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Tyrosine Phosphorylation of Crk-Associated Substrate Lymphocyte-Type Is a Critical Element in TCR- and $\beta_1$ Integrin-Induced T Lymphocyte Migration

Yoshiyuki Ohashi, Satoshi Iwata, Kenjiro Kamiguchi, and Chikao Morimoto

Crk-associated substrate (Cas) lymphocyte-type (Cas-L) is a 105-kDa cytoplasmic protein consisting of Src homology-3 domain and multiple YXXP motifs (substrate domain). Our previous studies showed that Cas-L is tyrosine-phosphorylated following the ligation of TCR and $\beta_1$ integrins in T lymphocytes. Here we show that Cas-L is involved in T cell motility following the ligation of TCR and $\beta_1$ integrin. Peripheral T lymphocytes showed a marked increase of migration on fibronectin (FN) after the ligation of TCR. In contrast, the migrating Jurkat cells, in which Cas-L was marginally expressed, were less than one-tenth in number on the same condition. Transfection of wild-type Cas-L into Jurkat cells resulted in restoring CD3 plus FN-induced cell migration.

Furthermore, following the ligation of $\beta_1$ integrin alone, the Cas-L transfectants significantly migrated better than the vector control. Mutational analysis of Cas-L revealed that the substrate domain is required for both FN- and CD3-induced tyrosine phosphorylation of Cas-L, and cell migration caused by FN alone and CD3 plus FN. In contrast, the Src homology-3 domain is required only for the FN-induced tyrosine phosphorylation of Cas-L and cell migration, but not for CD3-induced tyrosine phosphorylation or CD3 plus FN-induced cell migration. These data strongly suggest that Cas-L is a key molecule in T cell migration induced by the ligation of CD3 and $\beta_1$ integrins and that tyrosine phosphorylation of Cas-L is essential for T cell migration. The Journal of Immunology, 1999, 163: 3727–3734.

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Abbreviations used in this paper: Cas, Crk associated substrate; Cas-L, Cas lymphocyte-type; SH, Src homology; FN, fibronectin; FAK, focal adhesion kinase; p-Tyr, phosphorytrosine; SD, substrate domain; Cas-LΔSH3, SH3 domain-deleted mutant of Cas-L; Cas-LΔSD, SD-deleted mutant of Cas-L; Cas-LF, Cas-L mutant with the substitution of C-terminal YDYVHL to PFDVHL; HPF, high-power fields; WASP, Wiskott-Aldrich syndrome protein; VLA, very late Ag;...
Materials and Methods

Cells and reagents

Human lymphoblastic T cell lines, Jurkat, SUP-T1, and HPB-ALL were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, and gentamicin (50 μg/ml) (complete medium). Human PBMCs were isolated from healthy volunteers by density-gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs were separated into E rosette-positive cells with sheep erythrocytes (15). Contaminating monocytes were depleted by adherence to plastic plates. Further depletion of monocytes was achieved by incubation with 5 mM l-leucine methyl ester hydrochloride. Anti-CD3 (OKT3), anti-CD28 (4B10), anti-CD29 (4B4), anti-CD49d (3G6), and anti-CD49e (2H6) mAbs were described previously (15). Anti-Crk and anti-ZAP70 mAbs were purchased from Transduction Laboratories, Lexington, KY. mAbs against phosphotyrosine (p-Tyr) and c-Myc epitope-tag were purchased from Upstate Biotechnology (Lake Placid, NY) and Oncogene Science (Cambridge, MA), respectively. A rabbit polyclonal Ab against Cas-L was described previously (14). Cholera toxin, trycotalasin D, genistein, and wortmannin were obtained from Sigma (St. Louis, MO).

Expression vectors and transfection

cDNAs encoding wild-type Cas-L, the deletion mutant of the SH3 domain (residues 1–61) (Cas-LΔSH3) or the SD (residues 63–401) (Cas-LΔSD), or the mutant with the substitution of C-terminal YDYYHL to FDFVHL was described previously (13). The wild-type and mutant Cas-L cDNAs were c-myc and epitope-tagged (13) and subcloned into the expression vector pBCMG-Hygro (Hygro). The expression vectors were transfected by electroporation at 250 V and 960 μF using the Gene Pulser (Bio-Rad, Hercules, CA). Cells were selected and maintained in the hygromycin-containing complete medium.

Migration assays

Cell migration was assayed using 6.5-mm-diameter Transwell inserts (Costar, Cambridge, MA) with polycarbonate filters (3-μm pore size). The filters were left uncoated or were coated with OKT3 overnight at appropriate concentrations in PBS. After washing, the membranes were coated with human plasma FN (Life Technologies, Gaithersburg, MD) at 5 μg/ml for 2 h at room temperature. Cells were washed and resuspended at 1 × 10^6 in RPMI 1640 containing 0.6% BSA (BSA medium). For inhibition assays, cells were preincubated for 1 h at 37°C in the presence of mAbs or reagents. The Transwell chambers were inserted into wells filled with 600 μl of the BSA medium, and cells were added to the upper chamber in a final volume of 100 μl. After incubation at 37°C for appropriate duration of time, the filters were removed, fixed in methanol, and stained with May-Grimsa solutions. Cells on the upper side of the filters were wiped off. The number of migrated cells on the lower side of the filters (designated as fully migrated cells) was counted microscopically in five high-power fields (HPF) per well at ×400 magnification. Each experiment was performed in duplicate wells. The data represent the mean of duplicate wells, and error bars represent the SD.

Adhesion assays

OKT3 mAb (5 μg/ml) and FN (5 μg/ml) were coated on 96-well plates as described for migration assays. The wells were rinsed three times with PBS and blocked for 1 h with 2.5% BSA in PBS at 37°C. Cells were washed, resuspended at 5 × 10^5 cells/ml in the BSA medium, and added to each well in a final volume of 100 μl. The plates were centrifuged for 5 min at 500 rpm and incubated for 30 min at 37°C by floatation in a water bath. The plates were washed three times with PBS. The number of adherent cells was quantified by the MTT assay (17). Each data point was calculated from triplicate wells and represents the mean ± SD.

Cell stimulation

Cells were washed three times with RPMI 1640 and then incubated on ice for 15 min with 500 μl of RPMI 1640 containing 10 μg/ml of OKT3 mAb. Following washing with RPMI 1640, cells were incubated with 10 μg/ml of anti-mouse Ig for 2 min at 37°C and subsequently suspended in ice-cold IMDM (Sigma) containing 5 mM EDTA, 10 mM pyrophosphate, 10 mM sodium fluoride, and 0.4 mM sodium vanadate. After centrifugation, cells were lysed in a 1% Nonidet P-40 lysis buffer as described (18). For stimulation with the extracellular matrix, cells were incubated for 1 h on plates coated with poly-L-lysine (Sigma) (5 μg/ml) or FN (5 μg/ml) and then solubilized in the lysis buffer.

Results

Our previous studies have shown that Cas-L is predominantly expressed in T lymphocytes and that, in addition to β1 integrin stimulation, Cas-L is tyrosine-phosphorylated upon the TCR/CD3 stimulation (9, 14). Therefore, it is conceivable that Cas-L could be involved in CD3-induced T cell function. Because β1 integrins play an important role in cell migration, we attempted to determine the function of Cas-L in CD3-induced T cell migration on FN. For

FIGURE 1. CD3-induced T cell migration on fibronectin (FN) and Cas-L expression in various T cells. A, T cells were incubated for 2 h on porous filters coated with FN (5 μg/ml) alone (▪) or FN (5 μg/ml) plus OKT3 (5 μg/ml) (●). The results are expressed as the number of fully migrated cells on the lower side of the filters. The numbers of cells were counted in five microscope fields. Bars represent the mean ± SD of duplicate samples. Data are representative of two independent experiments. B, Expression of Cas-L in T cells and transfectants. Whole-cell lysates (50 μg/lane) were subjected to Western blotting with indicated Abs.

Immunoprecipitation and immunoblotting

c-Myc-tagged proteins were immunoprecipitated with 9E10 and protein A-Sepharose (Pharmacia Biotech) as described (9). Immunoprecipitates and lysates were separated by SDS-PAGE and electrophoretically transferred onto the nitrocellulose membranes. Immunoblotting was performed with the indicated primary Abs, a HRP-conjugated anti-mouse or anti-rabbit Ab (Amersham, Arlington Heights, IL), and chemiluminescence reagents (NEN Life Science Products, Boston, MA) as described elsewhere (14). Tyrosine-phosphorylated proteins were detected with 125I-labeled anti-p-Tyr mAb (4G10), followed by autoradiography.

Flow cytometry

Cells were incubated for 30 min at 4°C with saturating concentrations of the indicated mAbs or a control Ab. Subsequently, cells were incubated for 30 min at 4°C with FITC-conjugated anti-mouse Ig, followed by another wash. The cells were suspended in 0.5 ml of PBS containing 1% paraformaldehyde and analyzed on an Epics Elite (Beckman Coulter, Miami, FL).
To clarify the requirement of Cas-L in CD3 plus FN-induced T cell migration, we established Jurkat cells stably expressing c-myc epitope-tagged wild-type Cas-L (Fig. 1B) and determined cell migration of those transfectants using the same assay. Flow cytometry analysis revealed that transfection of Cas-L did not alter the expression of CD3, CD49d, CD49e, and CD29 (data not shown). For this purpose, cells were incubated for 2 h on the filter coated with FN and OKT3. As shown in Fig. 2A, wild-type Cas-L-transfected Jurkat cells showed a marked increase of CD3 plus FN-induced cell migration, whereas only slight induction was observed in the cells transfected with a control vector (Fig. 2A; V1 and V2) or parent Jurkat cells. Similar results were obtained in three independent clones (Fig. 2A; A1, A11, and A12). It should be noted that Cas-L transfectants showed a slight increase of migration on the filters coated with FN alone. The migration of wild-type Cas-L-transfected Jurkat cells increased in number at higher coating concentrations of OKT3, whereas vector-transfected cells displayed only a slight increase of migration even at the concentration of 10 μg/ml of OKT3 (Fig. 2B), indicating that the Cas-L-mediated cell migration was induced by OKT3 in a concentration-dependent manner. In time course experiments using the filters

FIGURE 2. Cas-L-transfected, but not vector-transfected, Jurkat cells show an increase of migration on FN by the ligation of CD3. A, The parent Jurkat cells, vector transfectants (clones V1 and V2), and Cas-L transfectants (clones A1, A11, and A12) were incubated for 2 h on porous filters coated with FN (5 μg/ml) alone (□) or FN (5 μg/ml) plus OKT3 (5 μg/ml) (■). B, Dose dependency of OKT3 in migration of vector transfectants (□) and Cas-L transfectants (■). Cells were incubated for 2 h on porous filters coated with FN (5 μg/ml) and OKT3. C, Time course for migration of vector and Cas-L transfectants on FN (5 μg/ml) plus OKT3 (5 μg/ml) (squares) or OKT3 (5 μg/ml) alone (circles). D, Cells were incubated for 2 h on porous filters coated with FN (5 μg/ml) alone (□), FN (5 μg/ml) plus OKT3 (5 μg/ml) (■), or FN plus anti-CD28 mAb (5 μg/ml) (●). The results are expressed as the number of fully migrated cells on the lower side of the filters. The numbers of cells were counted in five microscope fields. Bars represent the mean ± SD of duplicate samples. Data are representative of at least two independent experiments.
coated with OKT3 and FN, the migration of wild-type Cas-L-transfected Jurkat cells were observed in 2 h after incubation and was further increased in number after 4-h incubation (Fig. 2C). In contrast, the kinetics of migration was slow in vector-transfected cells, showing a 6-fold decrease in number compared with wild-type Cas-L-transfectants in 4 h after incubation (Fig. 2C). As previously mentioned, no migration was observed on the filters coated with OKT3 alone in the transfectants even at 4 h. Furthermore, neither OKT3 plus type I collagen nor OKT3 plus BSA could induce Cas-L-mediated cell migration (data not shown). These results indicate that the expression of Cas-L plays an important role in T cell migration following the ligation of CD3 and that, in addition to the stimulation of CD3, ligation of $\beta_1$ integrin is required for this migration. The ligation of CD28 can provide signals into the parent Jurkat cells as well as Cas-L transfectants in TCR/CD3-mediated IL-2 production (19). We next determined whether CD28 stimulation may substitute for CD3 stimulation in Cas-L-mediated migration on FN. As shown in Fig. 2D, the wild-type Cas-L transfectants failed to migrate on filters coated with FN and anti-CD28 mAb, indicating that the signal from CD28 alone is insufficient for T cell migration on FN.

Because it has been reported that the $\beta_1$ integrins very late Ag (VLA)-4 (CD49d/CD29) and VLA-5 (CD49e/CD29) are responsible for T lymphocyte binding to FN, the role of VLA-4 and VLA-5 in CD3-induced cell migration was next determined. Cells were pretreated with various mAbs for 1 h at concentrations of 10 μg/ml and allowed to migrate for 2 h on filters coated with OKT3 and FN. As shown in Fig. 3, anti-CD49d mAb, as well as anti-CD28 mAb, strongly blocked cell migration (~70% inhibition as compared with a control mAb). In contrast, anti-CD49d mAb

**FIGURE 3.** The inhibitory effects of mAbs to $\beta_1$ integrins on CD3-induced cell migration on FN. Cells were incubated for 2 h on porous filters coated with FN (5 μg/ml) and OKT3 (5 μg/ml) in the presence of 10 μg/ml of the indicated mAbs. The results are expressed as the number of fully migrated cells on the lower side of the filters. The numbers of cells were counted in five microscope fields. Bars represent the mean ± SD of duplicate samples. Data are representative of two independent experiments.

**FIGURE 4.** Cas-L-transfected and vector-transfected Jurkat cells evenly adhere to FN following the ligation of CD3. Cells were incubated for 10 min on wells coated with FN alone (5 μg/ml) ($\square$), FN (5 μg/ml) plus OKT3 (5 μg/ml) ($\bigcirc$), or BSA plus OKT3 (5 μg/ml) ($\uparrow$). The adherent cells were quantified by the MTT assay. Each data point was calculated from triplicate wells and represents the mean ± SD. Data are representative of three independent experiments.

**FIGURE 5.** The SD of Cas-L is required for Cas-L-mediated cell migration on FN and Cas-L tyrosine phosphorylation following the ligation of CD3. A, Cells were incubated for 2 h on porous filters coated with FN (5 μg/ml) plus OKT3 (5 μg/ml). The results are expressed as the relative number of fully migrated cells to the number observed in vector-transfected cells. The numbers of cells were counted in five microscope fields. Bars represent the mean ± SD of duplicate samples in two independent clones. Data are representative of two independent experiments. B, Cells stimulated with 10 μg/ml of OKT3 for 2 min were lysed and then immunoprecipitated with 9E10. The immunoprecipitates were analyzed by Western blotting with anti-Cas-L Ab, followed by anti-pTyr. Data are representative of three independent experiments.
cells on the lower side of the filters. The numbers of cells were counted in the inserts and the results were expressed as the number of fully migrated cells on the lower side of the filters. The numbers of cells were counted in five microscopic fields. Bars represent the mean ± SD of triplicate samples. Data are representative of three independent experiments.

It is reported that the ligation of CD3 promotes an increase of the integrin avidity for extracellular matrix (20). To determine whether the Cas-L expression could alter the integrin avidity in CD3 plus FN-induced cell migration, the cell binding ability to FN was compared between vector control and wild-type Cas-L transfectants using adhesion assays. For this purpose, cells were incubated for 10 min on plates coated with FN in the presence or absence of OKT3. As shown in Fig. 4, the ligation of CD3 induced cell adhesion to FN in vector control and wild-type Cas-L-transfected cells. However, there was no significant difference in their binding ability to FN with or without CD3 stimulation. Similar results were obtained in the adhesion assays at the time period of 20 and 60 min (data not shown). These findings indicate that Cas-L-mediated cell migration on FN induced by the ligation of CD3 is not due to the change of the integrin avidity to FN.

To identify functional domains of Cas-L in CD3 plus FN-induced cell migration, the SH3 domain-deleted mutants (Cas-LΔSH3), the SD-deleted mutants (Cas-LΔSD), and the mutants with the substitution of C-terminal YDYVHL to FDFVHL (Cas-LF) were created and stably expressed in Jurkat cells. Comparable amounts of CD3, CD49d, CD49e, and CD29 were expressed on all the transfectants (data not shown). As shown in Fig. 5A, Cas-LΔSD failed to promote the CD3 plus FN-induced cell migration, whereas Cas-LΔSH3 and Cas-LF induced significant increases in cell migration at similar levels to that observed in wild-type Cas-L. Western blotting analysis with an mAb to p-Tyr revealed that Cas-LΔSD was significantly diminished in tyrosine phosphorylation following the ligation of CD3, although the transfectants expressed comparable amounts of proteins (Fig. 5B). In contrast, Cas-LΔSH3 and Cas-LF, as well as wild-type Cas-L, were tyrosine-phosphorylated after CD3 cross-linking. These results indicate that the Cas-L SD, but not the C-terminal YDYVHL sequence or SH3 domain, plays an important role in CD3 plus FN-induced cell migration and that the tyrosine phosphorylation of the SD is also critical for this migration.

We next determined molecules being involved in Cas-L-mediated cell migration by the ligation of CD3 and β1 integrin using inhibitors that specifically block intracellular signaling molecules. For this purpose, cells were treated with different reagents before the cell migration assays. As shown in Fig. 6, cell migration was blocked by wortmannin, inhibitors of phosphatidylinositol 3-kinase, in a dose-dependent manner. Cholera toxin, an ADP-ribosylating agent of the Gs protein, was previously reported to inhibit T cell migration toward FN (21). Cholera toxin also inhibited Cas-L-mediated cell migration induced by CD3 plus FN stimulation. These data suggest that the phosphatidylinositol 3-kinase and the Gs proteins are involved in this cell migration. Cytochalasin D, an inhibitor of actin polymerization, and genistein, an inhibitor of tyrosine phosphorylation, also showed significant inhibitory effects on cell migration, suggesting that actin polymerization and tyrosine phosphorylation are important for Cas-L-mediated cell migration induced by the ligation of CD3 and β1 integrin.

It has been shown that the binding of β1 integrins to FN alone also promotes migration of T lymphocytes (21, 22). In our assays, after 4-h incubation on the filters coated with FN only, wild-type Cas-L-transfected cells significantly migrated better than vector controls (Fig. 7A; 31.8 ± 3.3 vs 11.4 ± 3.0/HPF at 4 h, p < 0.01), suggesting that ligation of β1 integrin alone could provide migratory signals into cells through Cas-L. To further define the mechanism of FN induced cell migration, β1 integrin-mediated cell migration by FN was determined in Jurkat cells expressing Cas-L mutants. In the case of transfectants of Cas-LΔSH3 and Cas-LΔSD mutants, only slight migration comparable to vector control cells was observed (Fig. 7B). Notably, both of the mutants failed to be tyrosine-phosphorylated by FN stimulation (Fig. 7C). In contrast, Cas-LF as well as wild-type Cas-L could promote FN-dependent cell migration, and these proteins were tyrosine-phosphorylated by FN stimulation. These results indicate that, like the Cas-L SD, the Cas-L SH3 domain is necessary for FN-dependent T cell migration, and that Cas-L tyrosine phosphorylation is a critical event for cell migration. Taken together, these findings suggest that FN not only provides adhesion sites for T cells but also induces intracellular signals to Cas-L through β1 integrin, leading to T cell migration.

**Discussion**

In the present study, the molecular basis for CD3-induced T cell migration on FN was determined by modified Boyden chamber assays. Transfection of Cas-L restored the ability of cell migration following the ligation of CD3 and β1 integrin in Jurkat cells, suggesting that Cas-L is a critical molecule in T cell migration. Consistent with our previous data (14), Cas-L is tyrosine-phosphorylated following the ligation of TCR/CD3, and T cell migration depends on tyrosine phosphorylation of Cas-L. It should be noted that, in our system, interactions between FN and the β1 integrins VLA-4 and VLA-5 were critical events for T cell migration. Furthermore, we demonstrated that ligation of β1 integrin alone also provides migratory signals significantly through Cas-L for T cells, although the level of migration is lower than that of CD3 plus FN-induced T cell migration.

Previous studies using T lymphocytes from TCR transgenic mice that can be tracked in vivo following specific Ag stimulation have shown that Ag-specific T lymphocytes change in their localization through the body in response to foreign Ags (2). Kedl and Mescher (5) demonstrated that CD8 T cells recognize initial Ags in lymphoid organs such as draining LNs and spleen, resulting in the access of the sensitized cells to Ag deposition sites in the periphery. Moreover, CD4 T cells have been shown to move from paracortical T zones to B cell follicles following Ag stimulation (7). These observations indicate that Ag stimulation through
TCR/CD3 provides migratory signals for T cells. It has been well documented that the Ag-induced locomotion of lymphocytes can be regulated by changes of adhesion molecules in the expression levels and the affinity with the ligand (1, 2, 8, 23). Our studies demonstrated that, following CD3 cross-linking, Cas-L transfectants, but not vector controls, showed significant increases in cell migration through $\beta_1$ integrins, although both cells were increased in their binding activity to FN. Moreover, PMA enhanced cell adhesion to FN, but did not enhance migration through FN even at higher concentrations in Cas-L transfectants (data not shown). Therefore, it is concluded that interactions between $\beta_1$ integrins and FN are critical for CD3-induced T cell migration, but that the change of the integrin avidity is not sufficient for the cell migration.

FAK is a 125-kDa cytoplasmic protein that plays an important role in $\beta_1$ integrin-mediated signaling pathways (24, 25). FAK is localized to focal adhesions in adherent cells and exhibits tyrosine kinase activity (26, 27). Upon the ligation of $\beta_1$ integrin, FAK is activated and tyrosine-phosphorylated (28). Recently FAK has been reported to promote integrin-mediated cell migration in adherent cells (29), indicating that FAK also appears to be involved in T cell migration. However, our results showed that the Cas-L$\Delta$SH3 mutant, which fails to bind FAK, could promote T cell migration induced by the ligation of CD3 and $\beta_1$ integrin. Our previous study has shown that the Cas-L$\Delta$SH3 is tyrosine-phosphorylated after the ligation of CD3 (14), suggesting that Cas-L is tyrosine-phosphorylated after the ligation of CD3 in a FAK-independent manner. Because FAK is not tyrosine phosphorylated after the ligation of CD3 (14), our results strongly suggest that FAK may not be involved in signal transduction pathways that induce T cell migration following the ligation of CD3.

Previous studies have shown that the ligation of $\beta_1$ integrin triggers T cell motility (21, 22). We showed that interactions between $\beta_1$ integrins and FN trigger migratory signals, which are mediated by Cas-L. The immobilized CS1 domain, the ligand for VLA-4, also triggered Cas-L-mediated T cell migration, which was inhibited by an mAb against VLA-4 (data not shown). These data indicate that migratory signals can be induced by $\beta_1$ integrin through Cas-L. In contrast to the results of CD3 plus FN-induced migration, the Cas-L$\Delta$SH3 failed to promote T cell migration and was not tyrosine-phosphorylated upon the ligation of $\beta_1$ integrin. We previously reported that Cas-L binds to the FAK C terminus through the Cas-L SH3 domain and is tyrosine-phosphorylated by FAK upon the ligation of $\beta_1$ integrin (9, 13). These findings indicate that the FAK-Cas-L interaction is important for the signaling pathway in T cell migration triggered by the ligation of $\beta_1$ integrin alone. Taken together, Cas-L may receive signals through both TCR and $\beta_1$ integrins in FAK-independent and -dependent manners, respectively, which results in tyrosine phosphorylation of Cas-L to transfer migratory signals to downstream signaling molecules.

It has been reported that $\beta_1$ integrins provide costimulatory signals to CD3-dependent T cell proliferation and IL-2 production.

**FIGURE 7.** FN alone can provide migratory signals in Cas-L-mediated migration, in which the substrate domain (SD) and SH3 domains of Cas-L are involved. A, Cas-L-transfected (▲) and vector-transfected (△) Jurkat cells were incubated on porous filters coated with FN (5 µg/ml). The results are expressed as the number of fully migrated cells on the lower side of the filters. The numbers of cells were counted in five microscope fields. Each data point represents the mean ± SD of duplicate samples in two independent clones. Data are representative of two independent experiments.

B, Cells were incubated for 4 h on porous filters coated with FN (5 µg/ml). The results are expressed as the relative number of fully migrated cells to the number observed in vector-transfected cells. The numbers of cells were counted in five microscope fields. Bars represent the mean ± SD of duplicate samples in two independent clones. Data are representative of three independent experiments.

C, Cells stimulated with FN (5 µg/ml) or poly-L-lysine (PLL, 5 µg/ml) coated on plates for 1 h were lysed and then immunoprecipitated with 9E10. The immunoprecipitates were analyzed by Western blotting with anti-Cas-L Ab, followed by anti-pTyr. Data are representative of three independent experiments.
findings suggest that TCR and b1 integrin by FN would mediate costimulation to activate cells, resulting in an increase of T cell migration induced by CD3 stimulation. However, we observed no increase of Cas-L-mediated T cell migration by stimulation with anti-CD3 plus anti-CD28 mAbs (data not shown), which also can induce IL-2 production in the same cells (19). The results indicate that the costimulatory signal that can activate T cells is insufficient to T cell migration, suggesting that b1 integrins may provide specific signals for T cell migration in addition to costimulatory signals in T cells. Because Cas-L was significantly tyrosine-phosphorylated upon the ligation of either CD3 or b1 integrin, the two receptors can independently provide sufficient migratory signals through Cas-L. The CD3 plus FN-induced T cell migration occurred within 2 h, while b1 integrin-induced migration was observed 4 h after incubation. Cas-L is rapidly tyrosine-phosphorylated and dephosphorylated quickly after the ligation of CD3. In contrast, tyrosine phosphorylation of Cas-L after the ligation of b1 integrin occurs slowly and continues stably (14, 35). These findings suggest that TCR and b1 integrins may induce cell migration in different phases; TCR plus b1 integrin signals are mainly for an early phase, and b1 integrin signal alone for a late phase in the T cell migratory process. These differences in the time courses, of which mechanism is still unclear, made it difficult to evaluate tyrosine phosphorylation or cell migration at the same time point. It should be noted that there remains a possibility that difference in the time frame of examination might have influenced our experimental results.

Our study showed that the Cas-LΔSD mutant failed to promote cell migration. It is conceivable that the SD appears to provide binding sites for downstream molecules leading to migratory signal transduction. Crk, an adapter protein that consists of SH2 and SH3 domains, is one of the possible binding molecules to the SD of Cas-L (36). v-Crk was originally reported as the oncogene that can transform mammalian cells such as fibroblasts (36). Crk has been reported to bind to several signaling molecules including C3G, Dock180, and c- abl through the Crk SH3 domain (37–39). Crk may regulate cytoskeletal changes through these molecules. Alternatively, overexpression of Crk with Cas-L results in tyrosine phosphorylation of Cas-L without any stimulation (9), suggesting that Crk may play a role in the regulation of Cas-L phosphorylation. We reported that Nck also binds to tyrosine-phosphorylated Cas-L (9). Nck is an adapter protein consisting of one SH2 domain followed by three SH3 domains (40). This molecule has been reported to bind the p21-activated kinase that regulates actin reorganization. It appears to be involved in reorganization of actin cytoskeleton.

In conclusion, our results showed that Cas-L can regulate T cell migration in response to signals from TCR/CD3 and/or b1 integrin. Furthermore, the present study strongly suggests that Cas-L plays a critical role as a docking protein regulating the association of signaling molecules to promote T cell migration.

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References

INVIOLVEMENT OF Cas-L IN T LYMPHOCYTE MIGRATION


