The Journal of Immunology

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J Immunol 1999; 163:563-568; ;
http://www.jimmunol.org/content/163/2/563

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Cas-L Is Required for β_1 Integrin-Mediated Costimulation in Human T Cells[1]

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β_1 integrins provide a costimulus for TCR/CD3-driven T cell activation and IL-2 production in human peripheral T cells. However, this β_1 integrin-mediated costimulation is impaired in a human T lymphoblastic line, Jurkat. We studied the molecular basis of this impaired costimulation and found that Cas-L, a 105-kDa docking protein, is marginally expressed in Jurkat T cells, whereas Cas-L is well expressed in peripheral T cells. Cas-L is a binding protein and a substrate for focal adhesion kinase and is tyrosine phosphorylated by β_1 integrin stimulation. We here show that the transfection of wild-type Cas-L in Jurkat T cells restores β_1 integrin-mediated costimulation. However, Cas-L transfection had no effect on CD28-mediated costimulation, indicating that Cas-L is specifically involved in the β_1 integrin-mediated signaling pathway. Furthermore, transfection of the Cas-LΔSH3 mutant failed to restore β_1 integrin-mediated costimulation in Jurkat cells. Cas-LΔSH3 mutant lacks the binding site for focal adhesion kinase and is not tyrosine phosphorylated after β_1 integrin stimulation. These findings strongly suggest that the tyrosine phosphorylation of Cas-L plays a key role in the signal transduction in the β_1 integrin-mediated T cell costimulation. ***The Journal of Immunology,*** **1999, 163: 563–568.**

The β_1 integrins are the major adhesion molecules in T cells and are involved in the trafficking of T cells (1). In addition to their roles in cell adhesion, β_1 integrins transduce signals into the interior of cells and induce various biological events such as cell differentiation (2), proliferation (3, 4), survival (5), migration (6), and cytoskeleton organization (7). The binding of T cells to extracellular matrix through β_1 integrins provides costimulatory signals to the CD3-dependent T cell proliferation and IL-2 production (3, 4, 8–12). One of the major biochemical signals induced by β_1 integrin stimulation is tyrosine phosphorylation of various proteins, including focal adhesion kinase (pp125FAK),[3] paxillin, p59fyn/p56lck, mitogen-activated protein (MAP) kinase, phospholipase C-γ, and pp105/Cas-L in T cells (13–15). Tyrosine phosphorylation of these signaling molecules appears to be involved in β_1 integrin-mediated costimulation in T cells.

Recently, we cloned a Crk-associated substrate (p130Cas)-related protein, pp105/Cas-L (16, 17). Among Cas family proteins (p130Cas, Cas-L/Hef-1, and Sin/Efs), Cas-L is preferentially expressed in lymphocytes (16). Every Cas family protein contains an N-terminal Src homology (SH) 3 domain followed by a cluster of YXXP motifs and a YDYVHL motif, which are potential binding sites for the SH2 domains (18, 19, 20). Cas-L and p130Cas are tyrosine phosphorylated by the stimulation of β_1 integrins (13, 16, 21) and by oncoproteins such as v-Crk, V-Src, and v-Abl (16, 17, 20). These findings indicate that Cas family proteins are docking proteins that participate in tyrosine phosphorylation-mediated signal transduction. Cas family proteins bind to pp125FAK by their SH3 domains (16, 20, 22). Furthermore, we found that Cas-L and p130Cas are the substrate of pp125FAK (23, 24). Since pp125FAK is a tyrosine kinase that is activated by integrin stimulation, Cas family proteins appear to transduce integrin-mediated signals just downstream of FAK. The tyrosine-phosphorylated Cas-L binds to the SH2 domains of Crk, Nck, and SHP2 following β_1 integrin stimulation (16), suggesting the involvement of Cas-L in the integrin-mediated signal transduction by the recruitment of these signaling molecules. Despite of the significance of these biochemical findings, the biological functions of Cas-L in the β_1 integrin-mediated signal transduction during immune response have not been well characterized. In this report, we demonstrate that Cas-L plays a crucial role in β_1 integrin-mediated T cell costimulation of IL-2 production.

Materials and Methods

Reagents and Abs

Gentamicin and RPMI 1640 were obtained from Sigma Chemical (St. Louis, MO), Ninety-six-well flat-bottom plates from Becton Dickinson (Lincoln Park, NJ), and [^{3}H]thymidine and ^{125}I from DuPont-NEN (Boston, MA). Anti-CD3 (OKT3; IgG2a), anti-CD29 (integrin β_1 chain) (4B4; IgG1), and anti-CD49d (integrin α_4 chain) (3G6; IgG1) mAbs have been described previously (3, 15, 25). Costimulation assays of Jurkat, Jurkat transfectants, and peripheral T cells were as described (15).

GST-CS1 fusion protein, cDNA, and plasmids

The CS1 domain of human fibronectin (26 amino acids), which is the ligand for $\alpha_4\beta_1$ integrin, was generated as a GST fusion protein (9). The GST-CS1 fusion protein contains two identical repeat of CS1 domain. Cas-L cDNA was isolated in our laboratory (16). Wild-type Cas-L cDNA or the deletion mutant of the SH3 domain (Cas-LΔSH3) cDNA, was c-*myc* epitope-tagged (23) and inserted into pBCMG-hygro (26). Cas-LΔSH3 lacks the amino acid residues 1–61 of Cas-L.

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Received for publication April 2, 1998. Accepted for publication March 17, 1999.

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[1] This work was supported by National Institutes of Health Grants AR33713 and AI29530.

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[3] Abbreviations used in this paper: pp125FAK, focal adhesion kinase; MAP, mitogen-activated protein; Cas, Crk-associated substrate; SH, Src homology.

0022-1767/99/$02.00

Cell culture and transfection

Jurkat (E6-1) and CTLL-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Jurkat cells were maintained in RPMI 1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine and 50 μg/ml gentamycin (10% FCS-RPMI). Expression vectors containing Cas-L cDNAs were transfected by electroporation using Gene Pulser (Bio-Rad, Hercules, CA) at 250 V and 960 μF. After hygromycin selection, stable clones were established by limiting dilution culture and maintained in 10% FCS-RPMI containing hygromycin (250 μg/ml). Peripheral T cells were isolated from human peripheral blood as described previously (15). CTLL-2 cells were cultured in 10% FCS-RPMI containing 2-ME (5 μM) and human IL-2 (10 U/ml).

Detection of secreted IL-2

Jurkat transfectants (10^5 cells in 100 μl of culture media per well) were stimulated with or without indicated Abs and/or β_1 integrin ligands and incubated for 24 h. From each well, 50 μl of supernatant were collected and stored at −20°C until the measurement of IL-2 concentration. Secreted IL-2 was detected using CTLL-2 cells, which require IL-2 for proliferation. CTLL-2 cells (2000 cells/well in 50 μl of culture media without IL-2) were incubated with 50 μl of sample supernatants for 24 h. After 18 h, CTLL-2 cells were pulsed with [^{3}H]thymidine, cells were harvested, and [^{3}H]thymidine incorporation was measured. IL-2 concentrations were determined by incubating CTLL-2 cells with a serial dilution of control IL-2 as standard. Results are given as mean units IL-2 per ml of triplicate determinations.

Immunoprecipitation and immunoblotting

Cells were lysed in 1% Nonidet P-40 lysis buffer as described (23). The c-*myc*-tagged wild type or mutant Cas-L protein was immunoprecipitated with anti-c-*myc*-tagged mAb (9E10, Oncogene Science, Manhasset, NY) and protein A-conjugated beads (Pharmacia, Uppsala, Sweden) as described (16). Immunoprecipitates were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Immunoblotting was performed with a primary mAb, HRP-conjugated anti-mouse IgG Ab (Amersham, Arlington Heights, IL), and chemiluminescence reagents (Renaissance, DuPont-NEN, Boston, MA) or with ^{125}I-labeled anti-phosphotyrosine mAb (4G10, Upstate Biotechnology, Lake Placid, NY) as described (27). mAb against pp125FAK was purchased from Transduction Laboratories (Lexington, KY). The rabbit anti-Cas-L polyclonal Ab was described previously (23).

Results

β_1 integrin-mediated costimulation of IL-2 production in human peripheral T cells but not in Jurkat T cells

Previous studies have shown that β_1 integrin-mediated cell adhesions induce TCR/CD3-dependent IL-2 production (8). As shown in Fig. 1*A*, stimulation by the coimmobilized anti-CD3 mAb and anti-CD29 (integrin β_1 chain) mAb, anti-CD49d (integrin α_4 chain) mAb, or the GST-CS1 fusion protein (the ligand for $\alpha_4\beta_1$ integrin), or anti-CD28 mAb induced IL-2 production in human peripheral T cells. In the absence of anti-CD3 mAb, IL-2 production was not induced by the stimulation of the immobilized GST-CS1, anti-CD29 mAb, anti-CD49d mAb, or anti-CD28 mAb. In contrast, the β_1 integrin-mediated signaling pathway is selectively impaired in a human T lymphoblastoid cell line, Jurkat. As shown in Fig. 1*B*, ligation of β_1 integrin by Abs or GST-CS1 coimmobilized with anti-CD3 mAb did not induce IL-2 production in Jurkat cells that express CD3, CD49d, CD29, and CD28 on the cell surface. Since CD28 provided a costimulus for TCR/CD3-driven IL-2 production (Fig. 1*B*), the minimal cell machinery required for IL-2 production is intact in Jurkat cells. The ligation of β_1 integrin with higher doses of anti-CD3 mAb (1 and 10 μg/ml) could not induce IL-2 production in these Jurkat cells (data not shown).

Establishment of Jurkat transfectants that express Cas-L

Because we observed the normal expression of $\alpha 4\beta_1$ integrin on the surface of Jurkat cells, we presumed that the intracellular signaling pathway from β_1 integrin was impaired in Jurkat cells. We analyzed the expression of the molecules involved in the β_1 integrin-mediated signal transduction and found that the expression of Cas-L in Jurkat cells was marginal (16) (Fig. 2*A*, *lanes 1* and *2*), we hypothesized that the lack of β_1 integrin-mediated costimulation in Jurkat cells is due to insufficient expression of Cas-L. To elucidate this possibility, we developed Jurkat cell clones that express c-*myc* epitope-tagged wild-type or SH3-deleted mutant Cas-L (Cas-LΔSH3). As shown in Fig. 2*A*, Cas-L-transfected Jurkat cells express a significant amount of Cas-L wild-type (*lane 4*) or Cas-LΔSH3 (*lane 5*) proteins, whereas both parental and vector-transfected Jurkat cells express barely detectable levels of endogenous Cas-L protein (*lanes 2* and *3*). In contrast, the expression level of pp125FAK was almost the same among these transfectants and parental Jurkat cells (Fig. 2*B*). All Jurkat cell clones used in

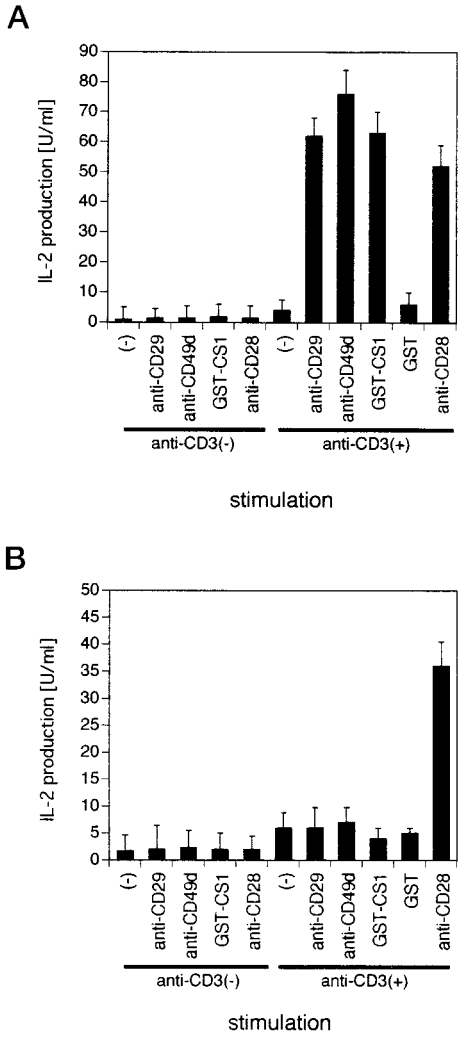

FIGURE 1. β_1 integrin-mediated costimulation of IL-2 production in T cells. *A*, β_1 integrin-mediated costimulation of IL-2 production in peripheral T cells. Human peripheral T cells (1×10^5) were cultured for 24 h on plates coated with a combination of anti-CD3 mAb (0.1 μg/ml), GST-CS1 fusion protein (10 μg/ml), anti-CD49d (integrin α_4 chain) mAb (10 μg/ml), anti-CD29 (integrin β_1 chain) mAb, or anti-CD28 mAb (10 μg/ml). The secreted IL-2 in the culture supernatant of peripheral T cells was measured with the use of CTLL-2 cells (CTLL-2 assay). *B*, Impaired β_1 integrin-mediated costimulation of IL-2 production in Jurkat cells. Jurkat cells (1×10^5) were cultured for 24 h on plates coated with a combination of anti-CD3 mAb (0.5 μg/ml), GST-CS1 fusion protein (10 μg/ml), anti-CD29 mAb (10 μg/ml), anti-CD49d mAb (10 μg/ml), or anti-CD28 mAb (10 μg/ml). The secreted IL-2 in the culture supernatant was measured by CTLL-2 assay.

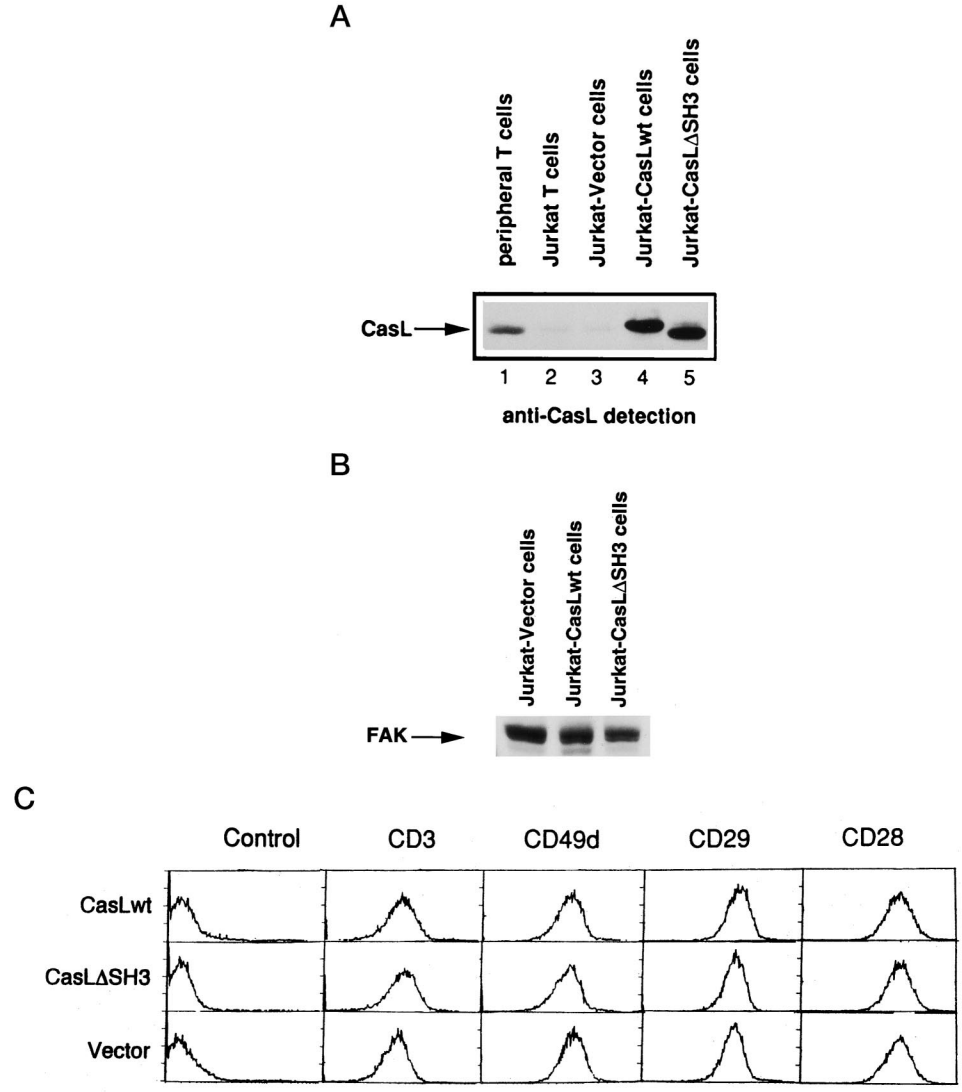

FIGURE 2. Establishment of Cas-L-transfected Jurkat cell clones. *A*, Expression of Cas-L in Jurkat transfectants. For each cell clone, 2×10^5 cells/lane were lysed in 1% Nonidet P-40 lysis buffer, separated by SDS-PAGE, and detected by immunoblotting with rabbit anti-Cas-L polyclonal Ab established in our laboratory. *B*, Expression of FAK in Jurkat transfectants. For each cell clone, 2×10^5 cells/lane were lysed in 1% Nonidet P-40 lysis buffer, separated by SDS-PAGE, and detected by immunoblotting with anti-FAK mAb. *C*, Expression of cell surface Ags expressed on Jurkat transfectants. Expression of CD3, CD49d, CD29, and CD28 on the cell surface of wild-type Cas-L (Cas-Lwt), SH3 domain-deleted Cas-L mutant (Cas-LΔSH3), and vector-transfected Jurkat cells was analyzed by indirect fluorescence using flow cytometry with OKT3 (anti-CD3), 3G6 (anti-α_4 chain), 4B4 (anti-β_1 chain), 4B10 (anti-CD28) mAbs, and mouse Ig as a negative control (control) plus FITC-conjugated goat anti-mouse IgG + IgM Ab.

this report express comparable amounts of CD3, CD49d, CD29, and CD28 on their surface, which was confirmed by flow cytometry (Fig. 2*C*).

Cas-L restores β_1 integrin-mediated costimulatory activity of IL-2 production

We first examined whether wild-type Cas-L restores the $\alpha_4\beta_1$ integrin-mediated costimulation in Jurkat cells (15). As shown in Fig. 3*A*, stimulation by the immobilized GST-CS1 protein and anti-CD3 mAb induced a marked enhancement of IL-2 production in the wild-type Cas-L-transfected Jurkat cells (Jurkat-Cas-Lwt cells, ■) in a dose-dependent manner, but not in the vector-transfected Jurkat cells (Jurkat-vector cells, □). At the dose of 10 μg/ml of the GST-CS1 protein, Jurkat-Cas-Lwt cells produced 5 times more IL-2 than the cells stimulated with anti-CD3 mAb alone. This increased IL-2 production in Jurkat-Cas-Lwt cells was inhibited by the addition of either soluble anti-CD49d or anti-CD29 mAb. Similarly, anti-CD49d mAb (clone 3G6) (15) also provided a dose-dependent costimulation in Jurkat-Cas-Lwt cells, but not in the control cells (Fig. 3*B*). Stimulation with anti-CD3 mAb alone caused no increase in IL-2 production, either in Jurkat-Cas-Lwt cells or in Jurkat-vector cells. In marked contrast, CD28-mediated costimulation is not altered by the transfection of Cas-L, suggesting that Cas-L is not involved in the CD28-mediated signal transduction. Taken together, these results indicate the involvement of Cas-L in $\alpha_4\beta_1$ integrin-mediated signal transduction.

To exclude the possibility that these observations were due to clonal variation, we selected five additional, independent clones of Jurkat-Cas-Lwt and Jurkat-vector cells. These clones were stimulated with GST-CS1 (10 μg/ml) plus anti-CD3 mAb (0.5 μg/ml). As shown in Fig. 3*C*, the average level of IL-2 production in wild-type Cas-L-transfected Jurkat cell clones was 15 times higher than that in the vector-transfected Jurkat cell clones (the mean ± SD is 35.4 ± 5.2 and 2.4 ± 1.3 U/ml in Cas-L-transfected and vector-transfected Jurkat cells, respectively). These findings indicate that: 1) the transfection of Cas-L restored β_1 integrin-mediated costimulation of IL-2 production in Jurkat cells; and 2) Cas-L appears not to be essential for the TCR/CD3- or CD28-mediated signaling pathways for IL-2 production in Jurkat cells.

Cas-LΔSH3 does not restore β_1 integrin-mediated costimulation of IL-2 production

To further study the function of Cas-L in β_1 integrin-mediated T cell costimulation, we analyzed Jurkat cell clones that expressed Cas-LΔSH3 mutant (Jurkat-ΔSH3 cells; Fig. 2*A*, *lane 5*). Cas-LΔSH3 lacks the SH3 domain and does not bind to pp125FAK (20, 23). As shown in Fig. 4*A*, stimulation of Jurkat-ΔSH3 cells (▨) with immobilized anti-CD3 mAb (0.5 μg/ml) plus either anti-

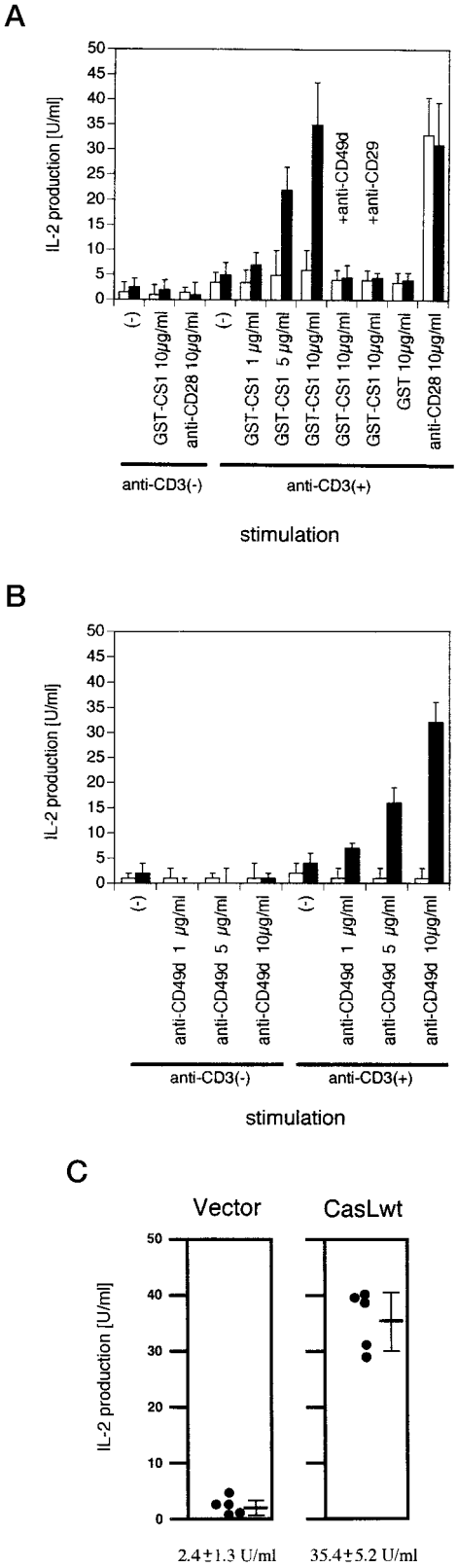

FIGURE 3. β_1 integrin-mediated costimulation of IL-2 production in wild-type Cas-L-transfected Jurkat cells. *A*, GST-CS1-mediated costimulation of IL-2 production in wild-type Cas-L-transfected Jurkat cells. Cas-L-transfected Jurkat cells (■) or vector-transfected Jurkat cells (□) were cultured for 24 h on plates coated with anti-CD3 mAb (0.5 μg/ml) and the indicated dose of GST-CS1 fusion protein. The secreted IL-2 in the culture supernatant was measured by CTLL-2 assay. Stimulation of GST-CS1 plus anti-CD3 mAb increased IL-2 production in Cas-L-transfected cells but did not increase IL-2 production in vector-transfected Jurkat cells. Soluble Abs (10 μg/ml) of anti-CD49d and anti-CD29 could inhibit IL-2 production. *B*, Anti-CD49d-mediated costimulation of IL-2 production in wild-type Cas-L-transfected Jurkat cells. Cas-L-transfected Jurkat cells (■) or vector-transfected Jurkat cells (□) were cultured for 24 h on plates coated with anti-CD3 mAb (0.5 μg/ml) plus the indicated dose of anti-CD49d mAb. The secretion of IL-2 was measured by CTLL-2 assay. *C*, GST-CS1-mediated costimulation of IL-2 production in wild-type Cas-L-transfected Jurkat cell clones. Five independent clones of Cas-L or vector-transfected Jurkat cells were cultured for 24 h on plates coated with anti-CD3 mAb (0.5 μg/ml) plus GST-CS1 fusion protein (10 μg/ml). The secreted IL-2 in the culture supernatant was measured by CTLL-2 assay.

CD49d mAb (10 μg/ml) or GST-CS1 (10 μg/ml) failed to induce significant levels of IL-2 production as opposed to levels observed in Jurkat-Cas-Lwt cells under the same conditions (■). On the other hand, stimulation with anti-CD3 plus anti-CD28 mAbs induced similar increases of IL-2 production in both transfectants. These findings suggest that the association of Cas-L with pp125FAK is essential for the function of Cas-L in the β_1 integrin-mediated T cell costimulation.

Cas-LΔSH3, which does not have a binding site to pp125FAK, failed to be tyrosine-phosphorylated following the stimulation with GST-CS1

To define the molecular basis of the difference in IL-2 production between wild-type Cas-L and Cas-LΔSH3, we examined tyrosine phosphorylation of these Cas-L proteins following stimulation by the immobilized GST-CS1 (10 μg/ml). As shown in Fig. 4*B*, adhesion of the transfectants to GST-CS1-coated plates induced tyrosine phosphorylation of wild-type Cas-L, but not Cas-LΔSH3. This finding indicates that the SH3 domain of Cas-L is required for its tyrosine phosphorylation. It is thus suggested that the binding of Cas-L to pp125FAK is crucial for β_1 integrin-mediated tyrosine phosphorylation of Cas-L and that tyrosine phosphorylation of Cas-L is critical in β_1 integrin-mediated costimulation of IL-2 production. The binding of these Jurkat transfectants to a GST-CS1-coated plate did not differ significantly among those clones (data not shown).

Discussion

IL-2 production in T cells requires costimulation delivered by TCR and the accessory molecules such as CD28 or β_1 integrins. There are many reports about the molecular mechanism of the TCR-mediated signal transduction, although little is known about the mechanism involved in β_1 integrin-mediated costimulatory signaling in T cells. In this paper, we demonstrated a requirement of Cas-L for the β_1 integrin-mediated costimulatory signaling in Jurkat T cells. Furthermore, we suggested that tyrosine phosphorylation of Cas-L is necessary for the β_1 integrin-mediated costimulation in Jurkat cells.

How does tyrosine-phosphorylated Cas-L transfer signals? Since the tyrosine-phosphorylated form of Cas-L recruits Crk, Nck, and SHP2 in an SH2 domain-dependent manner (16), these molecules may transduce signals downstream from Cas-L following β_1 integrin stimulation in T cells. One putative function of all these recruited signaling molecules is regulation of MAP kinase family kinases (15, 28, 29). Crk (30, 31), an oncogenic adapter protein, binds to tyrosine-phosphorylated Cas family proteins through its SH2 domain. Through its SH3 domain, Crk binds to C3G and Sos, which are guanine exchange factors for Rap1A and Ras, respectively (32). Since β_1 integrin-mediated cell adhesion induces Crk-mediated association of Cas family proteins with C3G and Sos (33), recruitment of these guanine exchange factors may

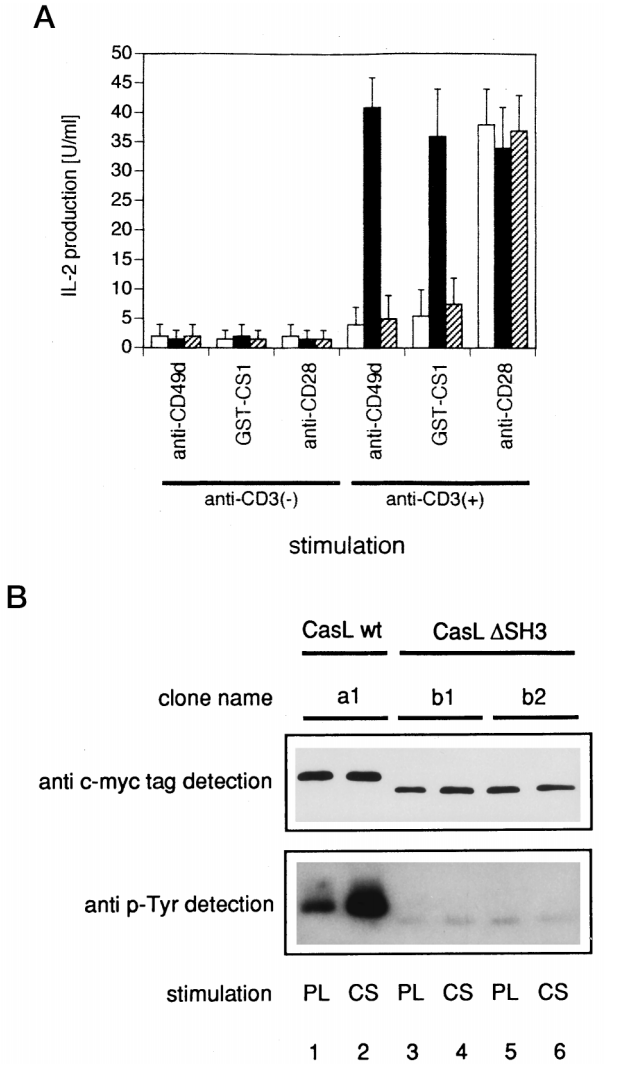

FIGURE 4. The SH3 domain of Cas-L is essential for β_1 integrin-mediated costimulation of IL-2 production and Cas-L phosphorylation. *A*, Absence of β_1 integrin-mediated costimulation of IL-2 production in Cas-LΔSH3-transfected Jurkat cells. Jurkat cell clones that were transfected with Cas-L wild type (■), Cas-LΔSH3 (▨), or vector (□) were cultured for 24 h on plates coated with anti-CD3 mAb (0.5 μg/ml) plus anti-CD49d (10 μg/ml), GST-CS1 fusion protein (10 μg/ml), or anti-CD28 (10 μg/ml). The secretion of IL-2 was measured by CTLL-2 assay. *B*, GST-CS1-mediated tyrosine phosphorylation of Cas-L in Jurkat transfectants. Jurkat transfectants (a1, Cas-L wild type; b1, b2, Cas-LΔSH3 mutant) were stimulated with poly-L-lysine (PL) control or GST-CS1 fusion protein (CS) for 1 h and lysed on plates using 1% Nonidet P-40 lysis buffer. Lysates were immunoprecipitated with anti-c-*myc* epitope Ab (9E10) and protein A-conjugated beads. Immunoprecipitates were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes, and immunoblotting was performed with 9E10 and anti-phosphotyrosine mAb.

regulate the activity of extracellular signal-regulated kinase through the activation of Rap1A and Ras (34, 35). Recently, it was shown that v-Crk and C3G are involved in the regulation of c-Jun N-terminal kinase (31). In addition, Nck and SHP2 were also demonstrated to be involved in the activation of extracellular signal-regulated kinase (24, 36). Therefore, it is possible that the recruited Crk, Nck, and/or SHP2 induce expression and activation of transcription factors through regulation of the MAP kinase family and subsequently result in IL-2 expression.

Cas-L also appears to be involved in β_1 integrin-mediated signal transduction in the other cell types. Cas-L/Hef-1 is tyrosine phosphorylated by the stimulation of β_1 integrin or Ag receptor in B cells and binds to Crk-L and Crk II (37, 38). We reported a transient tyrosine phosphorylation of Cas-L by CD3 cross-linking (39). Thus, Cas-L can be involved in the β_1 integrin-mediated signaling pathway and lymphocyte Ag receptor-mediated signaling pathways in both T cells and B cells. However, Cas-L appears not to be essential for the TCR signaling of IL-2 production in Jurkat cells, and the overexpression of Cas-L is not sufficient for the IL-2 production by the CD3 stimulation alone. Both wild-type and ΔSH3 mutant of Cas-L are transiently tyrosine phosphorylated by the cross-linking of CD3 molecules (39). The SH3 domain of a Cas family protein, which is the FAK binding site, is essential for the focal adhesion targeting (40). These findings strongly suggest that the localization of tyrosine-phosphorylated Cas-L and/or timing/duration of phosphorylation are very important for the function of Cas-L. The transfection of Cas-L did not alter the CD28-mediated signal transduction for IL-2 production. This difference in the effect of Cas-L in two costimulatory signaling pathways, β_1 integrins and CD28, indicates the divergence of T cell costimulatory signals.

In summary, we have shown that expression of wild-type Cas-L restored β_1 integrin-mediated costimulation of IL-2 production in Jurkat cells. The interaction between Cas-L and pp125FAK appears to be necessary for the costimulatory signaling pathway through β_1 integrin. Our present observation also provides the first evidence that establishes a biological function of Cas family proteins in T lymphocytes.

Acknowledgments

We thank Drs. Haruo Saito, Martin Hegen, and Toshio Honma for valuable discussions; Donny Cho for technical assistance; and Lisa Willis and Noriko Asahara for secretarial assistance.

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