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Janus Kinase 2 is Associated With a Box 1-Like Motif and Phosphorylates a Critical Tyrosine Residue in the Cytoplasmic Region of Cytotoxic T Lymphocyte Associated Molecule-4

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Abstract It is a consensus that a cytotoxic T lymphocyte associated molecule-4 (CTLA-4) transduces inhibitory signal for T cell activation under physiological condition, indicating that this molecule is an important regulator of T cell homeostasis in vivo. It has been reported that phosphorylation and dephosphorylation of tyrosine residue Y-165 in the cytoplasmic region of CTLA-4 play an important role in its negative signaling and cell surface expression. Some signaling molecules such as Src homology 2 protein tyrosine phosphatase 2 (SHP-2) and the p85 subunit of phosphatidylinositol 3 kinase (PI3 kinase) associate with phosphorylated tyrosine residue Y-165, through Src homology 2 (SH2) domains. On the other hand, the adapter complex proteins, AP-2 and AP-50 interact with the same tyrosine residue when unphosphorylated, resulting in clathrin-mediated endocytosis of CTLA-4 molecules. The objective of this study is to identify a tyrosine kinase that can directly bind and phosphorylate the critical tyrosine residue, Y-165 in the cytoplasmic domain of CTLA-4. Here, we demonstrated that 1) Janus Kinase 2 (Jak2) was directly associated with a box 1-like motif in the cytoplasmic tail of CTLA-4 molecule, 2) Jak2 phosphorylated Y-165 residue in the cytoplasmic region of CTLA-4 molecule, and 3) Jak2 was associated with CTLA-4 in HUT 78 T cell lines. J. Cell. Biochem. 78:241–250, 2000.

Key words: cytotoxic T lymphocyte associated molecule-4 (CTLA-4); Jak2

T cells require two signals for full activation [Jenkins et al., 1991; Johnson and Jenkins, 1992]. The first signal is provided by ligation of the T cell receptor (TCR) with the antigenic peptide plus major histocompatibility complex (MHC) molecule on antigen presenting cells (APC), and thus provides an antigen specificity to the immune responses. The second "costimulatory" signal is provided by binding of specific receptors on T cells with their ligands on APC. The best characterized costimulatory pathway is provided by CD28 on T cells interacting with B7 family molecules on APC [Allison, 1994; June et al., 1990; Thompson et al., 1989]. CTLA-4, a homologue of CD28 binds to B7 or related molecules on APC with higher avidity than CD28 does [Linsley et al., 1991]. CTLA-4 can not be detected on resting T cells, but after T cell activation, CTLA-4 mRNA was rapidly increased and peak expression of surface CTLA-4 molecules was observed after 24–72 h [Walunas et al., 1994]. Many reports have described the function of CTLA-4 signaling as a negative regulator for T cell activation. In vivo treatment of anti-CTLA-4 monoclonal antibody (mAb) led to the augmentation of anti-tumor immunity and exacerbation of autoimmune diseases [Perrin et al., 1996; Leach et al., 1996]. Additionally, Tivol and colleague demonstrated that CTLA-4-deficient mice rapidly developed lymphoproliferative disease with

Abbreviations used: APC, antigen presenting cell; CTLA-4, cytotoxic T lymphocyte associated molecule 4; GM-CSF, granulocyte-macrophage colony stimulating factor; Jak; Janus kinase; MAb, monoclonal antibody; MHC, major histocompatibility complex; PI3-kinase, phosphatidylinositol 3 kinase; Rlk, resting limphocyte kinase; SH, Src homology; SHP-2, Src homology 2 protein tyrosine phosphatase-2; TCR, T cell receptor.

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multiorgan lymphocytic infiltration and tissue destruction and died by 3–4 weeks of age [Tivol et al., 1995], indicating that CTLA-4 was one of the most important regulators of T cell homeostasis in vivo. The intracellular signaling pathway responsible for CTLA-4-mediated T cell inactivation has been analyzed. Recently, SHP-2 and PI3 kinase associating with Y-165VKM motif in the cytoplasmic region of CTLA-4 have been identified as candidates for signal transducing molecules of CTLA-4 [Schneider et al., 1995; Marengere et al., 1996]. Additionally, CTLA-4 was found to interact with the TCR complex ζ-chain and CTLA-4 ligation resulted in dephosphorylation of TCR ζ-molecules [Lee et al., 1998]. Because the interaction between CTLA-4 and SHP-2 or PI3 kinase is mediated through the binding of phosphotyrosine within CTLA-4 and SH2 domain within kinase or phosphatase, the identification of kinase(s) that phosphorylate tyrosine residue in the cytoplasmic region of CTLA-4 is critical. In this regard, four groups reported that resting lymphocyte kinase (Rlk) and Src family tyrosine kinases could phosphorylate the critical tyrosine residue, Y-165 by cotransfection of CTLA-4 and kinases in cell lines [Miyatake et al., 1998; Schneider et al., 1998; Chuang et al., 1999; Bradshaw et al., 1997]. A number of receptors, including cytokine receptors, lack catalytic domains in their cytoplasmic regions. However, ligand binding to these receptors initiates a protein tyrosin phosphorylation cascade and intracellular signaling by activating members of the Jak family of receptor associated kinases. These kinases constitutively associate with the membrane-proximal portions of cytokine receptor cytoplasmic domains, termed box 1 and box 2 and become activated upon ligand-induced homo- or heterodimerization [Tanner et al., 1995; Ihle, 1995; Murakami et al., 1991].

Here, we demonstrate that Jak2 is associated with a box 1-like motif in the cytoplasmic tail of CTLA-4 molecule and Jak2 but not Jak1 phosphorylates the critical tyrosine residue, Y-165. These results suggest that Jak kinases also regulate the signaling and the cell surface expression of CTLA-4 molecule.

MATERIALS AND METHODS

Recombinant DNA Constructs

Murine Jak 2 and Jak1 cloned into a mammalian expression vector pBOS were kindly provided by Dr. S. Nagata (Osaka University, Osaka, Japan). The cDNA construct for CTLA-4 was generated by PCR method, using a forward primer containing *Xho* I restriction endonuclease site just before amino-terminal region of CTLA-4 (5′-TTCTCG-AGATGGCTTGTCTTGGA-3′) and a reverse primer containing *Spe* I site following stop codon (5′-TTACTAGTTCAGTTGATGGGAATAAA-3′). Resulted fragment was sequenced and cloned into mammalian expression vector pME18S using *Xho* I and *Spe* I and was designated as CTLA-4 WT. The mutant CTLA-4 molecules lacking 5, 10, 15, 20, and 25 amino acid residues at the carboxy-terminal tail (desinated as CTLA-4-5, CTLA-4-10, CTLA-4-15, CTLA-4-20, and CTLA-4-25, respectively) were also constructed by PCR method, using a forward primer containing *Bam* HI site just before the transmembrane region of wild type molecule (5′-TTGGATCCTTGTCGCAGTTAG-CT-3′) and reverse primers containing stop codon at the suitable position, followed by *Spe* I site (for CTLA-4-5: 5′-TTACTAGTTCAATAAGGCTGAAATTG-3′, for CTLA-4-10: 5′-TTACTAGTTCACTTTTCACATTCTGG-3′, for CTLA-4-15: 5′-TTACTAGTTCACTCTGTTGGGGGCAT-3′, for CTLA-4-20: 5′-TTACTAGTTCATTTCACATAGACCCCTG-3′, and for CTLA-4-25: 5′-TTACTAGTTCATGTTGTAAGAGGACTTCT-3′). Resulted mutant fragments were sequenced and ligated with an extracellular portion of wild-type molecule. Additional mutant CTLA-4 constructs having point mutation(s) at the tyrosine residue(s) in the cytoplasmic region of CTLA-4 were constructed by a Sculptor in vitro mutagenesis system, version 3 (Amersham Pharmacia Biotech, Tokyo, Japan). Constitutively activated form of Lck (F505) [Shibuya et al., 1994] was kindly provided by Dr. H. Shibuya (Osaka University, Osaka, Japan). To prepare polyclonal antibody against CTLA-4, a fusion protein consisting of the CTLA-4 cytoplasmic portion and Glutathione-S-Transferase was generated. Using a forward primer containing *Bam* HI site (5′-AAGGATCCGCAAGATGCTAAAGAA-3′) and a reverse primer containing *Eco* RI site (5′-TTGAATTCAATCAGTTGATGGGAATAAAAT-3′), a cytoplasmic portion of CTLA-4 cDNA was amplified. Resulted fragment was sequenced and cloned into pGEX3X vector (Amersham Pharmacia Biotech, Tokyo, Japan) and a fusion protein was generated as de-

scribed previously [Katagiri et al., 1999]. A soluble form of B7-1, a natural ligand of CTLA-4 was generated as a fusion protein with a Fc portion of human immunogloblin G1, as described previously [Murakami et al., 1996]. Briefly,the extracellular region of mouse B7-1 was amplified using a forward primer (5′-CTAAGCTCCATT-GGCTCTAGATTC-3′) and a reverse primer (5′-ACAAGATCTTTCTTGCTATCAGGAGG-3′). Resulted PCR fragment was fused in-frame with cDNA of the human IgG1 heavy chain constant region in pBOS expression vector. COS-7 cells were transfected with the expression vector and a fusion protein desinated as B7-1 Ig was purified from culture supernatant using protein A sepharose affinity column.

Antibodies

Hamster anti-mouse CTLA-4 monoclonal antibody (mAb), UC10-4F10-11 (PharMingen, San Diego, CA), rabbit anti-mouse Jak1 or Jak2 antibody (Upstate Biotechnology, Lake Placid, NY), mouse anti-human CD8 mAb (Coulter Immunotech, Miami, FL), and anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY) were used in this study. For Western blot analysis, rabbit was immunized by a Glutathione-S-Transferase fused with the cytoplasmic portion of CTLA-4 (described above), and polyclonal anti CTLA-4 antibody was prepared.

Cells and Transfection

A human embryonic kidney epithelial cell line, 293T was provided from Dr. D. Baltimore (Massachusetts Institute of Technology, Cambridge, MA). 293T cells (3×10^5) were transfected with the plasmid DNA (3 μg of both CTLA-4 mutants and Jak kinases) using LIPOFECTAMINE (GIBCO BRL, Rockville, MD) reagent.

Flowcytometric Analysis

For direct immunostaining, 1×10^5 of transfectants were incubated with PE anti-mouse CTLA-4 mAb (1:300 dilution). To investigate association of CTLA-4 with the natural ligand, B7-1, cells were incubated with B7-1 Ig (10 μg/ml) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG1. Stained cells were analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ).

Immunoprecipitation and Western Blot

Transfected cells were lysed by Brij 97 buffer (1% Brij 97, 25 mM Tris pH7.6, 150 mM NaCl, 5 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 tablet of complete protease inhibitor cocktail (GIBCO BRL, Tokyo, Japan) per 50 ml of buffer). Lysates were centrifuged, precleared with protein A sepharose, and then immunoprecipitated by protein A beads coated with specific antibodies. All lysates were normalized by cell number and thus the sample corresponding to 1×10^6 cells was loaded onto the each lane of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% or 10–20% gradient gel, Daiichi-Kagaku Co, Tokyo), transferred to PVDF membrane, and Western blotted by antibody.

In Vitro Kinase Assay

After immunoprecipitation, precipitates were washed once with in vitro kinase buffer (40 mM HEPES pH 7.4, 10 mM $MgCl_2$, 3 mM $MnCl_2$). Kinase reactions were performed for 20 min at 37°C in 30 μl of the buffer containing 10 μCi of ^{32}P-γATP. Then the resulted beads were boiled in sample buffer and resulted samples were subjected to SDS-PAGE analysis. The gel was incubated in 1M KOH for 90 min at 60°C, fixed, dried up, and then visualized by autoradiography.

RESULTS

Jak2 was Associated With CTLA-4

To investigate whether Jak family kinases could directly associate and phosphorylate the critical tyrosine residue in the cytoplasmic region of CTLA-4, we cotransfected murine CTLA-4 with several Jak family kinases into 293T cells. Surface expression of CTLA-4 molecules was investigated by flowcytometer using B7-1 Ig. Mock transfectants did not express CTLA-4, however, both CTLA-4 plus Jak1 and CTLA-4 plus Jak2 transfectants expressed CTLA-4 (Fig. 1A). Then cells were subjected to immunoprecipitation with anti-CTLA-4 mAb, followed by Western blotting with anti-Jak antibody. As shown in Figure 1B, Jak2, but not Jak1 molecules were coimmunoprecipitated with CTLA-4 molecules, while equal amounts of CTLA-4 molecules were immunoprecipitated from both Jak1 and Jak2 transfectants. Jak3 was not associated with CTLA-4 (data not shown).

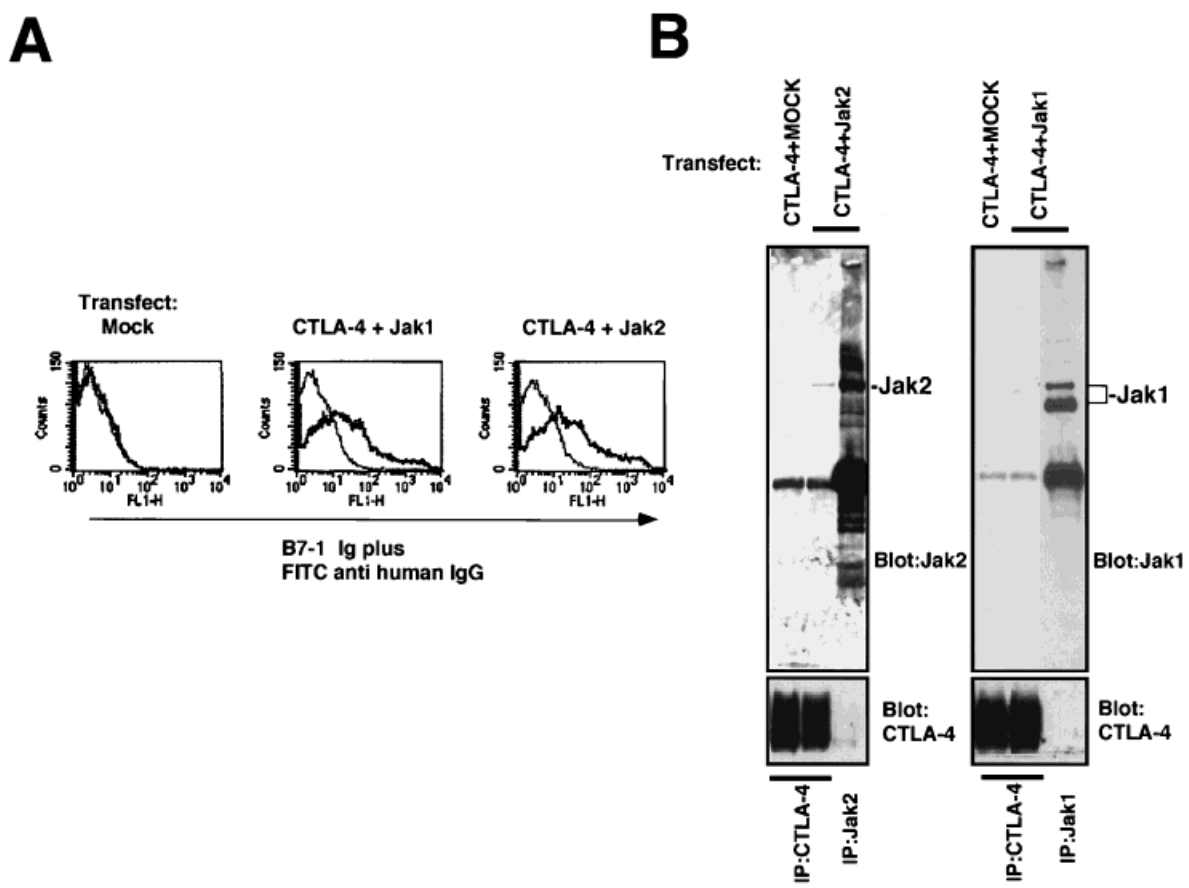

Fig. 1. Jak2 but not Jak1 was associated with CTLA-4 molecules. **A**: 293T cells (3×10^5) were transfected with the CTLA-4 expression vector. 48 hours after transfection, cells were stained with B7-1 Ig and FITC conjugated anti human IgG and were analyzed by flowcytometer. Mock; empty vector. **B**: Cell lysates derived from 1×10^6 of 293T cells, transfected with cDNA of CTLA-4 and Jak2 or Jak1 kinase were immunoprecipitated (IP) with anti-CTLA-4, anti-Jak2 or anti-Jak1 and Western blotted (Blot) with anti-Jak2 or anti-Jak1, and polyclonal anti-CTLA-4 antibodies. It should be noted that, in anti-Jak1 blot, 130 and 110 kilodalton bands were detected, which represented intact Jak1 molecule and its breakdown product, respectively. Mock; empty vector.

Jak2 was Associated With the Cytoplasmic Region of CTLA-4 in a T Cell Line

It is very important to confirm that Jak kinases were associated with CTLA-4 molecules in T cells. We used a T cell line, HUT-8CT, which was established by Dr. Weiss and colleague [Stein et al., 1994]. This cell line expressed a mutant CTLA-4 molecule which extracellular and transmembrane regions were replaced by those of CD8 moleucle. Mutant CTLA-4 molecules were immunoprecipitated by anti-CD8 mAb and Western blot analysis was preformed. As shown in Figure 2, Jak2 molecule was found in anti-CD8 immunoprecipitates in HUT-8CT, while in control T cells, HUT-CD8 which expressed only wild-type CD8, Jak2 was not found in anti-CD8 immunoprecipitates. To confirm the specificity, the same samples were blotted by anti-Jak1 antibody. Although the same amounts of samples were applied, no signal was detected around 130 kilodalton. Furthermore, we demonstrated that HUT-8CT cells expressed similar amounts of both Jak1 and Jak2 kinases (Fig. 2, lower panel). These results strongly suggested that Jak2, but not Jak1 molecules were associated with the cytoplasmic region of CTLA-4 molecules in T cells.

Mapping of the Jak2 Interaction Site in the Cytoplasmic Region of CTLA-4

Many receptors for cytokine and growth factor contained two motifs, box 1 and box 2 that were important for interaction with Jak ki-

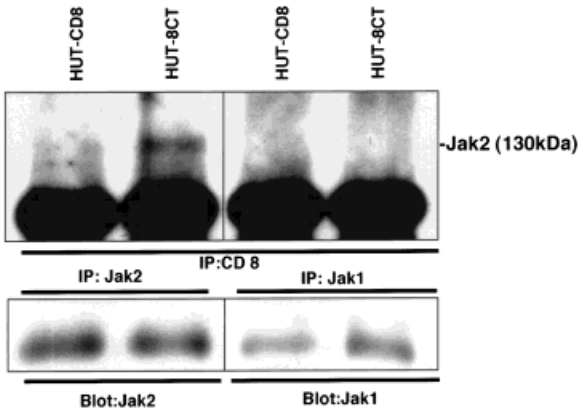

Fig. 2. Jak2 associated with CTLA-4 in HUT78 T cell line. HUT-8CT or HUT-CD8 cells (2.5×10^7) were solubilized and were immunoprecipitated (IP) with 10 μg/ml of anti-CD8 mAb (**upper**) or anti-Jak antibodies (**lower**). Western blot (Blot) analysis was then performed with anti-Jak2 or anti-Jak1 antibody.

nases [Tanner et al., 1995; Ihle, 1995; Murakami et al., 1991]. Box 1 is defined as a proline-rich portion that is most critical for the interaction of Jak kinases with the receptors. We searched for a proline-rich portion in the cytoplasmic region of CTLA-4. As shown in Figure 3A, we found the presence of a box 1-like proline-rich portion (PPTEP, amino acids 169–173). To ascertain the interaction between Jak2 and the box 1-like motif in the cytoplasmic tail of CTLA-4, we constructed a set of deletion mutant CTLA-4 molecules (Fig. 3A). The resulted mutant CTLA-4 and Jak2 cDNA were transfected into 293T cells. All mutant and wild-type CTLA-4 molecules were normally expressed and could bind to the soluble form of ligand, B7-1 Ig molecules (Fig. 3B). Then immunoprecipitation analysis was performed with anti-CTLA-4 mAb, followed by Western blotting with anti-Jak2 antiserum. Although similar amounts of CTLA-4 were precipitated from all transfectants, Jak2 was associated with the wild type and two mutant CTLA-4 molecules (CTLA-4-5 and CTLA-4-10) that contained box 1-like motif (Fig. 3C). In contrast, three other mutant CTLA-4 (CTLA-4-15, CTLA-4-20, CTLA-4-25) either completely or partially lacking PPTEP motif could not associate with Jak2.

Jak2 Phosphorylates CTLA-4

It was very important to investigate whether CTLA-4 could be phosphorylated by Jak kinases. cDNA of CTLA-4 and Jak or Lck kinase were cotransfected into 293T cells. After immunoprecipitation with anti-CTLA-4 mAb, Western blot analysis was performed by anti-phosphotyrosine mAb, 4G10. As shown in Figure 4, Jak2 kinase as well as constitutively active-Lck kinase but not Jak1 kinase phosphorylated CTLA-4. A molecule with approximate molecular weight of 130 kilodalton was coimmunoprecipitated and phosphorylated in CTLA-4 plus Jak2 lane. It was confirmed that this band was Jak2, by Western blot analysis using anti Jak2 antibody (data not shown).

Jak2 Phosphorylates the Critical Tyrosine Residue in the Cytoplasmic Region of CTLA-4

There are two tyrosine residues (Y-165 and Y-182) in the cytoplasmic region of CTLA-4. We constructed three point-mutant CTLA-4 molecules, Y165F, Y182F, and Y165/182F (Fig 5A). All mutant CTLA-4 moleucles were normally expressed on 293T cells and could bind to soluble form of B7 moleucles (Fig 5B). Then the resulted mutant constructs were cotransfected with Jak2 cDNA into 293T cells and immunoprecipitation was performed by anti-CTLA-4 mAb, followed by in vitro kinase assay using ^{32}P-γATP. Although Jak2 molecules were found within anti CTLA-4 immunoprecipitates, only wild type CTLA-4 and Y182F but not Y165F and Y165/182F were phosphorylated in vitro (Fig. 5C). However, we could not rule out the possibility that endogenous kinase(s) in 293T cells contributed to the phosphorylation of CTLA-4 molecule. As shown in Figure 5C, a weak background kinase activity was detected in the lane of CTLA-4 WT transfectants in the absence of Jak2. Interestingly, endogenous kinase(s) as well as exogenous Jak2 failed to phosphorylate Y165/182F CTLA-4 mutant. In contrast to exogenous Jak2, endogenous kinase weakly phosphorylated Y165F CTLA-4 mutant. In any event, these results demonstrated that Jak2 was associated with CTLA-4 and phosphorylated the critical tyrosine residue, Y-165.

DISCUSSION

Here, we demonstrated that 1) Jak2 was associated with box 1-like motif in the cytoplasmic tail of CTLA-4 molecule, 2) Jak2 phosphorylated Y-165 residue in the cytoplasmic tail of CTLA-4 molecule, and 3) Jak2 was associated with CTLA-4 in HUT78 T cell line. CTLA-4, a

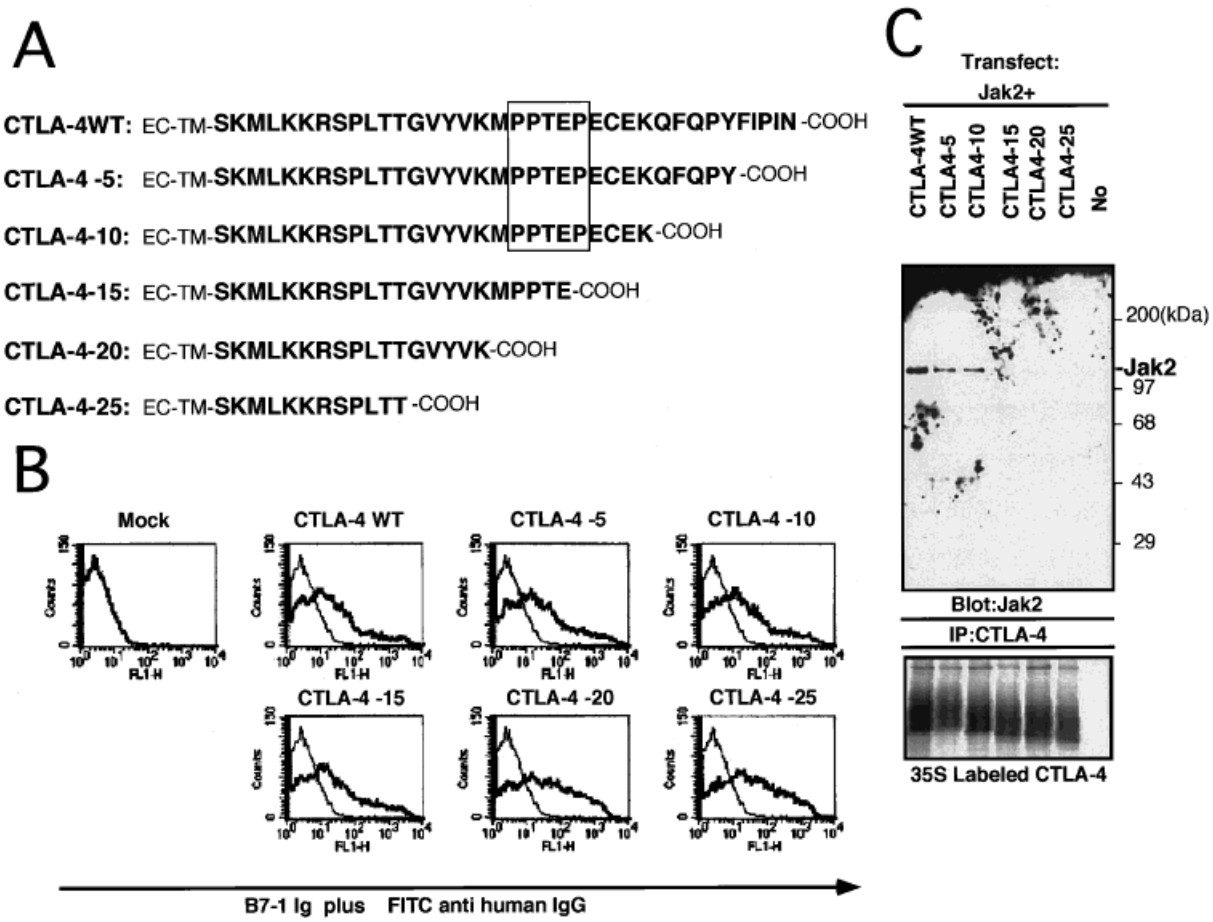

Fig. 3. CTLA-4 box 1-like domain mediates its association with Jak2. **A**: Schematic representation of wild-type (WT) and mutated CTLA-4 molecules. Box 1 like motif PPTEP was boxed. EC; extracellular portion. TM; transmembrane portion. **B**: Mutant CTLA-4 were transfected into 293T cells and 48 h after transfection, cells were stained with B7-1 Ig and FITC conjugated anti human IgG and were analyzed by flowcytometer. Mock; empty vector. **C**: Mutant CTLA-4 cDNA were cotransfected with Jak2 into 293T cells. No; cells were transfected only with Jak2. Then lysates from the resulted 293T cells were immunoprecipitated (IP) with anti-CTLA-4 mAb and blotted (Blot) with anti-Jak2. In parallel experiments, transfectants were internal labeled with ^{35}S and lysates were immunoprecipitated with anti-CTLA-4 mAb. Note that similar amounts of CTLA-4 molecules were detected in six different transfectants.

negative regulator of T cells, can not be detected on resting T cells, but after T cell stimulation, CTLA-4 mRNA was rapidly increased and peak expression of surface CTLA-4 molecules was observed after 24–72 h [Walunas et al., 1994]. It seems that several tyrosine kinases are activated after T cell activation and then these kinases phosphorylate many substrate molecules including CTLA-4 molecules. Before phosphorylation of Y-165 residue in the cytoplasmic region of CTLA-4, clathrin-associated adaptor complex proteins can associate with CTLA-4 molecules and induce the internalization of CTLA-4 molecules from the cell surface. After phosphorylation of Y-165 residue, several signaling molecules including SHP-2, PI3 kinase or tyrosine kinases themselves can replace clathrin-associated adaptor complex and interact with the Y-165 residue, resulting in the stable expression of CTLA-4 molecules on the cell surface of activated T cells. Thus, activated T cells expressing CTLA-4 become susceptible to the negative feedback mechanism of immune system through ligation of CTLA-4 molecules by B7 molecules on APC. Therefore, it is very important to define a tyrosine kinase that can phosphorylate the Y-165 residue. In this regard, four groups reported that Rlk and Src family tyrosine kinases could phosphorylate the Y-165 residue in the cytoplasmic region of CTLA-4 [Miyatake et al., 1998; Schneider et al., 1998;

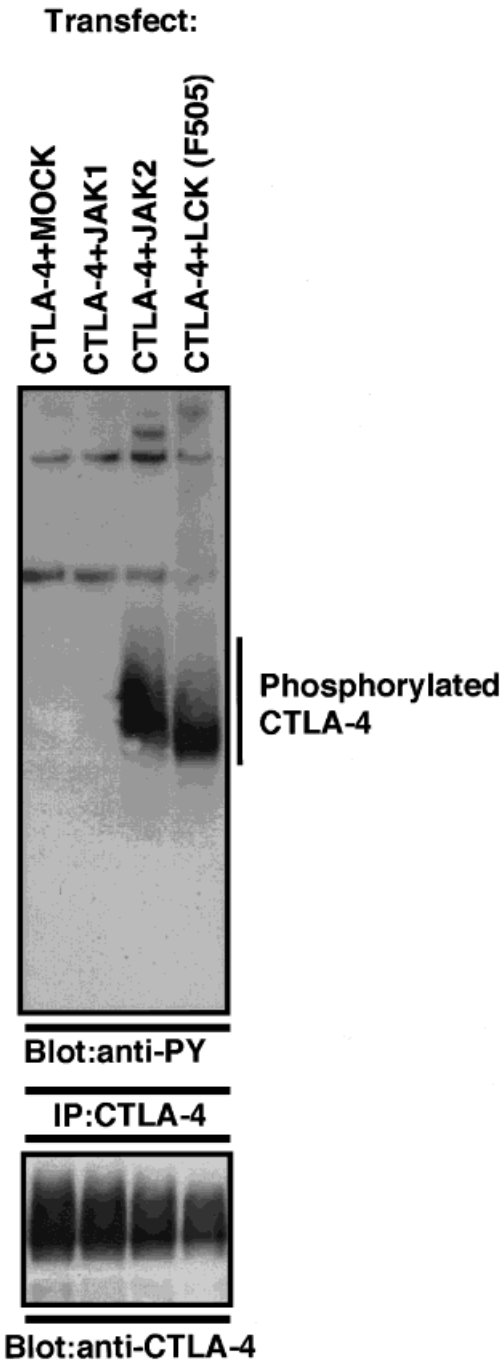

Fig. 4. Jak2 phosphorylates CTLA-4. 293T cells were cotransfected with cDNA of CTLA-4 and Jak or active form of Lck kinase. Then lysates from the resulted 293T cells were immunoprecipitaed (IP) with anti-CTLA-4 mAb and western blotted (Blot) with anti-phosphotyrosine (PY) mAb, 4G10. Note that similar amounts of CTLA-4 molecules were detected in all transfectants.

Chuang et al., 1999; Bradshaw et al., 1997]. One group described that a member of the Bruton tyrosine kinase family, Rlk could phosphorylate the critical tyrosin residue, Y-165 [Schneider et al., 1998]. However, Rlk failed to directly associate with CTLA-4 molecule. In addition, three independent groups reported that Src family tyrosine kinases such as Fyn and Lck phosphorylated both Y-165 and Y-182 residues in the cytoplasmic region of CTLA-4, and these kinases could also associate with CTLA-4 [Miyatake et al., 1998; Chuang et al., 1999; Bradshaw et al., 1997]. One emphasized the importance of SH2 domains within Src kinases which presumably bound to the Y-165VKM motif of CTLA-4 [Miyatake et al., 1998], while others emphasized the importance of SH3 domains [Chuang et al., 1999]. Both groups also pointed out the participation of as yet determined domains within kinases, which could bind to mutant CTLA-4 molecules lacking any tyrosine residues [Miyatake et al., 1998; Chuang et al., 1999]. We demonstrated that tyrosine kinase Jak2 could directly (not through phosphotyrosine residues in CTLA-4 molecules) associate with box 1-like motif (PPTEP) in CTLA-4 molecules. Since Jak2 molecules were expressed by various kind of cells including T cells [Ihle, 1995], it was reasonable to speculate that this kinase could associate constitutively with CTLA-4 molecules in T cells. Indeed, we demonstrated that Jak2 molecule was associated with CD8-CTLA-4 chimeric molecule in HUT78 cells (Fig. 2). We suggest that Jak2 tyrosine kinase is one of the most possible tyrosine kinases that phosphorylate the critical tyrosine residue, Y-165 in T cells. However, since CTLA-4 molecules are expressed only on activated T cells and activated T cells contain many activated tyrosine kinases including Fyn, Lck, and Rlk, these tyrosine kinases also play a role in CTLA-4 signal transduction in T cells. It should be pointed out that a transient expression system using 293T cells allowed us to investigate the association and phosphorylation of CTLA-4 by kinases. However, this system was unable to test whether phosphorylated CTLA-4 molecules at critical residue were indeed recruited to the cell surface, rather than staying in the cytoplasm through interaction with clathrin-associated adaptor molecules. This may be due to the efficient transcription of transfected CTLA-4

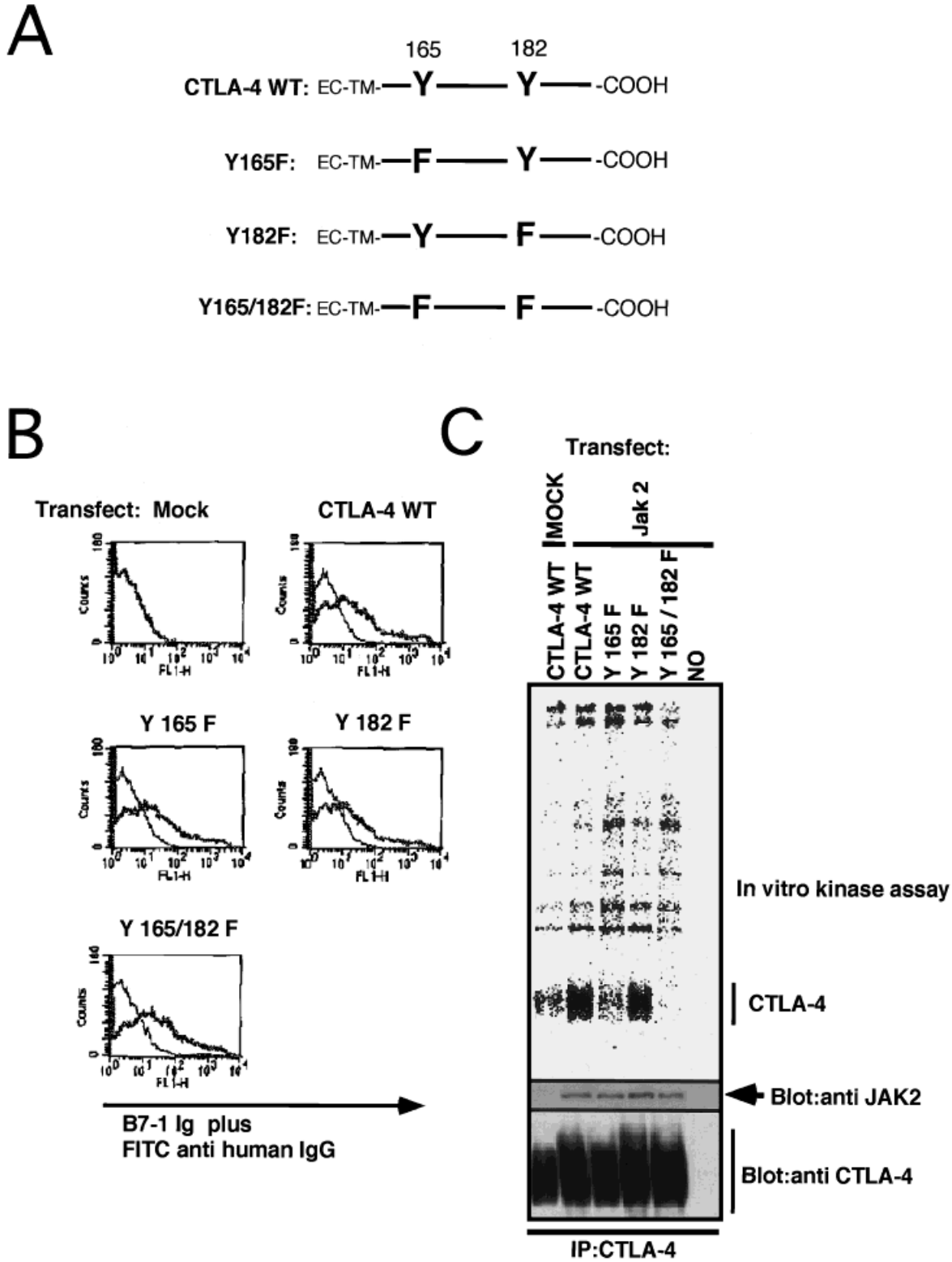

Fig. 5. Jak2 phosphorylates the critical tyrosine residue in the cytoplasmic region of CTLA-4. **A**: Three point mutant CTLA-4 constructs were generated. Tyrosine residue (Y) at position 165 and/or 182 was (were) replaced by phenylalanine (F). **B**: Three point mutants and wild-type CTLA-4 were transiently transfected to 293T cells. Cell surface expression of those CTLA-4 was analyzed by flowcytometer. Mock; empty vector. **C**: 293T cells were transfected with wild-type (WT) or mutant CTLA-4 plus Jak2 cDNA. Cell lysates were immunoprecipitated (IP) with anti-CTLA-4 mAb, followed by either Western blot (Blot) analysis with anti-CTLA-4 or anti-Jak 2 antibody or in vitro kinase assay using ^{32}P-γATP. No; no CTLA-4 construct cDNA. Mock; no Jak2 cDNA.

gene, which leads to the production of excess amounts of CTLA-4.

In cytokine receptor signal transduction, activated Jak kinases phosphorylate the receptor chains, generating docking sites for signal transducers and activators of transcription (STATs) and other intracellular signaling molecules [Ihle, 1995]. We demonstrated that Jak2 was associated with CTLA-4 molecules and could phosphorylate the tyrosine residue,Y-165 in the cytoplasmic domain of CTLA-4 moleucle (Figs. 1–5). It is possible that after phosphorylation of the tyrosine residue, Y-165, SHP-2, PI3 kinase, and SH2 containing tyrosine kinases such as Fyn and Lck can be effectively recruited to the phosphotyrosine residue, Y-165. In this regard, Jak2 is the most possible kinase that initially phosphorylate Y-165, because Jak2 can associate constitutively with non-phosphorylated form of CTLA-4. However, the initial stimuli that can activate Jak2 is yet to be determined. It is also possible that Jak kinases can regulate SHP-2 and PI3 kinase activities. In fact, recently, it was demonstrated that 1) p85 subunit of PI3 kinase was phosphorylated by Jak2 kinase in GM-CSF (granulocyte-macrophage colony stimulating factor) signaling cascade [Al-Shami, 1999] and 2) SHP-2 moleucles were associated and phosphorylated by Jak1 and Jak2 kinases in vitro [Yin et al., 1997].

In conclusion, we demonstrated that 1) Jak2 was associated with box 1 like motif in the cytoplasmic tail of CTLA-4 molecule, 2) JAK2 phosphorylated Y-165 but not Y-182 residue in the cytoplasmic portion of CTLA-4 molecule, and 3) Jak2 was associated with CTLA-4 in HUT78 T cell lines.

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