of the culture medium were incubated for 10 min at 37°C with 80 µl of 250 µM 7-methoxycoumarin-4-yl acetyl-GKPILFFRLK (DNP) γ,NH₂ in 0.5% DMSO and 10 ml of 50 mM sodium acetate buffer. The reaction was stopped by addition of 1 ml of 5% trichloroacetic acid, and fluorescence was measured at excitation 328 nm/emission 393 nm. The background level of cathepsin D was measured similarly to the assay for β-hex.

Results

Localization of mutant CTLA-4 in CD4⁺ T cells clones

In a previous study (23), we generated four different forms of mutant CTLA-4 as illustrated in Fig. 1A: three mutants with the alteration of either or both tyrosines in the cytoplasmic domain of CTLA-4 to glycine(s) (Y165G, Y182G, Y165/182G), and one deletion mutant lacking most of the cytoplasmic tail except for the membrane-proximal 7 amino acids (ΔCP7). These mutants were transfected into a murine CD4⁺ Th1 clone, 23-1-8, to analyze the

FIGURE 1. Localization of mutant CTLA-4 in Th1 clones. A. Schematic structures of WT and various mutant forms of CTLA-4. These constructs were transfected into murine Th1 clone 23-1-8 as previously described (23). Black boxes indicate the transmembrane region. EX, Extracellular; TM, transmembrane; CP, cytoplasmic domains; a.a., amino acid. B. Localization of CTLA-4 in various mutant CTLA-4-transfected Th1 clones (23-1-8). Each transfectant was fixed, permeabilized, stained with anti-CTLA-4 mAb (UC10) followed by FITC-conjugated secondary Ab, and analyzed by confocal microscopy.
CTLA-4-containing vesicles (Fig. 2). In contrast, anti-lgp110 Ab stained intracellular vesicles (Fig. 2B). Although brightly stained and large vesicles were prominent in WT and mutant CTLA-4 transfectants, it was noted that weakly stained and smaller vesicles as well as a perinuclear compartment were also stained with the Ab (Fig. 1B). In addition, the intracellular vesicles containing CTLA-4 were significantly reduced in T cells expressing Y165/182G mutant CTLA-4, whereas the cell surface became bright. In distinct contrast, these granules were greatly diminished in T cells transfected with ΔCP7. Although brightly stained and large vesicles were prominent in WT and mutant CTLA-4 transfectants, it was noted that weakly stained and smaller vesicles as well as a perinuclear compartment were also stained with the Ab (Fig. 1B). These data suggest that intracellular CTLA-4 was located mainly in large granules but was also distributed in some other organelles.

### Localization of CTLA-4 in lysosome

To define the characteristics of CTLA-4-containing vesicles in CD4⁺ T cells, we performed double staining for both CTLA-4 and several marker proteins that are well-defined and known to be specific for various organelles (Fig. 2). It has been suggested that CTLA-4 is partially colocalized with perforin (as in secretory granules) and transferin receptor (as in endocytic compartments) in CD8⁺ T cells (12). However, neither perforin nor typical secretory granules are present in CD4⁺ T cells. Because it has been reported that FasL was localized in the perforin⁺ lytic granules in CD8⁺ T cells (26) and FasL is induced even in CD4⁺ T cells upon activation, colocalization of CTLA-4 and FasL in these Th1 clones was examined after stimulation with Con A for 2–6 days. As shown in Fig. 2A, FasL was mainly present in the intracellular large vesicles and colocalized with CTLA-4 (Fig. 2A).

To analyze vesicles involved in the endocytic pathway, WT CTLA-4-transfected clones were immunostained for several marker proteins localized to the endocytic compartments, such as anti-Rab5a and anti-lgp110 Abs. Rab5a is an early endosomal marker and lgp110 is a late endosomal/lysosomal marker. Staining of Rab5a showed that Rab5a⁺ vesicles were very small and partially overlapped with the small type of CTLA-4-containing vesicles (Fig. 2B). In contrast, anti-lgp110 Ab stained intracellular large vesicles that were mostly colocalized with the large type of CTLA-4-containing vesicles (Fig. 2C). Because the preform of cathepsin D immediately changes into the active form and hardly detected, anti-cathepsin D has been thought to stain only the active form of cathepsin D, which is specific for lysosomes. Therefore, we stained for cathepsin D to discriminate late endosomes and lysosomes. The staining pattern of WT CTLA-4-transfected Th1 cells for cathepsin D was similar to that of lgp110 (Fig. 2D). From these data, we concluded that most of the intracellular large vesicles containing CTLA-4 in CD4⁺ Th cells were lysosomes.

### Degradation of intracellular CTLA-4 in lysosome

Because we found that intracellular CTLA-4 is accumulated mainly in lysosomes, we next tried to analyze the physiological significance of lysosomal localization of CTLA-4, whether CTLA-4 is accumulated in lysosomes for degradation or storage. To address this question, we treated T cells with cyclohexamide (CHX) to inhibit new synthesis of CTLA-4 and examined whether ready-made CTLA-4 could be stably maintained in lysosomes. The amount of intracellular CTLA-4 was significantly decreased during the 2-h treatment with CHX, and remarkably diminished after 4 h (Fig. 3A). This observation suggests that intracellular CTLA-4 was degraded in lysosomes in a relatively short period in the absence of any stimulation. Indeed, when we treated cells with several reagents, which are known to inhibit lysosomal degradation, such as NH₄Cl, methylamine, and bafilomycin, the cell surface expression (Fig. 3B) as well as intracellular expression (data not shown) of CTLA-4 was augmented with any of these reagents. In contrast, these treatments did not induce any surface expression of lysosomal proteins, such as lgp85 (data not shown).

Collectively, these results suggest that the degradation of CTLA-4 in lysosomes is one of the mechanisms to regulate the surface expression of CTLA-4. CTLA-4 may be degraded constantly, and it will be accumulated when the synthesis level overcomes degradation upon stimulation under physiological conditions.
B细胞被免疫固定化抗CD3 mAb刺激。WT CTLA-4转染细胞被用NH4Cl、甲基胺或巴氯和 CTLA-4在细胞表面的受体细胞（粗线）和非受体细胞（细线）被检测用流式细胞术。

图3. 降解调节CTLA-4表达。A. 表面和细胞内CTLA-4的减少。WT CTLA-4转染的T细胞在无CHX环境或CHX（10 μg/ml）的1、2或4 h中培养，固定并与ST646的mAb共孵育，然后用荧光显微镜检测。

图4. TCR刺激后CTLA-4和lgp85的细胞表面表达。

As shown in Fig. 5, the number of lysosomal granules was dramatically reduced upon stimulation, and the remaining granules were found to be localized to the proximal region of Ab-coated plates. These data indicate that lysosomes containing CTLA-4 indeed moved toward the plasma membrane and were secreted upon TCR stimulation.

Because such lysosomal secretion must be accompanied with secretion of lysosomal enzymes in the supernatant, we next analyzed the secretion of β-hex in the supernatant upon TCR stimulation. As shown in Fig. 6A, TCR stimulation with anti-TCR or anti-CD3 mAb induced β-hex release into the supernatant. Considering that these so-called lysosomal markers (lgp110, lgp85, and CD63, etc.) are present on both lysosomes and late endosomes and these granules also contain β-hex, the surface expression of lgp10/lgp85 and the secretion of β-hex does not necessarily reflect to lysosomal secretion. Therefore, we measured the activity of cathepsin D in the culture supernatant to confirm the lysosomal secretion, because the active form of cathepsin D is exclusively present in lysosomes. Enzymatic activity of cathepsin D was detected in the culture supernatants by TCR cross-linking (Fig. 6B). Thus, secretion of both β-hex and cathepsin D confirms lysosomal secretion upon TCR stimulation.

Degranulation of the secretory granules in mast cells or basophilic cells is known to be induced by the increase of intracellular Ca2+ concentration within a short period when treated with a high dose of Ca2+ ionophore. We questioned whether the observed lysosomal secretion in CD4+ T cells is also induced by the similar mechanism. To examine this possibility, we stimulated WT CTLA-4-transfected T cells with Ca2+ ionophore alone for a short period (10–120 min). Indeed, the secretion of both β-hex and cathepsin D was induced as quick as 10 min after stimulation (Fig. 6, C and D) with a high dose of Ca2+ ionophore, suggesting similar mechanism to the degranulation in mast cells.

Furthermore, we analyzed whether the regulation of CTLA-4 expression by lysosomal secretion is observed in normal T cells. Resting T cells do not possess CTLA-4 transcript, and CTLA-4 mRNA and protein are produced upon stimulation. CTLA-4-transfected Th clones, which express CTLA-4 within the cells, represent the cells equivalent to preactivated T cells. CD4+ splenic T cells were isolated and stimulated with Con A for 2 days to induce CTLA-4 within the cells as well as a low level of the cell surface expression. In the normal T cells, we confirmed that the most CTLA-4-containing vesicles are also stained with anti-lgp110 Ab.

Activation-induced secretion of lysosomes in CD4+ T cells

Lysosome is the acidic organelle containing a characteristic set of acid hydrolases, and is responsible for the degradation of internalized proteins from the endocytic pathways. However, it has been described that CTLs possess “secretory lysosomes” containing perforin and granzyme, which behave as secretory granules (27). Activation-induced extracellular release of hydrolytic enzymes towards the target cells is one of the mechanisms for target cytotoxicity. Such secretory lysosomes have been found widely in hemopoietic lineages, such as CTLs, NK cells, and granulocytes, but have not been analyzed in CD4+ T cells. To analyze the secretory lysosomes in CD4+ T cells, we examined whether CD4+ T cells secrete lysosomes upon physiological stimulation by using a lysosomal marker protein, lgp85, together with CTLA-4. Upon stimulation by cross-linking with anti-TCR or anti-CD3ε mAb, the surface expressions of both CTLA-4 and lgp85 were increased (Fig. 4), suggesting that CTLA-4-containing lysosomes were secreted and lysosomal membrane proteins appeared on the cell surface upon TCR stimulation.

We then examined whether the dynamic intracellular translocation of CTLA-4-containing lysosomes can be observed by confocal microscopy upon TCR stimulation. WT CTLA-4 transfectants were stimulated with immobilized anti-CD3ε mAb on a coverslip.
Thereafter, the preactivated T cells were restimulated by TCR cross-linking, and the surface expressions of CTLA-4 and lgp85 were analyzed. Similar to the results obtained on Th1 clones, stimulation of splenic CD4\(^{+}\) T cells induced enhancement of the cell surface expression of both CTLA-4 and lgp85 (Fig. 7B).

These results demonstrate that most CTLA-4 is accumulated in lysosomes in normal T cells upon T cell activation and also indicate the existence of activation-dependent secretory lysosomes in CD4\(^{+}\) T cells, and the cell surface expression of CTLA-4 is augmented by secretion of these lysosomes upon TCR stimulation.

**Discussion**

The CD28/CTLA-4 costimulation system for T cell activation has three major characteristics. One is that both share the same ligands, but CTLA-4 has 10-fold higher affinity; the second is that the expression of CD28 is constitutive, whereas CTLA-4 expression is induced by activation; and third, CD28 and CTLA-4 induce positive and negative signals for T cell activation, respectively. Due to these characteristics, once CTLA-4 is induced at a high level on the cell surface, T cell activation will be inhibited. Therefore, the expressionional regulation of CTLA-4 is critical for T cell function. Recent studies from several groups, including us, uncovered the mechanism of endocytosis of CTLA-4, which is mediated by the association of a tyrosine motif in the cytoplasmic tail of CTLA-4 with the \(\mu 2\) subunit of AP-2 complex (19–22). The mutation of this responsible tyrosine induced the higher expression of the surface CTLA-4.
In this study, we first analyzed the relationship between surface and intracellular CTLA-4. As previously shown (23), the majority of CTLA-4 is localized in intracellular large granules. Despite our expectation that the majority of Y165G mutant would be expressed on the cell surface, we surprisingly found that even Y165G mutant T cells accumulate intracellular large granules similarly to WT-CTLA-4; although the large vesicles containing CTLA-4 were reduced, they still remained to a significant degree in these Y165G-expressing cells. These data suggest that there is a mechanism other than the tyrosine motif/AP-2 system for internalizing and accumulating CTLA-4 in the large granules. In contrast, we found that large granules had almost disappeared in ΔCP7 transfectants. These results suggest a tyrosine-independent sorting signal within the cytoplasmic domain of CTLA-4 for targeting to lysosomes in addition to the tyrosine signals responsible for endocytosis through AP-2-binding. This feature of CTLA-4 resembles the regulation of FasL; the cytoplasmic tail of FasL may contain specific signal(s) for targeting to the lytic granules (26). Furthermore, considering that Y165G and Y165/182G mutant cells still possess CTLA-4-containing large granules within the cells, and that because the tyrosine phosphorylation of Y165, a physiologically resembling form of Y165G mutation, results in dissociation from AP-2 and induction of high expression of cell surface CTLA-4, these data suggest that the majority of the CTLA-4 protein is localized inside the cell even under the condition of maximal CTLA-4 expression on the surface of activated T cells (12, 28).

Confocal microscopic analysis revealed that CTLA-4 is localized in two distinct compartments, a majority in large granules and a minority in small vesicles. Because these small vesicles containing CTLA-4 were partially overlapped with Rab5a, a part of CTLA-4 was also localized in early endosomes, which is consistent with the endocytosis of CTLA-4. Although it has been generally believed that CD4+ T cells do not possess typical secretory granules, unlike CTL, NK cells, or granulocytes, we found that the large granules containing CTLA-4 were stained with various lysosome-specific markers, particularly for lgp110 and the active form of cathepsin D, and that they were secreted upon TCR stimulation. It has been described that “secretory lysosomes” are present in hemopoietic lineage cells, but, further, in the case of CTLs, cell activation induces extracellular release of hydrolytic enzymes present within the lysosomes toward the target cells, which might be one of the cytotoxic mechanism (29). Indeed, it has been shown that CTLA-4 is partly colocalized with lytic granules and endocytic compartments in CD8+ T cells (12).

Similar to these previous observations in CD8+ T cells, we observed that CTLA-4-expressing CD4+ T cells induced the cell surface expression of lgp85 upon T cell activation similarly to FasL. It was recently shown that the surface expression of CD63 was increased by Ca2+ ionophore treatment in T cells (26). However, because these marker proteins such as lgp110, lgp85, and CD63 are present in both lysosomes and late endosomes, it would be premature to conclude definitively that the increased expression of these markers reflects lysosomal secretion. In this study, therefore, we measured the activity of cathepsin D released into the culture medium because the active form of cathepsin D is exclusively present in lysosomes. The activity of cathepsin D was detected in the culture medium, in parallel with the increase of lysosomal markers and CTLA-4 on the cell surface upon TCR stimulation. Therefore, these observations demonstrated for the first time to our knowledge that the activation-induced secretion of lysosomes can be induced in CD4+ T cells. This may also partly explain the cytotoxic mechanism by CD4+ Th cells. These vesicles in CD4+ T cells contain CTLA-4 and are secreted upon T cell activation. Furthermore, we observed that CTLA-4-containing large granules moved to the proximity of the cell surface upon TCR engagement, which is consistent with the previous observation that CTLA-4 is localized toward the TCR engagement sites upon stimulation (12). The importance of the secretory granules for CTLA-4 expression has been recently suggested by the analysis of CD8+ T cells from patients with Chediak-Higashi syndrome (30). These cells failed to express CTLA-4 on the cell surface and resulted in lymphoproliferative disorder, whereas it is accumulated in the perforin+ giant secretory granules, suggesting that trafficking from the secretory granule is crucial for the surface expression of CTLA-4.

Most of our experiments were performed on various CTLA-4-transfected CD4+ Th clones that represent preactivated phenotype of T cells because these cells already accumulate intracellular CTLA-4 in lysosomes. In contrast, resting T cells do not have CTLA-4 within the cells. Upon TCR stimulation, CTLA-4 transcripts and then protein are synthesized and accumulated in intracellular lysosomes. Therefore, TCR stimulation of preexisting CTLA-4-containing lysosomes to induce secretion in Th clones is equivalent to restimulation of Con A-stimulated naive splenic T cells. We clearly demonstrated that lysosomal secretion was induced under such physiological condition.

It was recently reported that CTLA-4 also interacted with AP-1, which regulates trafficking from the Golgi to the lysosomal compartments (31). A part of CTLA-4 may be transported from the Golgi directly to lysosomes without targeting to the cell surface, although the exact contribution of each pathway needs to be determined. CTLA-4 transported to lysosomes either directly from the Golgi or by endocytosis from the cell surface was degraded in a short period. Because inhibition of this degradation resulted in an increase in the surface expression of CTLA-4, degradation of CTLA-4 in lysosomes appears to be one of the regulatory mechanisms for the surface expression of CTLA-4. In addition to the down-regulation by degradation in lysosomes, lysosomal targeting
of CTLA-4 has another advantage, in that activation-induced secretion of lysosomes can be utilized for regulating the surface expression of CTLA-4. Therefore, in combination with the previously described AP-2-mediated endocytosis of the cell surface receptor of CTLA-4, two additional mechanisms for regulating the cell surface expression of CTLA-4, degradation and lysosomal secretion, have been uncovered in this study. Furthermore, we previously found that tyrosine-phosphorylation of Y165 prevents AP-2-mediated endocytosis and keeps CTLA-4 on the cell surface (21). Because the level of tyrosine phosphorylation of CTLA-4 was very low, it is possible that tyrosine phosphorylation is induced only on the cell surface. Together with our recent finding that negative signals through CTLA-4 can be delivered in the absence of these tyrosines (23), phosphorylation may serve primarily to maintain CTLA-4 on the cell surface for signal transduction (32).

The molecular mechanism of lysosomal secretion is largely unknown. Similar to degranulation in mast cells and basophilic cells upon stimulation with Ca$^{2+}$ ionophore, CTLA-4-containing lysosomes were also secreted upon Ca$^{2+}$ ionophore stimulation within as quick as 10 min, as demonstrated by secretion of cathepsin D. As a summary, our current results together with previous observations allow us to make a new proposal concerning the dynamics of the sorting/expression regulation of CTLA-4. CTLA-4 may be mainly targeted to lysosomes and accumulated during the early stage of T cell activation. Although some proportion of CTLA-4 may be delivered to the cell surface, CTLA-4 present on the cell surface is immediately endocytosed by association with AP-2, keeping cell-surface CTLA-4 at a low level. CTLA-4 in lysosomes would be degraded in the absence of further phosphorylation, whereas CTLA-4-containing lysosomes may move toward the site of TCR engagement and be secreted upon stimulation, as a negative regulator of T cell activation. Immunity 1:405.


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