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Trafficking of LAG-3 to the Surface on Activated T Cells via Its Cytoplasmic Domain and Protein Kinase C Signaling

Joonbeom Bae,* Suk Jun Lee,[†] Chung-Gyu Park,[‡] Young Sik Lee,* and Taehoon Chun*

Lymphocyte activation gene-3 (LAG-3; CD223), a structural homolog of CD4, binds to MHC class II molecules. Recent research indicated that signaling mediated by LAG-3 inhibits T cell proliferation, and LAG-3 serves as a key surface molecule for the function of regulatory T cells. Previous reports demonstrated that the majority of LAG-3 is retained in the intracellular compartments and is rapidly translocated to the cell surface upon stimulation. However, the mechanism by which LAG-3 translocates to the cell surface was unclear. In this study, we examined the trafficking of human LAG-3 under unstimulated as well as stimulated conditions of T cells. Under the unstimulated condition, the majority of LAG-3 did not reach the cell surface, but rather degraded within the lysosomal compartments. After stimulation, the majority of LAG-3 translocated to the cell surface without degradation in the lysosomal compartments. Results indicated that the cytoplasmic domain without Glu-Pro repetitive sequence is critical for the translocation of LAG-3 from lysosomal compartments to the cell surface. Moreover, protein kinase C signaling leads to the translocation of LAG-3 to the cell surface. However, two potential serine phosphorylation sites from the LAG-3 cytoplasmic domain are not involved in the translocation of LAG-3. These results clearly indicate that LAG-3 trafficking from lysosomal compartments to the cell surface is dependent on the cytoplasmic domain through protein kinase C signaling in activated T cells. *The Journal of Immunology*, 2014, 193: 000–000.

Lymphocyte activation gene-3 (LAG-3; CD223), a member of the Ig superfamily, was originally identified as an inducible protein when T cells become activated (1). Later, LAG-3 expression has also been detected in other cells, including activated NK cells, invariant NKT cells, and B cells (2–4). LAG-3 is structurally similar to CD4; LAG-3 and CD4 contain four extracellular Ig-like domains with conserved structural resemblance, although they share <20% amino acid sequence similarity (5). The structural similarity between LAG-3 and CD4 suggests that they might arise as a result of gene duplication (1). Therefore, it has been speculated that LAG-3 may function as a coreceptor as does CD4. Indeed, LAG-3 was found to bind to MHC class II molecules (6, 7).

However, there are several characteristics that differ between LAG-3 and CD4. First, CD4 is constitutively expressed on T cells and invariant NKT cells, whereas LAG-3 expression is only observed in activated T cells and invariant NKT cells (1–4, 8). Second, LAG-3 has much higher binding affinity to MHC class II molecules than CD4 does (5, 6). Third, LAG-3 mediates signaling with a negative regulatory effect on T cells and invariant NKT cells, whereas CD4 costimulates and amplifies TCR signaling to activate those cells (3, 5, 9–12). In particular, LAG-3 associates with the CD3/TCR complex present on the surface of activated T cells and downregulates the CD3/TCR activation signaling pathway (9). Additionally, cross-linking of LAG-3 or introduction of LAG-3 impairs CD4^{+} T cell activation, as determined by altered calcium fluxes, IL-2 expression, and a reduction in CD3/TCR complex expression (5, 9). Moreover, the signaling mediated by LAG-3 inhibits the proliferation of T cells and invariant NKT cells (3, 10–12). Therefore, LAG-3 may play an important role in maintaining T cell homeostasis by transducing a negative signal to calm down the activated T cells. Supporting this idea, LAG-3 has been described not only as an important cell surface marker of regulatory T cells, but also as an essential protein for their function (13, 14).

Recent studies reported that most of LAG-3 is colocalized with microtubule-organizing center, early/recycling endosomes, and secretory lysosomes within unstimulated T cells (15). Intriguingly, it was also found that LAG-3 localized more rapidly to the surface of T cells upon stimulation, when compared with CD4 (15). However, the regulatory mechanism of LAG-3 trafficking and surface expression remains unknown.

In the current study, we have examined the intracellular localization and stimulation-dependent trafficking of human LAG-3 to the surface of T cells. We found that the majority of LAG-3 is localized and degraded within the lysosomal compartments in the unstimulated T cells. Upon stimulation, LAG-3 within the lysosomal compartments translocates to the cell surface, and LAG-3 trafficking to the cell surface is dependent on the cytoplasmic domain of LAG-3 and is mediated by protein kinase C (PKC) signaling. Our results revealed the detailed mechanism of trafficking and surface expression of LAG-3 that may provide a means to regulate T cell activity.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BIM, bisindolylmaleimide I; CHX, cycloheximide; CQ, chloroquine; EGFP, enhanced GFP; EP motif, Glu-Pro repetitive sequence; LAG-3, lymphocyte activation gene-3; MFI, mean fluorescence intensity; PKC, protein kinase C; RT, room temperature; STS, staurosporine; WT, wild-type.

Materials and Methods

Reagents

Purified anti-human LAG-3 Ab (goat IgG; catalogue AF2319), PE-conjugated anti-human LAG-3 Ab (goat IgG; catalogue FAB2319P), and PE-conjugated goat IgG isotype control (catalogue IC108P) were purchased from R&D Systems (Minneapolis, MN). Anti–β-actin Ab (mouse IgG; catalogue A5441) was purchased from Sigma-Aldrich (St. Louis, MO). Purified anti-Rab5a (goat IgG; catalogue sc-26566), anti-LAMP2 (goat IgG; catalogue sc-8100), anti-cathepsin D (goat IgG; catalogue sc-6486 and sc-6494), and anti-GFP Ab (mouse IgG; catalogue sc390394) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lyso-tracker Red DND-99 (catalogue L7528) and Alexa594-conjugated anti-goat IgG (catalogue A11058) were purchased from Invitrogen (Carlsbad, CA). PE-conjugated anti-human CD4 Ab (mouse IgG1; catalogue 550630), PE-conjugated anti-human CD69 Ab (mouse IgG1; catalogue 555531), and PE-conjugated mouse IgG1 isotype control (catalogue 556650) were purchased from BD Biosciences (San Jose, CA). HRP-conjugated anti-goat IgG (catalogue PA1-28805) and HRP-conjugated anti-mouse IgG (catalogue 31430) were purchased from Thermo Fisher Scientific (Waltham, MA). HRP-conjugated streptavidin (catalogue SA-5004) was purchased from Vector Laboratories (Burlingame, CA). Ammonium chloride (NH_4Cl), chloroquine (CQ), cycloheximide (CHX), ionomycin, and PMA were purchased from Sigma-Aldrich. Bisindo-lylmaleimide I (BIM), glutathione, GM6001, MG132, sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate, and staurosporine (STS) were purchased from Merck Millipore (Billerica, MA).

Cell culture and treatment

Jurkat cells (human T cells) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C and 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 2 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin. For stimulation, Jurkat cells were treated with 1 μg/ml ion-omycin and 10 ng/ml PMA for 24 h. In some experiments, the cells were treated with GM6001 (matrix metalloproteinase inhibitor), MG132 (proteasome inhibitor), CQ (lysosomal inhibitor), ammonium chloride (lysosomal inhibitor), CHX (protein synthesis inhibitor), BIM (PKC inhibitor), and STS (PKC inhibitor), as indicated in the figure legends.

Construction of DNA constructs and mutagenesis

The expression vectors encoding human LAG-3, CD4, LAG-3 deletion mutant lacking the cytoplasmic domain of LAG-3 (LAG-3ΔCY), LAG-3 deletion mutant lacking Glu-Pro repetitive sequence (EP motif) in the cytoplasmic domain of LAG-3 (LAG-3ΔEP), and CD4 deletion mutant lacking the cytoplasmic domain of CD4 (CD4ΔCY) were constructed by ligating PCR products cloning into XhoI and NotI restriction sites of the retroviral vector pLNCX2. To construct vectors expressing the human LAG-3 enhanced GFP (EGFP) fusion protein (LAG-3-EGFP), CD4-EGFP fusion protein (CD4-EGFP), LAG-3-EGFP fusion protein lacking the cytoplasmic domain of LAG-3 (LAG-3ΔCY-EGFP), LAG-3-EGFP fusion protein lacking the EP motif within the cytoplasmic domain of LAG-3 (LAG-3ΔEP-EGFP), and CD4-EGFP fusion protein lacking the cytoplasmic domain of CD4 (CD4ΔCY-EGFP), cDNA encoding each protein was PCR amplified and cloned into XhoI and NotI restriction sites of pLNCX2. PCR was performed using ion-omycin- and PMA-stimulated human PBMCs as a template. The primer pairs used for each expression vector are listed in Supplemental Table I. The expression vectors encoding two different types of fusion proteins between LAG-3 and CD4 (LAG-3-CD4 or CD4-LAG-3), LAG-3 mutants with a single amino acid substitution (LAG-3S484A, LAG-3S397A), and LAG-3 mutant with a double amino acid substitution (LAG-3S484, 497A) were constructed using PCR-mediated recombination of two DNA fragments amplified by PCR with the primers listed in Supplemental Table I (16, 17). Also, the expression vectors encoding two different types of LAG-3/CD4-EGFP chimeric fusion proteins (LAG-3-CD4-EGFP or CD4-LAG-3-EGFP), a LAG-3-EGFP fusion protein bearing a single amino acid substitution within LAG-3 (LAG-3S484A-EGFP, LAG-3S397A-EGFP), and a LAG-3-EGFP fusion protein harboring a double amino acid substitution within LAG-3 (LAG-3S484, 497A-EGFP) were constructed by the PCR-mediated recombination using the primers listed in Supplemental Table I. The schematic diagrams of individual proteins expressed from DNA constructs are shown in Supplemental Fig. 1. Each of the DNA constructs was then transfected into 293GPG cells using lipofectamine (Invitrogen). After 3 d, the supernatant from each transfectant was used to infect Jurkat cells using 1 μg/ml polybrene (Sigma-Aldrich). To establish stable cell lines, each group of infected cells was selected by 1.5 mg/ml neomycin. The expression of the corresponding gene in individual Jurkat cell lines was confirmed by RT-PCR and Western blot analyses.

Western blot analyses

Cell lysates were prepared and subjected to Western blot analyses with the appropriate Abs, as previously described (18). Briefly, the cells were washed in PBS, centrifuged, and resuspended in a cell lysis buffer (20 ml Tris-HCl [pH 7.4], 10 mM EDTA, 0.5% Triton X-100) containing PMSF and protease inhibitor mixture (Merck Millipore). The cell lysates were subsequently resolved by 10% SDS-PAGE and transferred onto the Hybond-P membranes (GE Healthcare, Piscataway, NJ). Membranes were blocked using 5% skim milk in TBST for 1 h and probed with anti-human LAG-3 Ab in blocking buffer overnight at 4°C. The membranes were subsequently incubated with HRP-conjugated anti-goat IgG Ab for 1 h at room temperature (RT). To detect GFP, cathepsin D, and β-actin, the membranes were incubated with anti-GFP Ab, anti-cathepsin D Ab (catalogue sc-6486), or anti–β-actin Ab in blocking buffer overnight at 4°C, followed by incubation with HRP-conjugated anti-mouse Ab for 1 h at RT. Immunoreactive bands were visualized using an ECL Western blotting detection kit (GE Healthcare), according to the manufacturer's instructions.

To measure stability of LAG-3, the cells were treated with or without 20 μg/ml CHX for indicated time and then subjected to Western blot analysis, as described above. Relative band intensity of protein expression was quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Flow cytometry analyses

Flow cytometry analyses were performed to determine the cell surface or intracellular expression of human LAG-3, CD4, and CD69. For surface staining of LAG-3 and CD4, cells were stained with PE-conjugated anti-human LAG-3 Ab or PE-conjugated anti-human CD4 Ab for 30 min on ice. PE-conjugated goat IgG control and mouse IgG1 control were used as isotype controls. To visualize CD69 surface expression, the cells were stained with PE-conjugated anti-human CD69 Ab. PE-conjugated mouse IgG1 control was used as an isotype control. For intracellular LAG-3 and CD4 staining, the cells were fixed with 4% paraformaldeyde for 20 min, permeabilized with 0.15% saponin in PBS for 20 min, and then stained with relevant Abs. After washing several times with PBS, all the stained cells were resuspended in PBS and analyzed by flow cytometry using a FACS-Calibur with the CellQuest software (BD Biosciences). LAG-3, CD4, and CD69 expression levels were quantitated using the mean fluorescence intensity of the staining.

Confocal microscopy

Cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then blocked with 3% BSA in PBS for 1 h at RT. After blocking, the cells were washed three times in PBS and stained with anti-human LAG-3, anti-Rab5A, anti–LAMP-2, and anti-cathepsin D (catalog sc-6494) Abs overnight at 4°C. Cells were then washed three times in PBS and stained with Alexa594-conjugated donkey anti-goat IgG Ab for 1 h at RT. For staining with Lysotracker Red DND-99, the cells were incubated at 37°C for 1 h with a culture medium containing 50 nM Lysotracker Red DND-99, followed by fixation with 4% paraformaldehyde. After washing with PBS, the cells were mounted in a solution containing DAPI (Vector Laboratories). Immunofluorescence was analyzed using a Zeiss LSM 5 EXCITER confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

Internalization and recycling assay

Biotin-based internalization and recycling assays were performed, as previously described, with a slight modification to the method (19). Briefly, the cells were stained with 1 mM sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate in HBSS at 4°C for 30 min, followed by washing out unbound biotin with prechilled HBSS. Biotinylated cells were then incubated in the culture medium at 37°C for 1 h to allow the internalization of the biotinylated cell surface proteins. Control cells were kept in prechilled HBSS until ELISA analyses were performed. Protein internalization was stopped by treating cells with cold HBSS. Biotin was stripped off from the residual cell surface proteins in a glutathione solution (50 μM glutathione, 75 μM NaCl, 1 μM EDTA, 75 μM NaOH, and 10% FBS) for two cycles of 20 min at 4°C. For the recycling assay, cell surface proteins were bio-tinylated and incubated in culture medium at 37°C for 1 h. After stripping of residual surface biotin, cells were incubated again in culture medium at 37°C for 1 h, followed by glutathione stripping of biotin-labeled recycled protein. Each cell was washed three times with HBSS prior to lysis in 1% Triton X-100. To determine the amount of biotinylated LAG-3 protein, the cell lysates were incubated in 96-well plates coated with anti-human LAG-3 Ab, followed by incubation with HRP-conjugated streptavidin. The

amount of internalized LAG-3 was determined by comparing the lysates of the stripped cells with those of unstripped control cells. The amount of recycled LAG-3 was calculated by comparing cells after two rounds of stripping with those after a single round of stripping.

Statistical analysis

Mean values were compared using Student's *t* test for independent variables. All the data are representative of three independent experiments and presented as mean ± SEM. Significant differences as determined by *p* values <0.05 and 0.01 are indicated with asterisks (*) and (**), respectively, on each graph.

Results

The majority of LAG-3 is retained in the late endosomal and lysosomal compartments in unstimulated T cells and translocated to the cell surface upon activation of T cells

To investigate the intracellular trafficking and cell surface expression of LAG-3 in human T cells, we overexpressed human LAG-3 in Jurkat cells. As assessed by Western blot analysis, endogenous LAG-3 protein was not detected in control cells transfected with empty vector (mock), whereas the significant amount of LAG-3 was detected in LAG-3–overexpressing Jurkat cells (LAG-3) (Supplemental Fig. 2A). After stimulation with PMA and ionomycin, total LAG-3 expression moderately increased in LAG-3–overexpressing Jurkat cells (LAG-3), unlike that in the cells before stimulation (Supplemental Fig. 2A). To monitor the activation status of Jurkat cells, surface expression of human CD69 on Jurkat cells was assessed by flow cytometry before or after stimulation. Induction of human CD69 expression on Jurkat cells was only observed in cells after stimulation (Supplemental Fig. 2B). Next, we examined cell surface and intracellular expression of LAG-3 in LAG-3–overexpressing Jurkat cells before or after stimulation. The cell surface expression of LAG-3 was barely detected in human LAG-3–overexpressing Jurkat cells before stimulation (Fig. 1A). However, the cell surface expression of LAG-3 in human LAG-3–overexpressing Jurkat cells upon stimulation was increased >2-fold compared with that of those cells before stimulation (Fig. 1A). The intracellular levels of LAG-3 in human LAG-3–overexpressing Jurkat cells were moderately increased after stimulation, compared with those of cells before stimulation (Fig. 1A). These results indicated that the majority of LAG-3 is retained within intracellular compartments of unstimulated T cells and translocated to the cell surface upon activation of T cells.

To visualize LAG-3 trafficking and surface expression on human T cells, LAG-3-EGFP was overexpressed in Jurkat cells. Overexpression of LAG-3 was confirmed by Western blot analysis (data not shown). We analyzed the cell surface and intracellular expression pattern of LAG-3-EGFP in such cells by flow cytometry analyses using anti-human LAG-3 Ab before or after stimulation. We found that the expression pattern of LAG-3-EGFP in Jurkat cells was the same as that of LAG-3 in human LAG-3–overexpressing Jurkat cells (data not shown). To rule out the possibility that EGFP influences the LAG-3 trafficking, we stained LAG-3-EGFP–overexpressing Jurkat cells using anti-human LAG-3 Ab and analyzed localization of EGFP and LAG-3 using confocal microscopy before or after stimulation. Without stimulation, EGFP and LAG-3 were mainly colocalized within intracellular compartments in LAG-3-EGFP–overexpressing Jurkat cells (Fig. 1B). After stimulation, colocalization of EGFP and LAG-3 was also observed in the cell surface as well as intracellular compartments (Fig. 1B). These results confirmed that a substantial amount of LAG-3 is retained in intracellular compartments in unstimulated T cells and translocates to the cell surface upon activation in T cells.

To identify specific organelles that contain LAG-3 in unstimulated T cells, unstimulated LAG-3-EGFP–overexpressing cells were stained with Abs that recognize various subcellular organelle markers, and colocalization was analyzed between LAG-3-EGFP and each specific subcellular organelle marker using confocal microscopy. LAG-3-EGFP was colocalized with LAMP-2 (late endosome and lysosome marker) and cathepsin D (lysosome marker), whereas LAG-3-EGFP was not merged with Rab5A (early endosome and cytoplasmic surface of the plasma membrane marker) (Fig. 1C). These results demonstrated that intracellular LAG-3 is located primarily within late endosome and lysosome of unstimulated T cells.

### Blockade of lysosomal enzyme activity enhances surface expression of LAG-3

Next, we investigated factors that influence LAG-3 trafficking from intracellular compartments to the cell surface. A previous study demonstrated that two transmembrane metalloproteases, ADAM10 and ADAM17, mediate the cleavage of LAG-3 expressed on the cell surface of T cells (20). To examine whether the activity of such metalloproteases influences the LAG-3 trafficking to the cell surface, we treated LAG-3–overexpressing Jurkat cells with a metalloprotease inhibitor, GM6001, and then analyzed the LAG-3 expression pattern. Secretion of soluble LAG-3 was inhibited by ~80% at 100 μM GM6001 (Fig. 2A). Therefore, we treated both unstimulated and stimulated cells with 100 μM GM6001 to monitor surface expression of LAG-3. As a result, inhibition of metalloprotease activity by GM6001 did not significantly influence the surface expression of LAG-3 in both unstimulated and stimulated T cells (Fig. 2B).

A nonlysosomal pathway of protein degradation by proteasome complex is often used to control the $t_{1/2}$ of proteins expressed on the cell surface (21). We therefore tested the possibility that the activity of proteasome complex influences the LAG-3 trafficking to the cell surface. Consistent with previous observation, treatment of unstimulated or stimulated LAG-3–overexpressing Jurkat cells with 20 μM MG132 led to an increase in the relative amount of cyclin D1 (22), whereas it had no effect on the total expression levels of LAG-3 (Supplemental Fig. 2C). These results indicated that cyclin D1 expression is largely dependent on the activity of proteasome complex, but the expression of LAG-3 is not. To confirm this observation, we treated either unstimulated or stimulated LAG-3–overexpressing Jurkat cells with 20 μM MG132 to monitor the cell surface expression of LAG-3. As a result, inhibition of proteasome activity by MG132 does not significantly influence the cell surface expression of LAG-3 in both unstimulated and stimulated T cells (Fig. 2C).

Because the majority of LAG-3 was retained within the lysosomal compartment in unstimulated T cells, we tried to clarify the role of lysosomal compartments in LAG-3 trafficking. We treated cells with either CQ or ammonium chloride to block the activity of lysosomal enzymes by neutralizing acidic environment within lysosomal compartments (23, 24). As an initial attempt to block lysosomal enzymes, we treated unstimulated or stimulated LAG-3–overexpressing Jurkat cells with 100 μM CQ for 24 h. Interestingly, both unstimulated and stimulated cells treated with CQ showed a significant increase in the cell surface expression of LAG-3 compared with untreated cells (data not shown). Subsequently, we treated unstimulated or stimulated LAG-3–overexpressing Jurkat cells with CQ for different time points (Supplemental Fig. 2D) and monitored the expression pattern of LAG-3 on cell surface. We observed that the cell surface expression of LAG-3 increased depending on the time interval of CQ treatment and was maximal after 24-h treatment of CQ irrespective of cell stimulation (Fig. 2D).

FIGURE 1. Subcellular localization of LAG-3 before or after stimulation of human T cells by PMA and ionomycin. Empty vector (mock) or LAG-3–expressing Jurkat cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. (**A**) Intracellular and cell surface levels of LAG-3 in mock transfectants or LAG-3 transfectants before or after stimulation. Cells were stained with PE-conjugated anti-human LAG-3 Ab. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates mean fluorescence intensity. (**B**) Before or after stimulation, LAG-3-EGFP transfectants were stained with anti-human LAG-3 Ab, followed by staining with Alexa594-conjugated donkey anti-goat IgG Ab. After staining, colocalization between EGFP and LAG-3 was analyzed under a confocal microscope. (**C**) Before or after stimulation, LAG-3-EGFP transfectants were stained with anti-Rab 5A Ab, anti–LAMP-2 Ab, or anti-cathepsin D Ab, followed by staining with Alexa594-conjugated donkey anti-goat IgG. The nucleus was visualized by DAPI staining. After staining, colocalization between EGFP and each Ab was analyzed under a confocal microscope. All of the data are representative of three independent experiments.
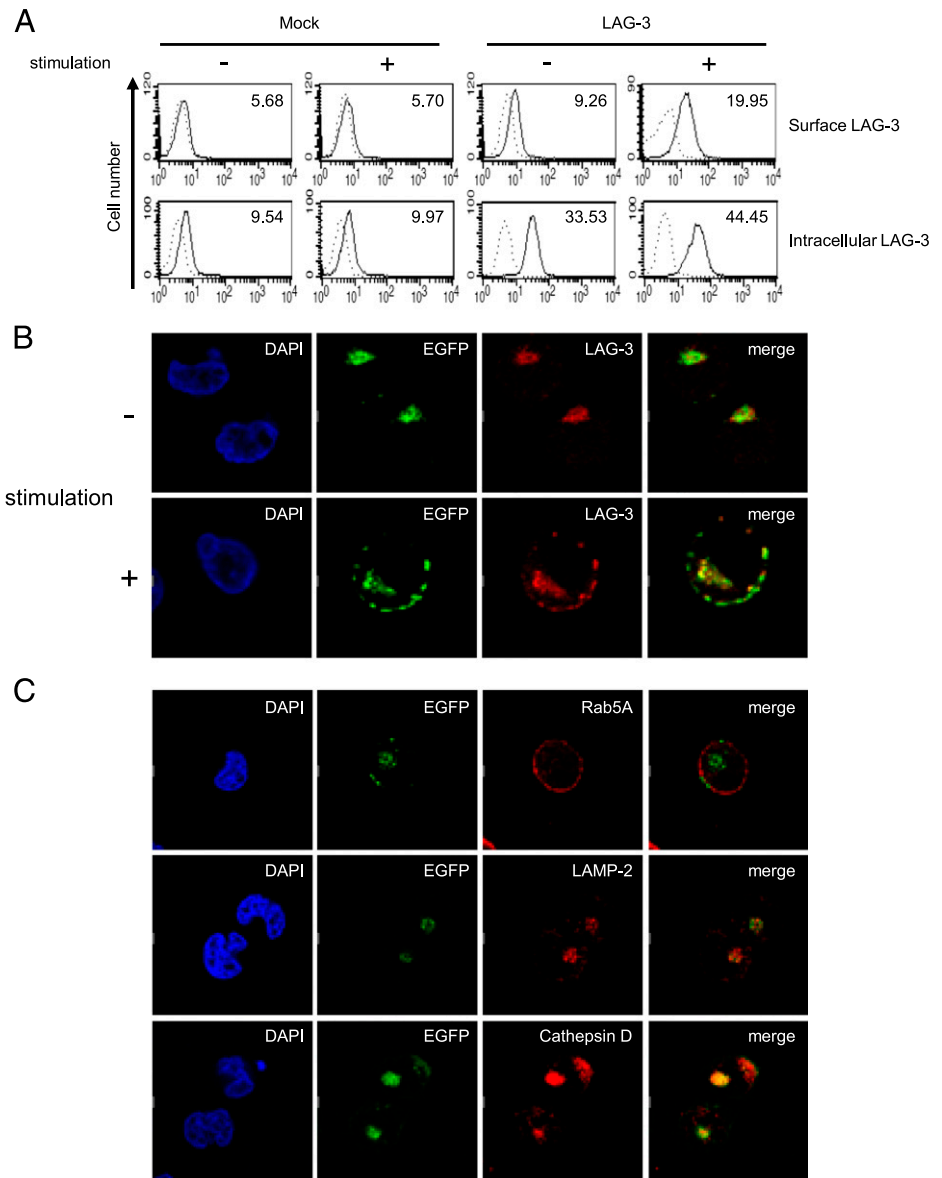
To confirm these results, we treated the cells with another lysosomal inhibitor, ammonium chloride (10 mM), for different time points (Supplemental Fig. 2D) and examined the expression pattern of LAG-3 on the cell surface. The results also demonstrated an inverse relationship between the lysosomal enzyme activity and the surface expression of LAG-3 on both unstimulated and stimulated cells (Supplemental Fig. 2E).

Therefore, it could be hypothesized that LAG-3 is degraded within the late endosomal and/or lysosomal compartments before it reaches the cell surface. To test this hypothesis, unstimulated LAG-3–overexpressing Jurkat cells were treated with 20 μg/ml CHX in the absence or presence of CQ, and we measured the levels of total LAG-3 by Western blot analyses. We found that total LAG-3 levels in the presence of CQ were increased compared with those in the absence of CQ (Fig. 2E), indicating LAG-3 is stabilized when lysosomal activity is blocked. We also measured the cell surface expression of LAG-3 in these samples. The surface expression of LAG-3 in the presence of CQ was slightly increased compared with that in the absence of CQ (Supplemental Fig. 2F). Collectively, these data suggested that localization and degradation of LAG-3 in lysosomal compartments is a major mechanism to inhibit its translocation to the cell surface.

The cytoplasmic domain without the EP motif of LAG-3 is required for cell surface translocation of LAG-3

To investigate the structural requirement of LAG-3 on the trafficking to the cell surface, we generated two deletion mutants, including a mutant lacking an entire cytoplasmic domain of LAG-3 (LAG-3ΔCY) and a mutant lacking only EP motif within the cytoplasmic domain of LAG-3 (LAG-3ΔEP) (Supplemental Fig. 1). Each of these deletion mutants or wild-type (WT) LAG-3 was expressed in Jurkat cells, and their cell surface expression was monitored. The levels of LAG-3 deletion mutants were comparable to those of WT LAG-3 (LAG-3) (Supplemental Fig. 3A), and the intracellular pool of LAG-3 did not show any significant difference between WT LAG-3 and its deletion mutants (Fig. 3A, 3B). However, the surface expression of LAG-3ΔCY was significantly decreased under stimulated condition (Fig. 3A, 3B). The reason for this observation may be that the relative endocytosis and recycling rate of LAG-3ΔCY are different from those of WT LAG-3 and LAG-3ΔEP after induction of cell surface expression upon stimulation. To rule out this possibility, we conducted the biotin-based endocytosis and recycling assay and found that the relative endocytosis and recycling rate of LAG-3ΔCY were not significantly impaired, compared with those of WT LAG-3 and LAG-3ΔEP after simulation (Fig. 3C).
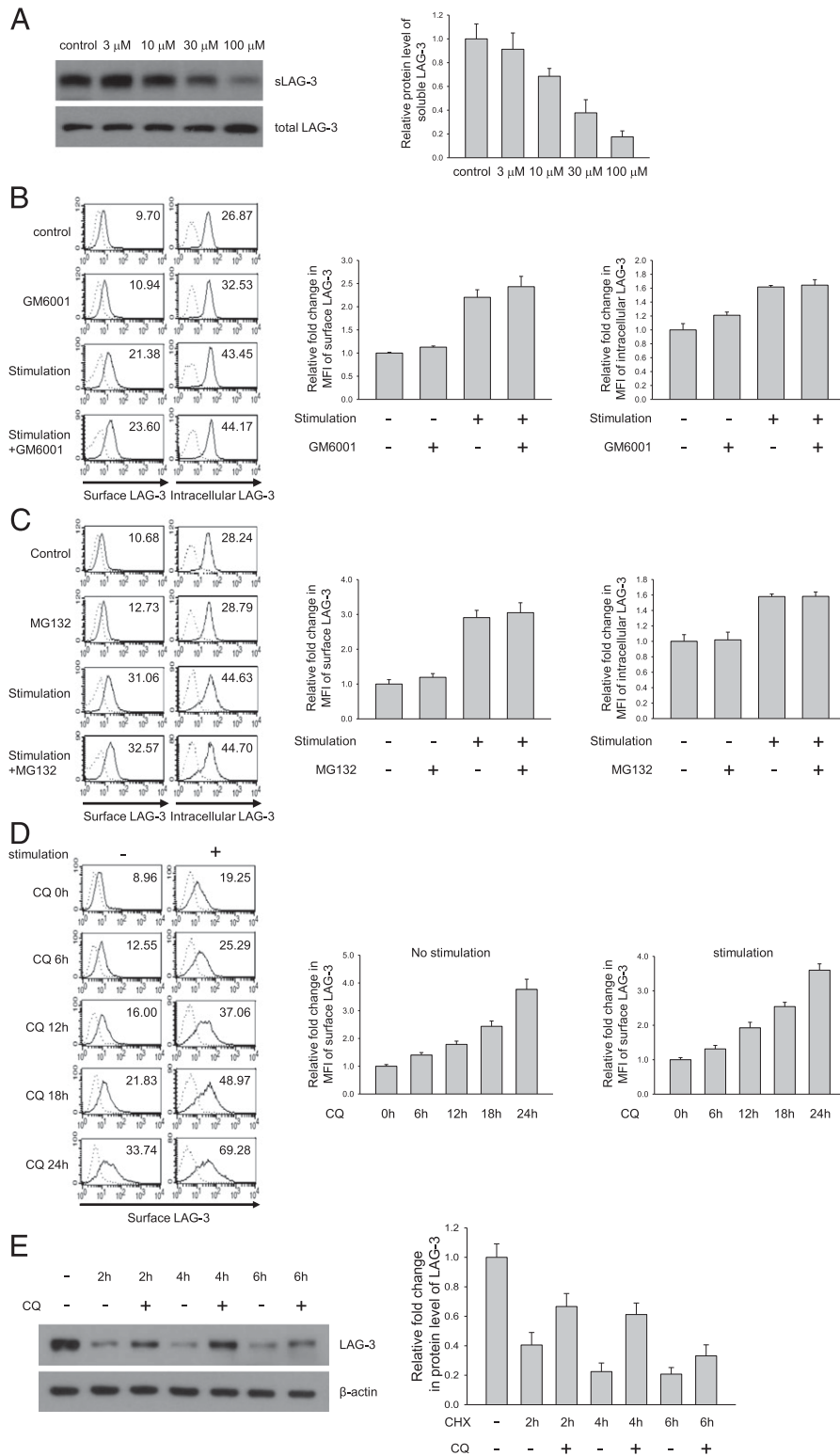
FIGURE 2. Lysosomal degradation is a major limiting step in translocating LAG-3 to the cell surface. (**A**) Inhibition of soluble LAG-3 secretion by GM6001. Stimulated LAG-3–overexpressing Jurkat cells were treated with various concentrations of GM6001, a metalloprotease inhibitor. After treatment with GM6001, Western blot analyses were performed to analyze the expression of soluble LAG-3 (sLAG-3) and total LAG-3 (*left panel*). Quantitation of inhibitory effect of GM6001 on the production of soluble LAG-3 (*right panel*). (**B**) The activity of metalloprotease does not influence the cell surface expression of LAG-3. LAG-3–expressing Jurkat cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) in the presence or absence of a metalloprotease inhibitor, GM6001 (100 μM). After stimulation, cells were stained with PE-conjugated anti-human LAG-3 Ab. Representative histograms are shown in the *left panels*. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates mean fluorescence intensity (MFI). Relative fold changes in MFI of LAG-3 surface expression are shown in the *right panels*. Data represent the mean ± SEM from three independent experiments. (**C**) The activity of the proteasome complex has no effect on the surface expression of LAG-3. LAG-3–expressing Jurkat cells were stimulated in the presence or absence of a proteasome inhibitor, MG132 (20 μM). After stimulation, cells were stained with the same Abs as those described in (B). Representative histograms (*left panels*) and relative fold changes in (*Figure legend continues*)

There are two possibilities that the cytoplasmic domain without the EP motif enhances cell surface expression of LAG-3 upon activation of T cells. The first possibility is that the cytoplasmic domain lacking the EP motif renders LAG-3 more resistant to the lysosomal degradation. The second possibility is that the cytoplasmic domain without the EP motif is critical for trafficking LAG-3 to the cell surface upon activation. To find out whether the cytoplasmic domain lacking the EP motif has an effect on the lysosomal degradation of LAG-3, we treated each of the three transfectants with CQ for different time points and measured the total amount of LAG-3 expression. As a result, total amount of LAG-3 expression among all of them was not significantly different and increased in the presence of CQ (Fig. 3D). Additionally, we monitored the cell surface expression of three types of LAG-3 in the presence of CQ by flow cytometry. Surface expression of WT LAG-3 and LAG-3ΔEP is gradually increased without or with stimulation of cells in the presence of CQ. However, surface expression of LAG-3ΔCY was hardly detected even in unstimulated cells treated with CQ. Under the stimulated condition, surface expression of LAG-3ΔCY was markedly reduced compared with those of WT LAG-3 and LAG-3ΔEP after CQ treatment (Fig. 3E, 3F). Therefore, we concluded that the cytoplasmic domain without the EP motif enhances cell surface expression of LAG-3 rather than protecting LAG-3 against the lysosomal degradation.

To confirm these results, LAG-3-EGFP, LAG-3ΔCY-EGFP, and LAG-3ΔEP-EGFP were constructed and overexpressed in Jurkat cells (Supplemental Fig. 1). Colocalization of LAG-3 (red fluorescence) and EGFP (green fluorescence) in these cells was analyzed by confocal microscopy. As shown in Fig. 3G, all three types of LAG-3 were colocalized with EGFP within the cytoplasm of unstimulated Jurkat cells. When cells were activated, colocalization of LAG-3 and EGFP was mainly observed in the cell surface in LAG-3-EGFP–expressing cells and LAG-3ΔEP-EGFP–expressing cells, whereas colocalization of LAG-3 and EGFP in LAG-3ΔCY-EGFP–expressing cells was mainly observed within the cytoplasm (Fig. 3G). The colocalization of EGFP (green fluorescence) and lysotracker (red fluorescence) was also observed by confocal microscopy. All three types of LAG-3 were colocalized with lysotracker-stained compartments under unstimulated condition (Fig. 3H). After stimulation, LAG-3-EGFP and LAG-3ΔEP-EGFP were mainly observed on the cell surface and were not colocalized with lysotracker. However, LAG-3ΔCY-EGFP was still colocalized with lysotracker, indicating it stayed within cytoplasm even after stimulation (Fig. 3H). Together, these results suggested that the cytoplasmic domain lacking the EP motif is critical for the translocation of LAG-3 to the cell surface.

The cytoplasmic domain without the EP motif of LAG-3 enhances cell surface expression of the CD4-LAG-3 chimeric protein

To further confirm the functional importance of the cytoplasmic domain without the EP motif on LAG-3 trafficking to the cell surface, we generated cDNA encoding several LAG-3/CD4 chimeric proteins and overexpressed in Jurkat cells. The LAG-3-CD4 chimeric protein contains the extacellular domain and trans-

membrane domain of LAG-3 combined with the cytoplasmic domain of CD4 (Supplemental Fig. 1). The CD4-LAG-3 chimeric protein has the extacellular domain and transmembrane domain of CD4 combined with the cytoplasmic domain of LAG-3 (Supplemental Fig. 1). After confirming the proper expression of each chimeric protein by Western blot analyses using anti-EGFP Ab (Supplemental Fig. 3B), we monitored the cell surface expression of LAG-3 or CD4 chimeric protein, compared with those of WT LAG-3, WT CD4, and their ΔCY mutants (Supplemental Fig. 1) by flow cytometry analyses using either anti–LAG-3 Ab or anti-CD4 Ab. As expected, the LAG-3ΔCY mutant (LAG-3ΔCY-EGFP) showed a defect in translocation to the cell surface after stimulation, compared with that of WT LAG-3 (LAG-3-EGFP) (Fig. 4A, 4B). Also, the LAG-3-CD4 chimeric protein (LAG-3-CD4-EGFP) behaved in a similar manner as observed with the LAG-3ΔCY mutant (LAG-3ΔCY-EGFP) (Fig. 4A, 4B). These results also indicated that the cytoplasmic domain without the EP motif of LAG-3 is a necessary component for the translocation of LAG-3 upon activation and that replacement of the cytoplasmic domain of LAG-3 to that of CD4 does not allow LAG-3 to properly localize to the cell surface upon activation.

In contrast to the expression of LAG-3, CD4 is constitutively expressed on the surface of T cell under unstimulated condition, and its cell surface expression is decreased via phosphorylation of serine residues on the cytoplasmic domain under stimulated condition (25, 26). As we expected, endogenous CD4 (mock) was constitutively expressed on the surface of unstimulated Jurkat cells, which was decreased upon stimulation (Fig. 4C, 4D). The similar results were also observed when a WT CD4 (CD4-EGFP) was overexpressed in Jurkat cells (Fig. 4C, 4D). The cell surface expression levels of CD4ΔCY mutant (CD4ΔCY-EGFP) in Jurkat cells were increased compared with those of WT CD4 (CD4-EGFP) upon stimulation (Fig. 4C, 4D). The expression levels of CD4-LAG-3 chimeric protein (CD4-LAG-3-EGFP) on the surface of Jurkat cells were ~2-fold higher compared with those of WT CD4 (CD4-EGFP) in the absence of stimulation (Fig. 4C, 4D). Upon stimulation, the surface expression levels of CD4-LAG-3 chimeric protein (CD4-LAG-3-EGFP) on Jurkat cells were increased by ~6-fold compared with those of WT CD4 (CD4-EGFP) without stimulation and by nearly 3- fold increased when compared with those of CD4-LAG-3 chimeric molecule (CD4-LAG-3-EGFP) under unstimulated condition (Fig. 4C, 4D). Combined results strongly suggested that the cytoplasmic domain of CD4 mediates downregulation of the surface expression of CD4 upon stimulation of T cells and that the cytoplasmic domain without the EP motif of LAG-3 upregulates the surface expression of LAG-3 upon stimulation of T cells.

PKC inhibitors block LAG-3 trafficking to the surface of activated Jurkat cells regardless of possible phosphorylation within the cytoplasmic domain of LAG-3

Based on our observation, the cytoplasmic domain without the EP motif of LAG-3 is important for translocation of LAG-3 to the cell surface. Therefore, we hypothesized that the cytoplasmic domain without the EP motif of LAG-3 may receive the signal for traf-

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MFI of LAG-3 surface expression (*right panels*) are shown. Data represent the mean ± SEM from three independent experiments. (**D**) Lysosomal inhibition by CQ increases the cell surface expression of LAG-3. LAG-3–expressing Jurkat cells were stimulated in the presence or absence of CQ (100 μM) for different time points. After stimulation, cells were stained with the same Abs as those described in (B). Representative histograms (*left panels*) and relative fold changes in MFI of LAG-3 surface expression (*right panels*) are shown. Data represent the mean ± SEM from three independent experiments. (**E**) Stability of LAG-3 is increased in the presence of CQ. LAG-3–expressing Jurkat cells were treated with CHX (20 μg/ml) in the presence or absence of CQ (100 μM). After treatment, cell lysates were immunoblotted with anti-human LAG-3 Ab and anti–β-actin Ab. Data are representative of three independent experiments.
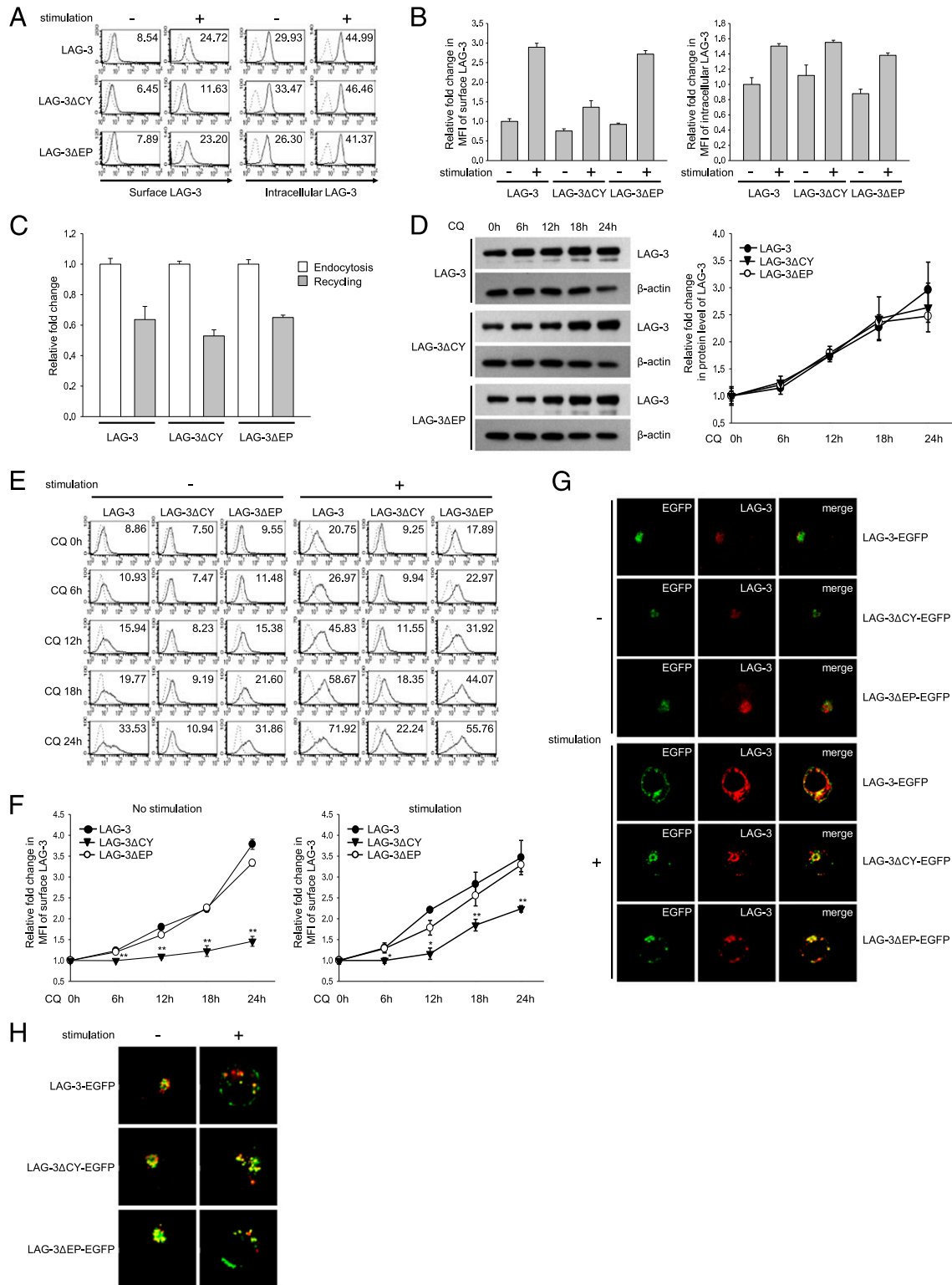
FIGURE 3. The cytoplasmic domain without the EP motif of LAG-3 is required for the translocation of LAG-3 to the cell surface. (**A**) Intracellular and cell surface levels of WT LAG-3 (LAG-3) and its deletion mutants (LAG-3ΔCY and LAG-3ΔEP) before or after stimulation with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. (**B**) Relative fold changes in surface and intracellular MFI of WT LAG-3 (LAG-3) and its deletion mutants (LAG-3ΔCY and LAG-3ΔEP) before or after stimulation. Data represent the mean ± SEM from three independent experiments. (**C**) Relative fold changes in endocytosis and recycling of WT LAG-3 (LAG-3) and its deletion mutants (LAG-3ΔCY and LAG-3ΔEP) after stimulation. Endocytosis and recycling of these proteins were measured using a biotin-based internalization and recycling assay, as described in *Materials and Methods*. Data represent the mean ± SEM from three independent experiments. (**D**) Expressions of WT LAG-3 (LAG-3) and its deletion mutants (LAG-3ΔCY and LAG-3ΔEP) were not significantly different and increased in the presence of CQ. Each Jurkat cell expressing these proteins was treated with CQ (100 μM) for 24 h. After treatment, cell lysates were immunoblotted with anti-human LAG-3 Ab and anti–β-actin Ab. Data are representative of three independent experiments. (**E**) Cytoplasmic domain without the EP motif of LAG-3 is critical for the induction of LAG-3 on the cell surface. Each Jurkat cell expressing WT LAG-3 (LAG-3) and its deletion mutants (LAG-3ΔCY and LAG-3ΔEP) was stimulated in the presence or absence of CQ (100 μM) for different time courses. After stimulation, cells   (*Figure legend continues*)

FIGURE 4. The cytoplasmic domain of LAG-3 effectively increases cell surface expression of the CD4-LAG-3 chimeric protein. Several LAG-3/CD4 chimeric proteins fused to EGFP were constructed and expressed in Jurkat cells, as described in *Materials and Methods* (Supplemental Fig. 1). Each transfectant was stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for 24 h. (**A**) Intracellular and cell surface levels of mock, WT LAG-3-EGFP (LAG-3-EGFP), LAG-3ΔCY-EGFP, and LAG-3-CD4-EGFP before or after stimulation of Jurkat cells. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. (**B**) Relative fold changes in surface and intracellular MFI of mock, LAG-3-EGFP, LAG-3ΔCY-EGFP, and LAG-3-CD4-EGFP before or after stimulation. Data represent the mean ± SEM from three independent experiments. (**C**) Intracellular and cell surface levels of mock, WT CD4-EGFP (CD4-EGFP), CD4ΔCY-EGFP, and CD4-LAG-3-EGFP before or after stimulation. Dotted line, background staining of isotype control Ab; thin line, specific staining of CD4. Each number in the histograms indicates MFI. (**D**) Relative fold changes in surface and intracellular MFI of mock, CD4-EGFP, CD4ΔCY-EGFP, and CD4-LAG-3-EGFP before or after stimulation. Data represent the mean ± SEM from three independent experiments.
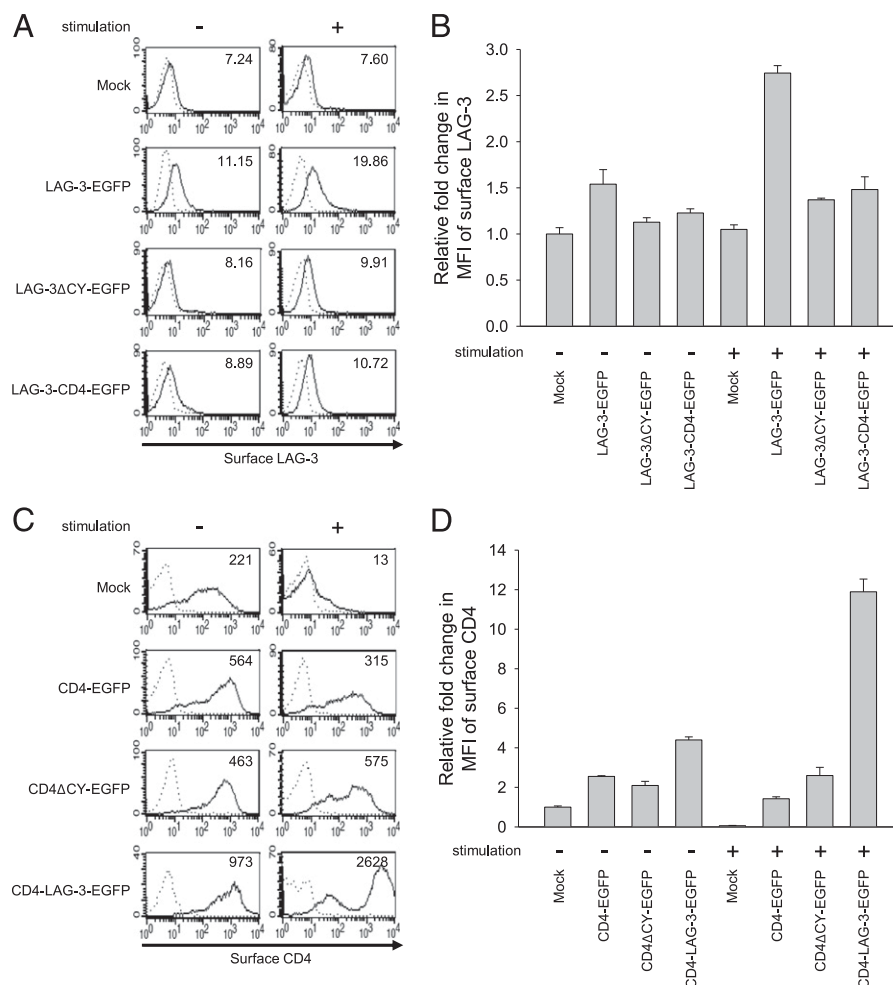
ficking to the cell surface after stimulation with PMA and ionomycin. To test this hypothesis, we first examined which signal is important to translocate LAG-3 on the surface of T cells. After stimulation of LAG-3–overexpressing Jurkat cells with PMA in the absence of ionomycin, we found that the cell surface expression of LAG-3 is increased (Fig. 5A, 5B). However, stimulation with ionomycin alone failed to translocate LAG-3 on the cell surface of Jurkat cells (Fig. 5A, 5B). Also, ionomycin had no additive effect with PMA on the translocation of LAG-3 to the cell surface of Jurkat cells (Fig. 5A, 5B). These results demonstrated that the treatment of PMA alone is sufficient to induce LAG-3 expression on the surface of T cells.

Because PMA triggers PKC signaling, we treated cells with two PKC inhibitors, STS and BIM, to examine whether they can block the translocation of LAG-3 on the surface of Jurkat cells (27–29). The level of total LAG-3 expression was not changed after treatment of various concentrations of STS and BIM as assessed by Western blot analyses (Supplemental Fig. 3C). After treatment

of STS and BIM, the surface expression of LAG-3 on PMA-activated Jurkat cells was gradually decreased in a dose-dependent manner (Fig. 5C, 5D). However, the intracellular pool of LAG-3 in PMA-activated Jurkat cells was not significantly decreased when treated with STS and BIM (Fig. 5C, 5D).

The cytoplasmic domain of human LAG-3 contains only two serine residues without threonine and tyrosine residues (Fig. 6A) (30, 31). Therefore, only two serine residues are possibly phosphorylated if the cytoplasmic domain of human LAG-3 is modified by signal transduction. One of serine residues (Ser[484]) in human LAG-3 is conserved in mouse LAG-3 and may be phosphorylated by PKC (30, 31). To estimate the functional importance of two possibly phosphorylated serine residues within the cytoplasmic domain of LAG-3 during LAG-3 trafficking to the cell surface, we constructed three different mutants (Ser[484] to Ala[484], Ser[497] to Ala[497], Ser[484] and Ser[497] to Ala[484] and Ala[497]) with amino acid substitutions (Supplemental Fig. 1). After verifying the proper expression of each mutant protein in

were stained with PE-conjugated anti-human LAG-3 Ab. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. (**F**) Relative fold changes in surface and intracellular MFI of WT LAG-3 (LAG-3) and its deletion mutants (LAG-3ΔCY and LAG-3ΔEP) before or after stimulation in the presence of CQ (100 μM). Data represent the mean ± SEM from three independent experiments. (**G**) Before or after stimulation, Jurkat cells expressing WT LAG-3-EGFP (LAG-3-EGFP), LAG-3ΔCY-EGFP, and LAG-3ΔEP-EGFP were stained with anti-human LAG-3 Ab, followed by staining with Alexa594-conjugated donkey anti-goat IgG Ab. After staining, colocalization between EGFP (green fluorescence) and LAG-3 (red fluorescence) was analyzed under a confocal microscope. Data are representative of three independent experiments. (**H**) Before or after stimulation, Jurkat cells expressing WT LAG-3-EGFP (LAG-3-EGFP), LAG-3ΔCY-EGFP, and LAG-3ΔEP-EGFP were stained with lysotracker, as described in *Materials and Methods*. After staining, colocalization between EGFP (green fluorescence) and lysotracker (red fluorescence) was analyzed under a confocal microscope. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$.
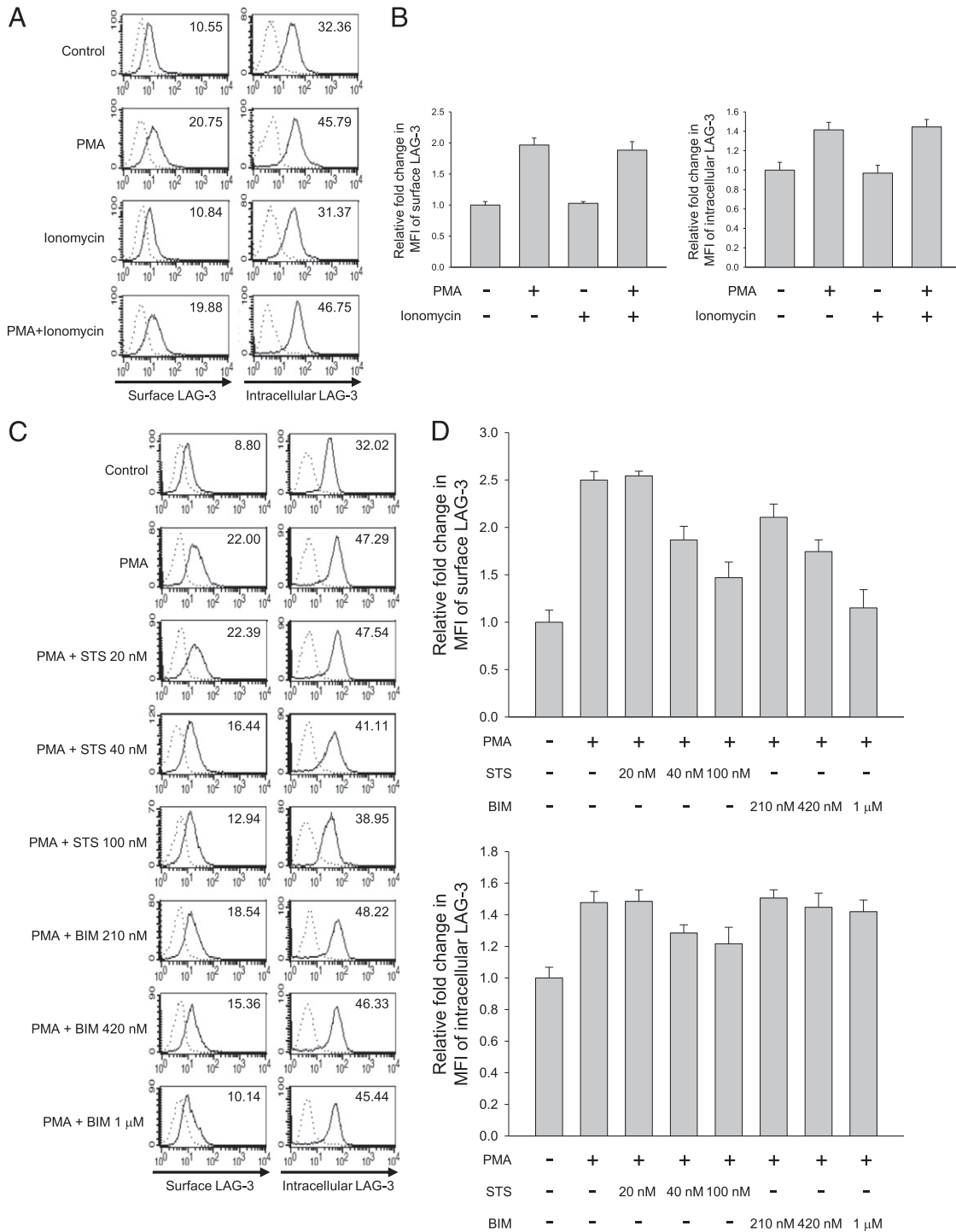
FIGURE 5. Inhibition of PKC signaling blocks surface expression of LAG-3. (**A**) LAG-3–expressing Jurkat cells were stimulated with PMA (10 ng/ml) and/or ionomycin (1 μg/ml) for 24 h. After stimulation, cells were stained with PE-conjugated anti-human LAG-3 Ab, and surface and intracellular LAG-3 were measured by flow cytometry. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates mean MFI. (**B**) Relative fold changes in surface and intracellular MFI of LAG-3 before or after stimulation. Data represent the mean ± SEM from three independent experiments. (**C**) Intracellular and cell surface levels of LAG-3 before or after stimulation of PMA with various concentrations of STS or BIM. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. (**D**) Relative fold changes in surface and intracellular MFI of LAG-3 before or after stimulation of PMA with various concentrations of STS or BIM. Data represent the mean ± SEM from three independent experiments.

Jurkat cells by Western blot analyses using anti–LAG-3 Ab (Supplemental Fig. 3D), we compared the cell surface expression of each amino acid substitution mutant with that of WT LAG-3 (LAG-3), LAG-3ΔCY, and LAG-3ΔEP with or without stimulation of cells with PMA. Among them, only LAG-3ΔCY

mutant showed a defect in the surface expression of LAG-3 on Jurkat cells (Fig. 6B, 6C). Consequently, the two serine residues within the cytoplasmic tail of LAG-3 are not involved in the translocation of LAG-3 to the cell surface of T cells after PKC signaling.

FIGURE 6. Potential serine phosphorylation sites within the cytoplasmic domain are not involved in the translocation of LAG-3 to the cell surface. (**A**) Sequence comparison between human LAG-3 and mouse LAG-3 cytoplasmic domains. Boxes indicate three conserved regions of the LAG-3 cytoplasmic domain, as follows: potential serine phosphorylation site at S^{484}, the KIEELE motif, and the EP motif. Serine residues are indicated with an asterisk located in S^{484} and S^{497} in the human LAG-3 cytoplasmic domain and S^{454} in the mouse LAG-3 cytoplasmic domain. (**B**) Several LAG-3 mutant molecules were constructed and expressed in Jurkat cells, as described in *Materials and Methods* (Supplemental Fig. 1). Each transfectant was stimulated with PMA (10 ng/ml) for 24 h, and intracellular and cell surface levels of WT LAG-3 (LAG-3) and its mutants were measured before or after stimulation. Dotted line, background staining of isotype control Ab; thin line, specific staining of LAG-3. Each number in the histograms indicates MFI. (**C**) Relative fold changes in surface and intracellular MFI of LAG-3 and its mutants before or after stimulation. Data represent the mean ± SEM from three independent experiments.

Discussion

LAG-3 is considered as an important receptor that provides a negative signal in activated T cells to regulate T cell homeostasis (3, 10–14). Identification of the mechanism that regulates molecular trafficking of LAG-3 may thus be essential to better understand T cell homeostasis. Two independent studies have shown that metalloprotease activity is important to regulate cell surface expression of LAG-3 and that the majority of LAG-3 is retained in the lysosomal compartment in unstimulated murine T cells and rapidly translocated to the cell surface upon stimulation (15, 20).

However, it was unclear why the majority of LAG-3 is retained in the lysosomal compartment in unstimulated T cells, which domain of LAG-3 is critical to trafficking to the cell surface after stimu-

lation, and which signal triggers LAG-3 trafficking to the cell surface. In this study, we tried to answer those questions and yielded several new findings on the mechanism of LAG-3 trafficking to the cell surface. First, our data demonstrated that lysosomal degradation of LAG-3 is the major limiting step to prevent cell surface expression of LAG-3. Consistent with previous observation (20), our result indicated that the majority of LAG-3 is localized in lysosomes in resting cells and translocates to the cell surface upon stimulation. Additionally, we found that LAG-3 is degraded within lysosomal compartments before trafficking to the cell surface. Therefore, inhibition of lysosomal degradation of LAG-3 enhances the surface expression of LAG-3 on either unstimulated or stimulated T cells. We also tested the possibility

that metalloprotease-dependent cleavage or proteasomal degradation may influence the surface expression of LAG-3. However, such activities were found to have no effects on the cell surface expression of LAG-3.

Secondly, we tested which domain of LAG-3 is critical for trafficking to the cell surface after stimulation. Our experiments with several LAG-3–truncated mutants revealed that the cytoplasmic domain without the EP motif is important for trafficking LAG-3 to the cell surface. This observation was further confirmed by analyzing the expression pattern of several LAG-3/CD4 chimeric proteins on the surface of T cells. For example, a CD4-LAG-3 chimeric protein that contains the extracellular domain and transmembrane domain of CD4 combined with the cytoplasmic domain of LAG-3 showed significantly enhanced expression of the protein on the cell surface compared with that of CD4 molecule before or after stimulation.

Our last question addressed which signal initiates the translocation of LAG-3 to the cell surface. We found that the treatment of PMA alone was sufficient to induce the trafficking of LAG-3 to the cell surface. Given that PMA induces PKC signaling, we treated cells with two different inhibitors of the signaling and found that it could indeed block translocation of LAG-3 after PMA stimulation. Our results clearly showed that PKC signaling initiates the translocation of LAG-3 to the cell surface.

The cytoplasmic domain of LAG-3 has three conserved regions between human LAG-3 and murine LAG-3 (Fig. 6A) (10). The first conserved region contains a potential serine phosphorylation site that may be involved in binding directly to PKC, homologous to that of CD4 (30, 31). The second conserved region contains the KIEELE motif, which is essential for LAG-3 downstream signaling (10). The third conserved region contains the EP repetitive sequence that binds to LAG-3–associated protein; however, the molecular function of the EP motif remains elucidated (32). Within the cytoplasmic tail of LAG-3, two serine residues could be phosphorylated (Fig. 6A). Because PKC signaling initiates translocation of LAG-3 to the cell surface, we tested whether potential serine phosphorylation sites are involved in the translocation of LAG-3. Accordingly, we made several mutants of LAG-3 to replace serine residue with alanine residue in the cytoplasmic domain of LAG-3 and monitored surface expressions of these LAG-3 mutants. The results indicated that neither of the potential serine phosphorylation sites is involved in LAG-3 trafficking. Therefore, it appears that PKC signaling does not induce a direct conformational change of the cytoplasmic domain of LAG-3.

Based on our results, downregulation of CD4 and upregulation of LAG-3 on the cell surface of T cells after stimulation use the same signaling pathway. In other words, PKC signaling can downregulate the expression of CD4 but upregulate the expression of LAG-3 on the cell surface. In the case of CD4, PKC directly binds to phosphorylated serine residues, thereby inducing endocytosis (26, 31). Supporting this idea, lack of phosphorylated serine residues within the cytoplasmic domain of CD4 inhibits the induction of endocytosis (31). In the case of LAG-3, PKC may modify the structure of the adaptor molecule that interacts with LAG-3, rather than directly modify the structure of LAG-3. After conformational change of this adaptor molecule, LAG-3 may translocate to the cell surface. Therefore, we propose that PKC signaling may act as one of the switches to turn off T cell activation to achieve T cell homeostasis.

In conclusion, lysosomal compartments are important organelles for LAG-3 stability and trafficking, and the cytoplasmic domain of LAG-3 is necessary for the surface expression of LAG-3. Moreover, PKC signaling can induce the translocation of LAG-3 to the cell surface without modification of the cytoplasmic domain.

Accordingly, we hypothesize that the conformational change of a certain molecule by PKC signaling allows the translocation of LAG-3 to the cell surface. Further study is required to identify which proteins are involved in LAG-3 trafficking to the cell surface by interacting with the cytoplasmic domain of LAG-3. Together with these insights, our observation would be helpful to clarify the surface expression mechanism of LAG-3 and may lead to therapeutic intervention to regulate T cell homeostasis.

Disclosures
The authors have no financial conflicts of interest.

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