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Molecules and Cells

LIME Mediates Immunological Synapse Formation through Activation of VAV

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Lck Interacting Membrane protein (LIME) was previously characterized as a transmembrane adaptor protein mediating TCR-dependent T cell activation. Here, we show that LIME associates with Vav in response to TCR stimulation and is required for Vav guanine nucleotide exchange factor (GEF) activity for Rac1. Consistent with this finding, actin polymerization at the immunological synapse (IS) was markedly enhanced by overexpression of LIME, but was reduced by expression of a LIME shRNA. Moreover, TCR-mediated cell adhesion to ICAM-1, laminin, or fibronectin was downregulated by expression of LIME shRNA. In addition, in the IS, LIME but not LAT was found to localize at the peripheral-supramolecular activation cluster (p-SMAC) where the integrins were previously shown to be localized. Together, these results establish LIME as a transmembrane adaptor protein linking TCR stimulation to IS formation and integrin activation through activation of Vav.

INTRODUCTION

When T cells recognize antigenic peptide conjugated with major histocompatibility complex (pMHC) on an antigen-presenting cells (APCs), various signaling molecules involved in TCR and coreceptor-mediated signaling pathway are recruited and reorganized in a specialized structure termed immunological synapse (IS) at the interface between T cell and APC (Saito and Yokosuka, 2006). In the initial stage of T cell conjugation with APCs, TCR-mediated signaling is initiated in hundreds of microclusters containing TCR and signaling molecules including Lck and PKCθ (Bunnell et al., 2001; Saito and Yokosuka, 2006; Yokosuka et al., 2005). Within several minutes, the initial TCR-mediated activation signal drives the movement of TCR-containing microclusters toward the center of the interface to form central supramolecular activation cluster (c-SMAC), while integrin lymphocyte function-associated antigen (LFA-1) and talin, actin cytoskeleton-associated proteins, are translocated to the peripheral-SMAC ring that surround the c-SMAC (Varma et al., 2006; Yokosuka et al., 2008).

Localization of integrins in p-SMAC is critical for TCR-mediated T cell adhesion. TCR-stimulation generates an "inside-out" signal that leads to conformational changes and clustering of the integrin LFA-1, which increase the affinity and avidity towards intracellular adhesion molecules (ICAM)-1 and -2 on antigen presenting cells. (Burbach et al., 2007). In the mature synapse, the adhesion of LFA-1 in p-SMAC with its ligand on APCs enhances and stabilizes the conjugation between T cells and APCs (Kaizuka et al., 2007; Monks et al., 1998). Other integrins bearing beta1 chain is also activated by inside-out signaling and drives T cell adhesion towards extracellular matrix substrates such as laminin and fibronectin, leading to T cell migration.

The formation of IS as well as inside-out signals from the TCR to integrin activation requires reorganization of the actin cytoskeleton (Billadeau et al., 2007). It is well characterized that guanine nucleotide exchange factor (GEF) Vav plays an essential role in actin polymerization (Fischer et al., 1998). Vav1-deficient T cells are inefficient in forming APC-T cell conjugates due to a failure to cluster LFA-1 (Ardouin et al., 2003). The observations that Vav1-deficient T cells are defective in TCR clustering and IS formation also support that Vav is a essential regulator of cytoskeletal rearrangements during T cell activation (Krawczyk et al., 2002).

During T cell activation, transmembrane adaptor proteins (TRAPs) play important roles by recruiting SH2 domain-containing signaling proteins including Vav from the cytoplasm to the membrane-proximal region (Horejsi, 2004; Leo et al., 2002). Currently, at least seven TRAPs are expressed in T cells (Horejsi, 2004; Hur et al., 2003). Among them, the importance of LAT in TCR signaling has been well characterized. Upon TCR stimulation, LAT is phosphorylated by ZAP-70 and associates with various signaling molecules such as phospholipase Cγ-1, Gads-SLP-76, Grb2, and PI3K (Wange, 2000; Zhang et al., 2000). Vav is recruited near the plasma membrane by Gads-SLP-76 complex and converts Rac1 and Cdc42 to their active GTP-bound forms (Crespo et al., 1997), which promote actin polymerization (Bunnell et al., 2001; Zhang et al., 2000) and integrin clustering (Gakidis et al., 2004; Tuosto et al., 1996). By confocal microscopic analysis, LAT is contained in the microclusters and involved in the initial TCR-mediated signaling, and its distribution is gradually concentrated in c-SMAC and internal vesicles in mature IS (Lee and Dominguez, 2010).

Previously, we and others identified LIME as another TRAP which mediates TCR signaling. LIME associates with Lck (Hur et al., 2003) or CD4/CD8 coreceptors (Brdickova et al., 2003).

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Upon TCR-stimulation or crosslinking of CD4 or CD8 coreceptors, LIME is phosphorylated presumably by Lck and Fyn (Brdickova et al., 2003; Hur et al., 2003). Phosphorylated LIME serves as a platform for the recruitment of signaling molecules such as the PI3K p85 subunit, Grb2, Gads, SHP-2, and Csk (Brdickova et al., 2003; Hur et al., 2003). In T cell line, The LIME-mediated signalosome is involved in TCR-mediated activation of ERK and JNK, intracellular calcium mobilization, as well as IL-2 transcription, which are essential for T cell activation (Brdickova et al., 2003; Hur et al., 2003).

We report here that, in response to TCR stimulation, LIME directly associates with Vav and mediates the TCR stimulation-dependent activation of the GEF activity for Rac1. Unlike LAT, LIME is predominantly localized in p-SMAC in mature IS, where integrins reside. By promoting Vav GEF activity, LIME induces actin-rearrangement and TCR-dependent integrin clustering. These findings demonstrate the essential roles of LIME in IS formation and integrin-mediated T cell adhesion.

MATERIALS AND METHODS

Plasmids

The expression plasmids encoding murine LIME-FLAG, LIME-GFP, and tyrosine mutants of LIME have been described previously (Hur et al., 2003). The expression plasmids encoding Lck wild-type (wt) or mutant (F505) have been described previously (Choi et al., 1999). The pEFneo plasmid encoding human Vav-myc was kindly provided by Dr. Yun-Cai Liu (Kaminuma et al., 2001). The *E. coli* expression construct for the GST-Rho binding domain (RBD) of PAK1 was kindly provided by J.H. Kim (Korea University, Korea). The plasmids encoding human LIME shRNA were constructed as previously described (Ahn et al., 2006). Three 19-mer sequences corresponding to nucleotides 408-427 (GGGTGCGCTGGCCTCGAGG), 522-541 (GG GACCCATCGCAGTCCCC), and 560-579 (GACTGAGGTGA CCCC GGCC) in the coding sequence of human LIME were selected as targets for the construction of shRNA-expressing constructs using pSUPER plasmids (Brummelkamp et al., 2002). The most effective construct was that corresponding to nucleotides 408-427. Therefore, this construct was used for all subsequent experiments. The plasmid encoding HA-tagged SHP-2 (pRC/CMV-SHP2-HA), phosphatase inactive mutant of SHP-2 (pRC/CMV-SHP2 C-S-HA) and the plasmid encoding p85 subunit of PI3K (SRα-wild type p85) were from Dr. Masato Kasuga (Noguchi et al., 1994). Human Gads cDNA was obtained from Dr. Naoto Ishii (Kikuchi et al., 2001) and were used for the construction of expression vector in pcDNA3.1 myc/his.

Antibodies

Polyclonal rabbit and mouse anti-LIME sera (Hur et al., 2003) and polyclonal rabbit anti-Lck sera (Choi et al., 1999) have been described previously. The other antibodies used in this study, monoclonal anti-Lck, anti-phosphotyrosine (4G10), anti-Vav, anti-Rac1 (Upstate Biotechnology), anti-Myc, anti-HA (Santa Cruz), anti-mouse IgG, TRITC-anti-mouse IgG, anti-CD3, anti-CD28 (BD Biosciences), anti-FLAG M2, anti-talin, IgG-conjugated protein A-agarose (Sigma-Aldrich), anti-ERK (Cell Signaling), anti-PKCθ, anti-LAT (R&D Systems), and Alexa 568- or Alexa 488-conjugated secondary antibodies (Molecular Probes), were purchased from the manufacturers indicated.

Cells and transient transfection

Jurkat T cells stably expressing the CD8-LIME chimera have been described previously (Hur et al., 2003). For transient expression, Jurkat T cells were transfected by electroporation (BTX-co.). Briefly, 1.5×10^7 Jurkat cells were combined with a total of 20 μg of pEGFP-N1 encoding GFP or other constructs in an electroporation cuvette. The cells were pulsed once at 240 V for 25 msec and then transferred into 10 ml RPMI containing 10% FBS and incubated overnight at 37°C for further processing. We routinely obtained transfection efficiencies of greater than 60% as measured by FACS analysis of GFP-expressing cells.

Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western analysis were performed as described previously (Hur et al., 2003).

APC-T cell conjugation and fluorescence microscopy

APC-T cell conjugates were formed using Raji B cells as APC. Prior to conjugation, Raji B cells were incubated in RPMI 1640 with 5 μg/ml Staphylococcus enterotoxin E (SEE, Toxin Technology) at 37°C for 1 h. Jurkat T cells and Raji B cells, mixed at a 1:1 ratio and a density of 10^6 cells/ml, were immediately transferred to poly-L-lysine coated slides, and incubated at 37°C for 5 min, 15 min, or 30 min. Slides were fixed in 3.7% formaldehyde for 20 min at room temperature and permeabilized in 0.1% Triton X-100 for 4 min at room temperature. Slides were incubated with the indicated antibodies diluted to 1:100 or 1:50 in 3% BSA/PBS for 30 min at 30°C. Subsequently, slides were exposed to Alexa 568- or Alexa 488-coupled secondary antibodies or Alexa 568-phalloidin to visualize filamentous actin (Molecular Probes) and incubated for 30 min at 30°C. After mounting, images were obtained using a LSM510 META confocal microscope (Carl Zeiss Co.). Images were automatically assessed for the distribution of specific proteins along a single 2D optical section (along the x-y axis) or the entire cell contact (along the x-z axis). The 3D views of the conjugates were generated as orthographic projections.

Analysis of Rac1 GTPase activity

Rac1 GTPase activity was measured as described previously (Benard et al., 1999). Jurkat cells (1.5×10^7) were serum starved for 3 h and stimulated with anti-CD3 for 5 min. Cells were lysed in Mg^{2+} lysis/wash buffer (Pierce) and cell lysates were incubated with purified GST-PAK-RBD fusion protein conjugated to glutathione-sepharose 4B beads in binding buffer (25 mM Tris-HCl (pH 7.5), 1 mM DTT, 30 mM $MgCl_2$, 40 mM NaCl, 0.5% NP-40) for 1 h at 4°C. The bead pellet was washed two times with binding buffer containing 1% NP-40 and then washed in binding buffer lacking NP-40. GTP-γS or GDP-loaded cell lysates were used as controls. Precipitated GST-PAK-RBD bound proteins were probed with anti-Rac1 antibody.

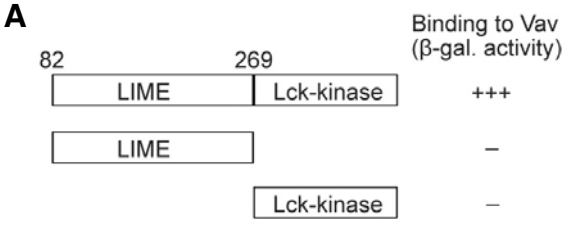
Adhesion assay

Jurkat T cells (2×10^5) stably expressing LIME or transfected with plasmids encoding LIME shRNA were transferred into 96-well flat-bottom plates coated with 10 μg/ml BSA, laminin, fibronectin, or recombinant ICAM-1 (R&D Systems) and stimulated with anti-CD3 (5 μg/ml) or PMA (100 ng/ml) for 1 h at 4°C. Subsequently, samples were incubated at 37°C for 30 min and washed with 2% BSA in PBS. Adherent cells were stained with crystal violet in 1% SDS and analyzed by measuring absorbance at 540 nm.

RESULTS

LIME associates with Vav upon TCR stimulation

Previously, LIME was shown to form a complex with signaling molecules such as PI3K, Gads, Grb2, and SHP-2 (Hur et al., 2003). To understand LIME function further, we searched for



B

Binding partners of LIME	No. of colonies
Grb2	2
Gads	5
PI3K p85α subunit	1
Nck	3
STAT5A	1
Vav	2
Lad/TSAd	1
SAP	20
SHPTP-2	1
P56Lck	3

Fig. 1. Identification of LIME-binding proteins by yeast two-hybrid screening. (A) Bait constructs for the yeast two-hybrid assay are shown. Vav interacted with the LIME-Lck kinase fusion protein but not with LIME or Lck kinase alone. (B) List of proteins identified as binding partners of LIME through yeast two-hybrid screening. Of approximately 60 positives sequenced, the SH2 domain-containing molecules are listed.

additional binding partners of LIME using the tyrosine phosphorylation-dependent yeast two-hybrid screening method described previously (Park and Yun, 2001). In this screening, the cytoplasmic domain of LIME is fused to the kinase domain of Lck and used as bait (Fig. 1). After screening of 2×10^6 clones of a murine T cell lymphoma cDNA library and subsequent characterization of positive colonies, Vav was identified as a potential binding partner of LIME (Fig. 1B). In addition, the PI3K P85 subunit, Gads, Grb2, SHP-2, and Lck, which have been previously identified as LIME binding proteins (Hur et al., 2003), were also positives in our screen. Subsequently, the binding of Vav to LIME was confirmed in Jurkat T cells stably expressing LIME (Fig. 2A). Previously, this cell line has been used for the analysis of signaling regulated by LIME proteins (Hur et al., 2003). After stimulation with anti-CD3 mAb for 5 min, LIME was immunoprecipitated from cell lysates and the precipitate was analyzed by Western blotting with anti-Vav. Vav was detected in LIME precipitates from CD3 stimulated cells, showing that Vav associates with LIME in response to TCR stimulation.

Next, we determined whether phosphorylation of LIME by Lck is required for Vav binding. LIME and Vav were co-expressed with wild-type Lck (Lck wt) or a constitutively active form of Lck (Lck F505) by transient transfection in HEK 293T cells. As shown in Fig. 1B, LIME and Vav coimmunoprecipitated in the presence of Lck F505 but not Lck wt showing that LIME phosphorylation by Lck is necessary for Vav binding. Subsequently, the LIME motifs responsible for binding Vav were mapped by co-expressing tyrosine mutants of LIME with Lck F505. The Y175F (tyrosine 175 substituted for phenylalanine) mutant failed to bind Vav (Fig. 2C) suggesting that phosphorylation of tyrosine 175 is required for Vav binding. In addition, following the same approach, we mapped the motif of LIME involved in binding other signaling molecules. The LIME residues responsible for binding to Gads, SHP-2, and the PI3K p85 subunit were mapped as tyrosine 137, 207, and 242/261, respectively (Fig. 3). In these experiments (Figs. 2C and 3), migration of LIME mutants in acryl amide gel was slightly different from each other probably because the tyrosine phosphorylation status of each mutant is altered due to the mutation of tyrosine residues. The binding site mapping results are summarized in Fig. 3F.

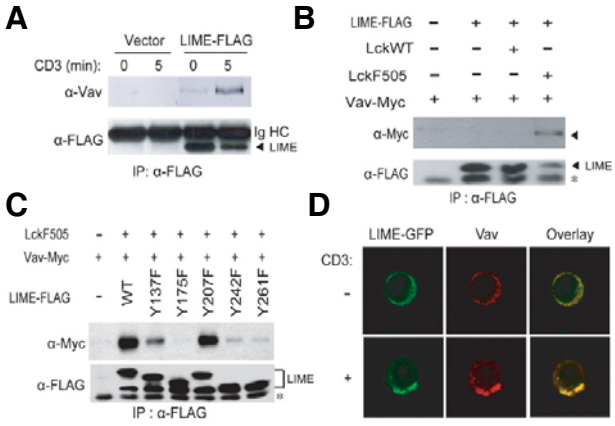


Fig. 2. LIME associates with Vav. (A) Vav coimmunoprecipitates with LIME. Jurkat T cells stably expressing FLAG-tagged LIME were stimulated by CD3-crosslinking for 5 min, cell lysates were collected and analyzed by immunoprecipitation with anti-FLAG and subsequent Western blotting with anti-Vav or anti-FLAG. (B) The association of Vav with LIME requires tyrosine phosphorylation by Lck. HEK 293T cells were transfected with the indicated combinations of expression plasmids encoding LIME-FLAG, Lck wt, LckF505, or Vav-myc. Anti-FLAG immunoprecipitates were analyzed by Western blotting with anti-Myc or anti-FLAG antibody. Nonspecific band (*) was also detected by anti-FLAG antibody slightly below LIME protein. (C) Tyrosine 175 in the cytoplasmic tail of LIME serves as the binding site for Vav. FLAG-tagged tyrosine site mutants of LIME were coexpressed with Vav-myc and Lck F505 in 293T cells by transient transfection. Anti-FLAG immunoprecipitates were analyzed by Western blotting with anti-myc or anti-FLAG. LIME proteins ( ]) were differently migrated by its phosphorylation status depending on the point mutation of tyrosine residues above the non-specific band (*). (D) Vav colocalizes with LIME at the CAP structure. Jurkat T cells were transfected with plasmids expressing LIME-GFP. Subsequently, cells were stimulated with anti-CD3 and stained with anti-Vav (red).

In addition, to ascertain the interaction of Vav with LIME, we determined whether these proteins co-localize after TCR stimulation (Fig. 2D). LIME-GFP expressing Jurkat T cells were stimulated with anti-CD3 and stained with anti-Vav. After stimulation with anti-CD3, LIME-GFP and Vav co-localized at the capping site. These results further support that Vav associates with LIME in response to TCR stimulation.

LIME induces the phosphorylation and activation of Vav GEF

Having identified the association of Vav with LIME, we examined whether LIME mediates TCR stimulation-dependent activation of Vav. We first tested the effects of LIME shRNA on TCR-mediated phosphorylation of Vav. Jurkat T cells were

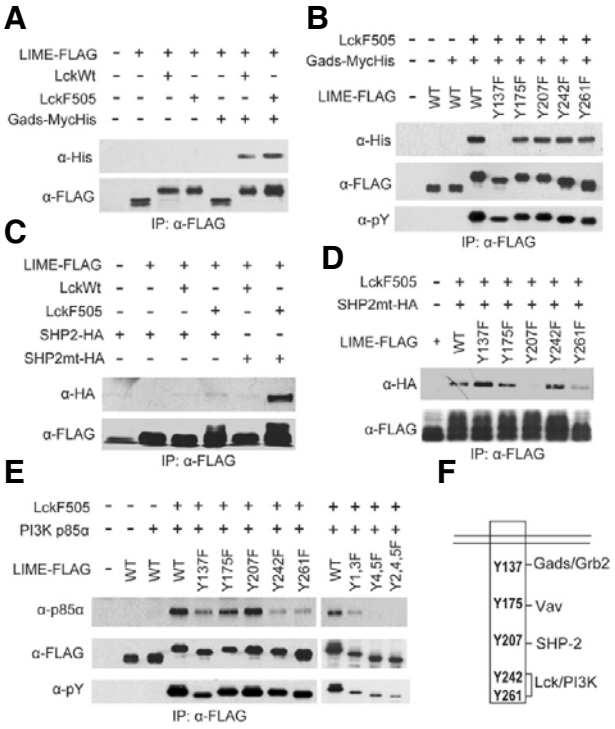


Fig. 3. Mapping of the LIME binding sites for association with Gads, SHP2, and the PI3K p85 subunit. Using the same methods described in the legends of Fig. 2C, LIME-binding sites of indicated signaling molecules were analyzed. (A, B) LIME associates with GADS through the pY137 motif. (C, D) LIME associates with SHP-2 through the pY207 motif. In the SHP-2 mt, the active site cysteine in the phosphatase domain is mutated to phenylalanine. (E) LIME associates with the PI3K p85 subunit through the pY242/pY261 motif. (Y1,3F = Y137/207F; Y4,5F = Y242/261F; Y2,4,5F = Y175/242/261F.). (F) Summary of the LIME binding sites for the indicated signaling molecules.

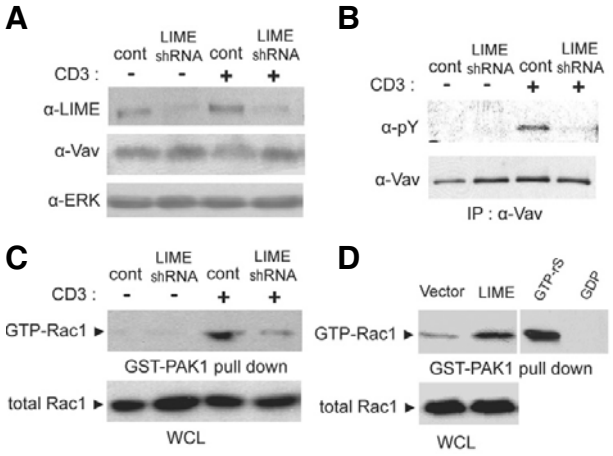


Fig. 4. LIME is required for the TCR stimulation-dependent GEF activity of Vav. (A) LIME expression is effectively blocked by the expression of shRNA. Jurkat T cells were transfected with control or shRNA-expressing plasmids and stimulated with anti-CD3 mAb for 5 min. Whole cell lysates were analyzed by Western blotting with the indicated antibodies. (B) TCR-mediated phosphoylation of Vav is blocked by the expression of LIME shRNA. Cells were processed as described in (A). Cell extracts were subjected to immunoprecipitation with anti-Vav and subsequent immunoblotting with anti-pY mAb (4G10). The same precipitates were analyzed with anti-Vav Ab. (C) TCR-mediated Rac1 activation is blocked by LIME shRNA. Cells were processed as described in (A) and the lysates were subjected to affinity precipitation using a GST-PAK-RBD fusion protein. The precipitates were analyzed by immunoblotting with anti-Rac1. (D) In Jurkat T cells stably expressing LIME were stimulated with anti-CD3 mAb for 5min and Rac1 activity was determined as described in (C). GTP-γS or GDP-loaded Jurkat cell lysates were used as positive and negative controls, respectively.

transfected with plasmid encoding LIME shRNA and stimulated by TCR crosslinking for 5 min. As shown in Fig. 4A, introduction of LIME shRNA efficiently blocked the expression of LIME but not Vav. Subsequently, endogenous Vav was immunoprecipitated from the cell lysates, and the precipitates were analyzed by immunoblotting with an anti-phosphotyrosine antibody (Fig. 4B). Phosphorylation of Vav upon T cell activation was blocked in LIME shRNA-expressing cells demonstrating that LIME is required for the TCR stimulation-dependent phosphorylation of Vav.

Secondly, we tested whether LIME was required for the TCR-mediated activation of Vav GEF. The GEF activity of Vav was examined using the Rac-binding domain (RBD) of PAK as a probe to isolate the active GTP-bound form of Rac1 (Crespo et al., 1997). Jurkat T cells were transfected with plasmids encoding LIME shRNA and stimulated by CD3 crosslinking. Subsequently, GTP-bound Rac1 precipitated with GST-RBD domain beads was separated by SDS-PAGE, and analyzed by Western blotting with anti-Rac1 Ab. As shown in Fig. 4C, the level of GTP-bound Rac1 significantly decreased in cells expressing LIME shRNA. In addition, we measured the GEF activity of Vav in Jurkat T cells stably expressing LIME and found that, upon TCR stimulation, the level of GTP-bound Rac1 was significantly higher than the control (Fig. 4D). These data show that LIME mediates TCR-dependent phosphorylation of Vav and activation of Vav GEF activity.

LIME mediates TCR-dependent actin polymerization and IS formation

Because Vav is required for TCR-mediated rearrangement of the actin cytoskeleton (Fischer et al., 1998) and IS formation (Krawczyk et al., 2002), we examined whether LIME controls actin polymerization. Jurkat T cells were transfected with plasmids encoding GFP, LIME-GFP, or the LIME Y175F mutant-GFP (mutant in Vav binding motif) and allowed to conjugate with SEE-pulsed Raji B cells for 15 min to induce mature IS formation. The conjugates were stained with Alexa 568-phalloidin and F-actin was visualized using confocal microscopy (Fig. 5A). In T cells overexpressing LIME-GFP, the F-actin content at the IS was markedly enhanced and a larger, stable F-actin structure was detected (Fig. 5A, middle panels). However, this enhancement in F-actin was not detected in T cells overexpressing the LIME Y175F mutant-GFP, in which the Vav binding site has been disrupted (Fig. 5A, bottom panels). These results suggest that LIME mediates actin polymerization through its association with Vav.

To further assess the involvement of LIME in actin-rearrangement, we investigated the effect of LIME shRNA on actin polymerization and IS formation. Jurkat T cells were transfected with the plasmid encoding LIME shRNA and total cellular F-actin content following TCR ligation was examined by FACS analysis. As shown in Fig. 5B, the TCR stimulation-dependent increase in F-actin content was blocked in cells expressing

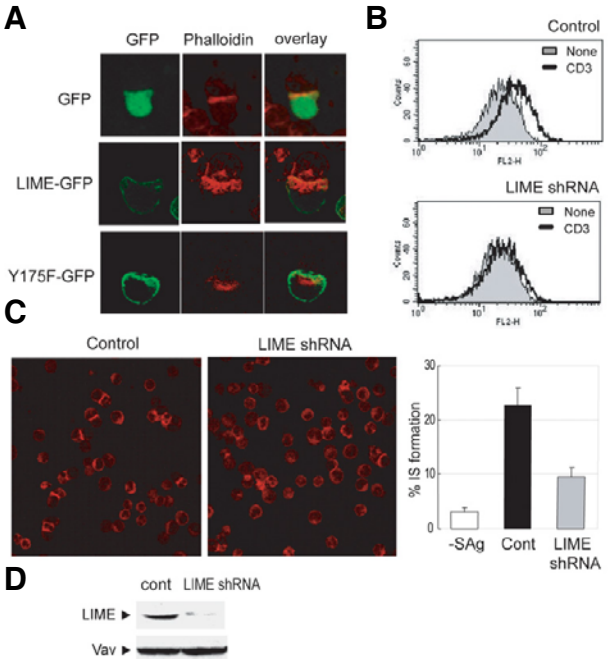


Fig. 5. LIME is required for actin polymerization and IS formation. (A) Overexpression of LIME leads to enhanced actin polymerization at the IS. Jurkat T cells were transiently transfected with the expression plasmids encoding GFP alone (upper panel), LIME-GFP (middle panel) or Y175F mutant LIME-GFP (bottom panel) and allowed to conjugate with SEE-pulsed Raji B cells. After 15 min, cells were stained with Alexa 568-conjugated phalloidin and analyzed by confocal microscopy. (B) Jurkat T cells were transfected with control or the plasmid encoding LIME shRNA and stimulated by CD3 crosslinking. Subsequently, cells were stained with Alexa 568-conjugated phalloidin and the intracellular F-actin content was analyzed by FACS. Data shown are representative of three independent experiments. (C) Jurkat T cells transfected with control vector or plasmid encoding LIME shRNA were allowed to conjugate with SEE-pulsed Raji B cells. Conjugates were stained with anti-talin (left panels) and the number of conjugates exhibiting talin accumulation at the contact site was counted and is shown as the percentage of IS-forming cells among all cells examined (right panel). The control was incubated in the absence of SEE (-SAg). (D) The efficiency of LIME shRNA was assessed by immunoblotting with anti-LIME or anti-Vav antibodies.

LIME shRNA.

To test the effect of the shRNA on IS formation, Jurkat T cells expressing LIME shRNA were allowed to conjugate with SEE-pulsed Raji B cells at a 1:1 ratio and the extent of IS formation was analyzed by counting the conjugates in which talin, a marker for the mature synapse, had accumulated (Fig. 5C). In cells expressing LIME shRNA, the number of T cells showing talin accumulation at the IS was significantly decreased compared to control cells. The experiments were repeated four times and one hundred conjugates per transfection were analyzed. The percentage of IS formation was reduced to 9% in Jurkat T cells expressing shRNA, compared to 25% in control cells (Fig. 5C, right panel). These data demonstrate that LIME is required for rearrangement of the actin cytoskeleton and IS formation.

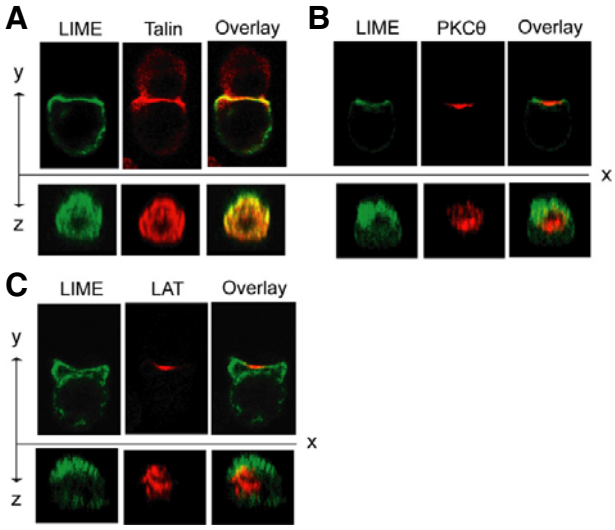


Fig. 6. LIME localizes to the peripheral-SMAC following conjugation with APC. Jurkat T cells were transfected with expression plasmids encoding LIME-GFP and allowed to conjugate with Raji B cells pulsed with SEE for 15 min. The cell conjugates were labeled with anti-talin (A), anti-PKCθ (B) or anti-LAT (C). A single 2D optical section of the center of the cell conjugates is shown in the upper panels. The 3D reconstructed view of the T cell-APC contact site is shown in the lower panels. Talin and PKC-θ are markers for the p-SMAC and c-SMAC, respectively. Note that LIME-GFP colocalized with talin, but not with PKCθ or LAT. (n = 45: total number of conjugates examined for each antibody).

LIME translocates to the p-SMAC following T cell-APC conjugation

Having found that LIME mediates IS formation, we examined the subcellular localization of LIME. Jurkat T cells were transfected with constructs expressing LIME-GFP and allowed to conjugate with APC for 30 min. The conjugated cells were then stained with antibodies against talin, PKCθ, or LAT. T cell-APC contact sites are shown in the upper panels, whereas single 2D optical sections through the center of cell conjugates are shown in the lower panels. Interestingly, in the mature synapse, formed by 15 min after conjugation, LIME co-localized with the ring-shaped distribution of talin (Fig. 6A), representing the p-SMAC, surrounding PKCθ (Fig. 6B), a c-SMAC marker. In contrast to the localization of LIME in the p-SMAC, LAT was found in the c-SMAC in the mature synapse (Fig. 6C). These results show that, in the mature synapse, LIME localizes to the p-SMAC, which serves as an adhesion zone.

LIME mediates TCR stimulation-dependent integrin activation

In addition to its role in IS formation, Vav controls integrin-mediated adhesion of T cells to ECM proteins and ICAM-1 (Ardouin et al., 2003). Therefore, we investigated whether LIME mediates integrin activation. The role of LIME in modulating TCR-induced integrin avidity was analyzed by an adhesion assay with Jurkat T cells stably expressing LIME (Fig. 7A) or transfected with a LIME shRNA-expressing plasmid (Fig. 7B). Cells were stimulated with anti-CD3 or PMA and were transferred to plates coated with BSA, laminin, fibronectin, or recombinant ICAM-1. After washing the wells, the adherent cells were quantified. Upon CD3 stimulation or PMA treatment, the adhesion of Jurkat T cells to each integrin ligands were increased,

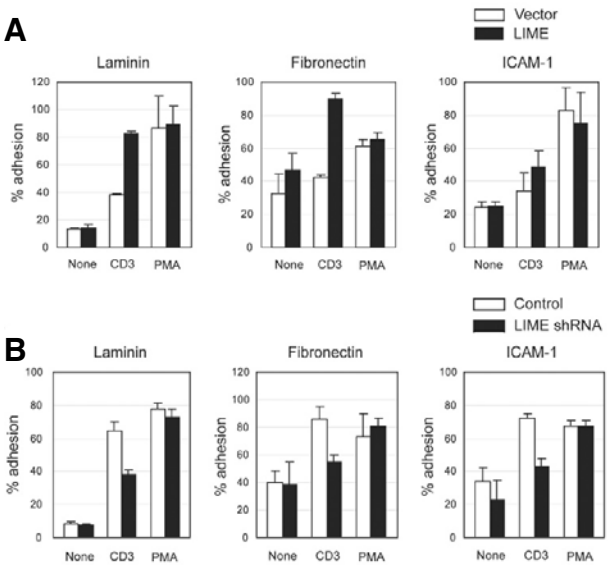


Fig. 7. LIME is required for TCR-induced cell adhesion. Jurkat T cells stably expressing LIME (A) or transfected with the plasmid encoding LIME shRNA (B) were transferred to 96 well plates coated with BSA, laminin, fibronectin, or ICAM-1 and were treated with anti-CD3 or PMA. Adherent cells were stained with crystal violet and quantified by measuring absorbance at 540 nm. The results shown represent four independent experiments performed in triplicate.

showing that integrins were activated upon TCR stimulation. Upon CD3 stimulation, the adhesion of Jurkat T cells stably expressing LIME to each integrin ligands were significantly increased compared to control Jurkat T cells stably transfected with vector plasmids (Fig. 7A). On the other hand, Jurkat T cells expressing LIME shRNA exhibited significantly less adhesion to laminin, fibronectin, and ICAM-1 following CD3 stimulation (Fig. 7B). The adhesion to each integrin ligands was unaffected by the ectopic expression or knockdown of LIME protein in response to PMA treatment, which indicates that LIME is involved in the membrane-proximal TCR signaling events upstream of PKC. These results show that LIME is critical in transducing the TCR signal to modulate integrin activation.

DISCUSSION

In this report, we have shown that LIME mediates actin-polymerization and integrin-mediated adhesion following TCR stimulation. These findings establish LIME as a raft-associated transmembrane adaptor protein linking TCR stimulation to IS formation by regulating actin polymerization and integrin clustering.

With respect to a molecular mechanism for LIME-mediated actin polymerization, we have shown that LIME recruits Vav to the membrane through a direct interaction mediated by pY175. Once recruited to the LIME complex, Vav becomes activated and catalyzes the exchange of GDP for GTP, to activate Rac1. Activated Rac1 mediates actin polymerization presumably through WASP-Arp2/3, which strengthens conjugation with the APC (Lee and Dominguez, 2010; Zeng et al., 2003). Previously, TCR stimulation-dependent actin polymerization was shown to be mediated by Vav recruitment to the membrane by a mechanism involving the LAT-Gads-SLP-76 complex (Tuosto et al., 1996). Vav recruitment by LIME, as demonstrated in this report, provides an alternative mechanism for Vav activation and subsequent actin polymerization. Although supporting data are insufficient at this stage, it is likely that these two mechanisms may operate at different stages of T cell activation since LIME expression is induced upon T cell activation whereas LAT expression is constitutive (Wange, 2000; Zhang et al., 1999). While LAT-mediated Vav recruitment is important for initial activation of resting T cells (Perez-Villar et al., 2002; Tuosto et al., 1996), LIME-mediated Vav recruitment may regulate events in activated/effector T cells.

In addition to its role in actin polymerization, LIME was shown here to mediate integrin clustering through recruitment of Vav. Again, this finding provides an alternative mechanism for TCR stimulation-dependent integrin clustering in addition to the previously reported LAT-mediated mechanism (Wange, 2000). In addition to direct recruitment of Vav, LIME may mediate integrin clustering through recruitment of the Gads-SLP76-Vav complex and subsequent activation of Rac1, as LIME has the ability to bind Gads through pY137 (Fig. 3). Both direct interaction with Vav and indirect recruitment of Vav via Gads-SLP-76 may cooperate to induce LIME-mediated integrin clustering. Again, LIME-mediated integrin clustering may function in activated/effector T cells as LIME expression is induced upon T cell activation.

Although both LIME and LAT are expressed in T cells and recruit a similar set of SH2 domain-containing signaling molecules, these proteins differ in several respects. First, LIME is phosphorylated by Lck (Hur et al., 2003), while LAT is phosphorylated by ZAP-70 (Zhang et al., 1998), which suggests that LIME and LAT mediate the signaling of a specific kinase, Lck or ZAP-70, respectively. Secondly, LIME expression is induced upon TCR stimulation (Hur et al., 2003), while LAT expression is constitutive in resting T cells, suggesting that these proteins operate at different stages of T cell activation. Thirdly, in this study we found that, in the mature synapse, LIME and LAT localize in different regions, namely the p-SMAC and c-SMAC, respectively (Fig. 6). The observation that the p-SMAC is enriched with integrins, including LFA-1 (Kaizuka et al., 2007), is consistent with the finding in this report that LIME is required for integrin clustering.

Recent analysis showed that the movement of microclusters from peripheral to central region and its down regulation is modulated by Myosin II, whereas actin flow is restricted to peripheral region of SMAC and critical for the sustained activation of TCR signaling (Hashimoto-Tane et al., 2011; Yu et al., 2010). However, the signaling modulator that involved in actin polymerization especially in p-SMAC has not been elucidated. LIME may function as a unique TRAP which promotes Vav-mediated actin polymerization at p-SMAC leading to the sustained T cell-APC conjugation and integrin activation through inside-out signaling.

Previously, LIME was shown to be involved in BCR-mediated B cell activation (Ahn et al., 2006). In relation to this, microcluster formation in the immunological synapse was recently shown to be required for B cell activation (Depoil et al., 2008; Lee and Dominguez, 2010). Therefore, it should be further examined whether LIME is also involved in BCR-mediated IS formation and B cell activation.

Despite the importance of LIME in TCR- and BCR-mediated signaling (Ahn et al., 2006; Hur et al., 2003) and IS formation *in vitro* here, however, LIME$^{-/-}$ mice were recently reported to have no discernible phenotype (Gregoire et al., 2007). The activation and proliferation of T cells were normal in these mice. This phenomenon may be due to functional complementation by

other TRAPs. Alternatively, the expression level of LIME protein in naive T cells is not high enough to affect the initial T cell activation in resting T cells. Previously, we observed that LIME protein is barely detectable in murine naïve T cells, and is gradually expressed following differentiation to effector T cells in the presence of IL-2 cytokine (Hur et al., 2003). In conclusion, we have identified a role of LIME in actin polymerization and integrin activation, which are important for IS formation.

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