

LIME: A New Membrane Raft-associated Adaptor Protein Involved in CD4 and CD8 Coreceptor Signaling

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Abstract

Lymphocyte membrane rafts contain molecules critical for immunoreceptor signaling. Here, we report identification of a new raft-associated adaptor protein LIME (Lck-interacting molecule) expressed predominantly in T lymphocytes. LIME becomes tyrosine phosphorylated after cross-linking of the CD4 or CD8 coreceptors. Phospho-LIME associates with the Src family kinase Lck and its negative regulator, Csk. Ectopic expression of LIME in Jurkat T cells results in an increase of Csk in lipid rafts, increased phosphorylation of Lck and higher Ca²⁺ response to CD3 stimulation. Thus, LIME appears to be involved in regulation of T cell activation by coreceptors.

Key words: membrane microdomains • Lck • Csk • signal transduction • phosphorylation

Introduction

Membrane microdomains, also called lipid rafts or glycolipid-enriched microdomains, are small areas of cell membranes that can be distinguished from the rest of the membrane by unique lipid and protein composition. They are enriched in lipids containing long saturated fatty acid residues (mainly sphingomyelin and glycosphingolipids) and cholesterol, glycosylphosphatidylinositol-anchored proteins, and several cytoplasmic proteins associated with the inner leaflet of the membrane via covalently attached fatty acid residues. Among these cytoplasmic proteins are, e.g., Src family kinases (possessing NH₂-terminal double acylation) and heterotrimeric G proteins. The integrity of membrane rafts seems to be maintained by lateral interactions of their constituent lipids which form, together with cholesterol, a more organized “liquid ordered” phase in the membrane. Membrane rafts can be easily isolated due to their relative insolubility in solutions of common mild detergents of the polyoxyethylene type (such as Triton X-100, Brij series, NP-40) at

low temperature and their ability to float when such detergent lysates are ultracentrifuged in sucrose density gradient. Conversely, membrane rafts are readily solubilized by alkyl-glycosidic type of detergents (for general review on the rafts and their role in immunoreceptor signaling see references 1–3). Most transmembrane proteins are excluded from lipid rafts, exceptions being few molecules palmitoylated on cytoplasmic membrane-proximal cysteine residues, such as the pre-TCR (4), the CD4 and CD8 coreceptors (5, 6), and the three recently discovered transmembrane adaptor proteins, linker for activation of T cells (LAT) (7, 8), phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) (9), or Csk-binding protein (Cbp) (10) and non-T cell activation linker (NTAL) (11) or linker for activation of B cells (LAB) (12).

Because of the presence of several key signal-transducing molecules (Src family kinases, transmembrane adaptor proteins LAT, NTAL/LAB, and PAG/Cbp), membrane rafts have been shown recently to play an essential role in initiation of immunoreceptor (TCR, BCR, FcR) signaling (1–3). To identify novel lipid raft-associated transmembrane adaptor

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Preliminary reports (abstracts, poster, and oral communications) on some aspects of this work were presented at the ELSO2002 Meeting, Nice, June 29–July 3, 2002, 5th EFIS Tatra Immunology Conference, Tatranske Zruby, September 7–11, 2002, and 15th EFIS Meeting, Rhodes, June 2003.

Abbreviations used in this paper: Cbp, Csk-binding protein; LAT, linker for activation of T cells; LAB, linker for activation of B cells; LIME, Lck-interacting molecule; NTAL, non-T cell activation linker; PAG, phosphoprotein associated with glycosphingolipid-enriched microdomains; PBL, peripheral blood leukocytes; PHA, phytohemagglutinin; PLC γ , phospholipase C γ ; PTK, protein tyrosine kinase; P-Tyr, phosphotyrosine; SH Src homology; TRAP, transmembrane adaptor protein; TRIM, TCR-interacting molecule.

proteins, we have searched public databases for molecules sharing the structural features characteristic of raft-targeted transmembrane adaptor proteins. One of the proteins identified by this approach was Lck-interacting molecule (LIME), a molecule of unknown function whose amino acid sequence has been deposited in the databases since 1998. In the present study we describe the first biochemical and functional characterization of LIME.

Materials and Methods

Cells and Antibodies. T, B, and NK cells and monocytes were obtained from buffy coats by Ficoll centrifugation and preparative cell sorting using a FACS Vantage™ flow cytometer (Becton Dickinson) and PE-conjugated mAbs to CD3 and CD19 (Serotec), biotinylated anti-CD14 (Serotec), fluoresceinylated (FITC) streptavidin (BD Biosciences), unlabeled CD56 mAb MEM-188, and fluorescein-conjugated F(ab)₂ fragments of goat anti-mouse Ig (Caltag). Single-positive (CD4⁺8⁻, CD4⁻8⁺), double-positive (CD4⁺8⁺), and double-negative (CD2⁺CD4⁻CD8⁻CD19⁻) thymocytes were isolated by flow sorting using anti-CD4-FITC (MEM-241), anti-CD8-PE (BD Biosciences), anti-CD2-PE (Immunotech), anti-CD4-biotin (Serotec), anti-CD8-biotin (Serotec), anti-CD19-biotin (Serotec), and Streptavidin-FITC (BD Biosciences) mAbs. Purity of all sorted thymocyte subpopulations used in subsequent experiments was >99%. In some experiments, the peripheral blood leukocytes (PBLs) obtained by Ficoll centrifugation were passed through nylon wool column, the nonadhering T and NK cells were recovered and are referred to as peripheral blood T cells. 293T cells were from the cell line collection of the Institute of Molecular Genetics. T cell line J77 (CD4⁺ Jurkat) was provided by Dr. O. Acuto (Institut Pasteur, Paris, France).

Antiserum to LIME was produced in the Prague laboratory by immunization of rabbits with bacterially expressed and Talon purified (CLONTECH Laboratories, Inc.) cytoplasmic fragment of human LIME (described under constructs), and mouse mAbs to LIME were prepared using standard techniques from splenocytes of mice immunized with the same bacterially produced LIME fragment. In addition, antipeptide mAbs were prepared against the COOH-terminal peptide comprising residues 281–296 of the human molecule (purchased from PolyPeptide Laboratories) conjugated to keyhole limpet hemocyanin using a commercial kit (Pierce Chemical Co.). Rabbit antiserum to LAT (13) was provided by Dr. L. Samelson (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD).

The sources of the other antibodies used were as follows: mAb to phosphotyrosine (4G10; UBI), rabbit polyclonal antibodies to Erk1/2 (Promega), phospho-Lck (Tyr⁵⁰⁵) and phospho-Src family (Tyr⁴¹⁶) (New England Biolabs, Inc.), Csk (Santa Cruz Biotechnology, Inc.), mAbs to CD3 (OKT3; Ortho), CD28 (IgM isotype; provided by Dr. U. Moebius, University of Heidelberg, Germany), and CD4 (B66.1; donated by Dr. C.T. Baldari, University of Siena, Siena, Italy). mAbs to Lck (LCK-01), CD4 (MEM-16, MEM-115, MEM-241), CD8 (MEM-31), CD3ε (MEM-92, MEM-57), CD20 (MEM-97), and CD56 (MEM-188) were all prepared in the Prague laboratory.

Immunoprecipitation, In Vitro Kinase Assay, and Other Biochemical Methods. LIME and LIME-containing complexes were immunoprecipitated from postnuclear supernatants of cells solubilized by a detergent effectively disrupting lipid rafts (laurylmaltoside [*N*-dodecyl β-D-maltoside]; Calbiochem) (lysis buffer: 1% lau-

rylmaltoside in 20 mM Tris, pH 7.5, containing 100 mM NaCl, 10% glycerol, 1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 10 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄) using CNBr-Sepharose beads (Amersham Biosciences) coupled with mAbs purified by protein A-Sepharose affinity chromatography. The lysates were passed through minicolumns (30–50 μl packed volume) of such immunosorbents; after washing with 10 column volumes of lysis buffer, bound proteins were eluted with 2 column volumes of 2× concentrated SDS sample buffer and the flow-through, and eluted fractions were analyzed by SDS-PAGE followed by Western blotting.

In vitro kinase assays were performed as described before (14). Briefly, cells were solubilized in ice cold 1% laurylmaltoside lysis buffer (see previous paragraph), and the postnuclear supernatants were incubated in plastic wells coated with appropriate mAbs. After washing, the kinase solution (20 mM Hepes, pH 7.4, 5 mM MgCl₂, 5 mM MnCl₂, 0.1% detergent Igepal [Sigma-Aldrich]) containing 0.1 μCi [γ-³²P]ATP (ICN) per well was added, and the proteins phosphorylated by kinases present in the immunoprecipitates were resolved by SDS-PAGE followed by autoradiography and immunoblotting. In some cases, purified cytoplasmic domain of TCR-interacting molecule (TRIM) protein (15) (30 μg/ml) was used as an exogenous Lck substrate and added together with 0.5 μCi [γ-³²P]ATP per well. At the end of the test, the content of the wells was taken and processed for SDS-PAGE and autoradiography. For control, the wells were then washed, and phosphorylated proteins in immunoprecipitates were resolved as in the standard kinase assay. Lipid raft separation by sucrose gradient ultracentrifugation was performed essentially as described before, using 3% Brij-58 detergent for membrane solubilization; the density gradient fractions were analyzed by SDS PAGE and Western blotting (9). In some experiments, cells were directly solubilized in 2× concentrated SDS sample buffer, ultracentrifuged (250,000 g, 30 min), and the supernatant was analyzed by SDS-PAGE and Western blotting. Biosynthetic labeling with ³H-palmitate, SDS-PAGE, and Western blotting were performed as described (11).

PCR. Nested PCR with two sets of primers (first 5′-GAGAATTCTGCACAAAGACCTTCCTGG-3′ plus 5′-GAG-AATTCACAGGTCCTTGAGTTCAG-3′ and then the same 5′ end primer plus 5′-GCAGCACCTAGAACCCAGA-3′ primer) was used to detect the LIME cDNA in the Multiple Tissue Panels I and II and the Immune System Panel (CLONTECH Laboratories, Inc.).

DNA Constructs and Transfections. The coding region of human LIME was amplified from human leukocyte cDNA library (CLONTECH Laboratories, Inc.) (primers: 5′-GAGAATTCTGCACAAAGACCTTCCTGG-3′ and 5′-GAGAATTCACAGGTCCTTGAGTTCAG-3′). The PCR product was cloned into EcoRI site of pBluescript SK vector (Stratagene) and sequenced. For bacterial expression, the LIME intracellular fragment corresponding to aa 141–295 was cloned into XhoI and BamHI sites of pET-15b expression vector (Novagen), generating a construct with NH₂-terminal histidine tag. For expression in eukaryotic cells, LIME coding sequence was subcloned into EcoRI site of pEFIRES-N vector (16) (provided by Dr. S. Hobbs, Institute of Cancer Research, London, UK). For transient transfection of 293T cells, Lipofectamine 2000™ reagent (Invitrogen) was used according to manufacturer's instructions. For transfection experiments in 293T cells, the following cDNA constructs were used: Flag-tagged Lck and Myc-tagged ZAP-70 inserted into pcDNA3 vector (donated by Dr. R. Abraham, Mayo Clinic, Rochester, Minnesota), Fyn cloned in pSRα expression

vector (provided by Dr. A. da Silva, Dana Farber Cancer Institute, Boston, MA), Csk in pEF-BOS vector (9), Syk cloned into the pRK5 vector (provided by Dr. W. Kolanus, Gene Center, Munich, Germany), Myc-tagged Lyn in pcDNA3.1 vector (provided by Dr. S. Watson, University of Oxford, Oxford, UK), and Flag-tagged Hck in pcDNA1 vector (provided by Dr. G. Langsley, Institut Pasteur, Paris, France).

J77 cells (Jurkat T cell line expressing CD4; essentially devoid of endogenous LIME expression) were transfected with the LIME-pEFIRES-N construct by electroporation, and stable transfectants were selected by growing the cells in 96-well plate in selective medium containing 1 mg/ml G418 (Calbiochem). After 3 wk, oligoclonal G418-resistant populations were expanded and checked for LIME expression by Western blotting. Single Tyr→Phe mutants were produced by site-directed mutagenesis using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. COOH-terminally FLAG-tagged NTAL was produced by site-directed mutagenesis using the same kit; FLAG-tagged LIME construct was generated by PCR using the primer containing sequence for FLAG. All mutated nucleotide sequences were verified by full-length sequencing and subcloned to pEFIRES-N.

For overexpression in peripheral blood T cells, LIME was subcloned into EcoRI site of pXJ41 vector (17), and the transfection was performed using the nucleofector apparatus and human T cell nucleofector kit (Amaxa Biosystems) according to the manufacturer's instructions.

Confocal Microscopy. J77-LIME transfectants were spun on coverslips coated with poly-L-lysine (Sigma-Aldrich), and fixed and permeabilized 3 min in -20°C methanol and then 5 s in cold acetone. After washing in PBS, the slides were blocked in PBS containing 1% BSA and incubated 45 min with mouse mAb to LIME (LIME-1, 50 $\mu\text{g}/\text{ml}$), followed by 45 min incubation with Alexa 488 goat anti-mouse IgG (Molecular Probes, 500 \times diluted). Nuclei were stained with propidium iodide (Molecular Probes, 10 min, 0.5 $\mu\text{g}/\text{ml}$). The samples were mounted in PBS and viewed on a Laserscan microscope (Leica TCS SP). Incubation with irrelevant primary antibody and staining of nontransfected J77 cells served as a negative control.

Cell Activation. Peripheral blood T cells were stimulated by 2 min incubation with anti-CD3 (MEM-92) or anti-CD4 (MEM-16) IgM mAbs (~ 10 $\mu\text{g}/\text{ml}$) at 37°C . In some experiments, a combination of anti-CD4 mAbs (MEM-16 + MEM-115 [IgG] + MEM-241 [IgG]; ~ 10 $\mu\text{g}/\text{ml}$ each) for 2 min at 37°C was used. When IgG mAbs alone were used (anti-CD4 [MEM-241], anti CD8 [MEM-31]), the cells were first preincubated 30 min on ice with the primary antibody (~ 10 $\mu\text{g}/\text{ml}$), washed with ice cold medium, and stimulated by cross-linking with 10 $\mu\text{g}/\text{ml}$ goat anti-mouse polyclonal antibody (ICN) for 2 min at 37°C . The cells were then lysed, subjected to LIME immunoprecipitation, and LIME phosphorylation was determined by anti-phosphotyrosine (P-Tyr) immunoblotting. In some experiments the cells were pretreated for 7–20 min with 2.5–10 $\mu\text{g}/\text{ml}$ of the Src kinase family inhibitor PP2 (Calbiochem).

For long term activation, culture dishes were precoated with purified sterile anti-CD3 mAb MEM-57 (IgG2a; final concentration 10 μg per ml of 0.1 M Tris, pH 9.5) for 4 h at 37°C . Dishes were washed three times with RPMI medium without serum, and 5 million T cells, resuspended in 5 ml RPMI supplemented with FCS and 500 IU IL-2 (Chiron), were seeded per 5-cm dish. Cells were then cultured, harvested, and lysed at indicated time points. Where indicated, cells were removed from the anti-CD3-coated dish, washed, and cultured in a new uncoated dish in the

presence of IL-2 (100 IU/ml). For phytohemagglutinin (PHA) stimulation, PBLs were cultured for 3 d in RPMI medium with 10% FCS and PHA (Wellcome Reagents; commercial stock solution diluted for optimal activity 50 \times).

To measure Ca^{2+} response to anti-CD3 activation, J77 cells were electroporated either with FLAG-tagged LIME or NTAL expression constructs or with an empty pEFIRES-N vector. After 48 to 72 h in culture, the cells were transferred to Hepes-buffered Hank's balanced salt solution with 1% FCS and loaded with Fluo-4 and Fura Red dyes (Molecular Probes, each 1 $\mu\text{g}/100$ μl containing 10^6 cells) for 20 min at room temperature. The cells were washed in the same buffer incubated for 10 min at room temperature and then warmed for 10 min at 37°C . The measurement was performed on the FACSsort™ flow cytometer (Becton Dickinson) in a heated holder (37°C); after measuring the baseline, the anti-CD3 IgM mAb MEM-92 (10⁵ diluted ascitic fluid) was added, and eventually, when the response subsided the calcium ionophore ionomycin (Molecular Probes) was used to determine the absolute maximal response. The analysis was performed using the FlowJo program (Tree Star, Inc.). From a kinetic curve constructed from ratios of Fluo-4 to FuraRed fluorescence, the average baseline, the maximum after anti-CD3 stimulation, and the average response to ionomycin were deduced.

Results

LIME, A Novel Lipid Raft-Associated Transmembrane Adaptor Protein. Public databases were searched for proteins possessing the following structural features: (a) an NH_2 -terminal peptide consisting of 5–50 amino acids followed by a transmembrane helix; (b) a palmitoylation motif (CxxC) starting 0–4 amino acids downstream from the transmembrane helix; and (c) at least one tyrosine-based motif (Yxx[I/L/V]) in the COOH-terminal (presumably cytoplasmic) portion of the protein. This approach yielded a number of candidate proteins, most of them being false positives (e.g., proteins with NH_2 -terminal signal peptide). However, the search identified also the already known transmembrane adaptors PAG/Cbp and NTAL/LAB. Among several other potentially interesting molecules we identified a protein carrying the GenBank/EMBL/DDBJ accession no. BAA91148. BLAST search revealed that the sequence of its murine homologue, LIME (standing for "Lck interacting molecule") is also available under GenBank/EMBL/DDBJ accession no. AAG35210. Although Hur et al. (18) submitted the sequence to the database already in 1998, no biochemical or functional characterization of LIME has been reported up to now.

The human LIME cDNA codes for a strongly basic polypeptide (predicted pI 9.7) of 295 amino acid residues and a predicted molecular weight of 31,229 daltons (Fig. 1). The leaderless type III protein consists of a very short NH_2 -terminal extracellular peptide (4 aa), a single putative hydrophobic transmembrane domain (23 aa), which is followed by a potential palmitoylation site (a CxxC motif) and two arginine residues. The predicted cytoplasmic domain contains a total of five tyrosines, all potentially phosphorylated by Src family kinases. Two of the tyrosine-based signaling motifs (Y²⁰⁰ and Y²³⁵, respectively) potentially represent immunoreceptor tyrosine-based inhibition motifs.

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1  MGLP[VSWAPP ALWVLGCCAL LLSLWAI]CTA CRRPEDAVAP
41  RKRARRQRAR LQGSATAAEA SLLRRTHLCS LSKSDTRLHE
81  LHRGPRSSRA LRPASMDLLR PHWLEVS RDI TGPQAAPSAF
121  PHQELPRALP AAAATAGCAG LEATYSNVGL AALPGVSLAA
161  SPVVAEYARV QKRKGTHRSP QEPQQGKTEV TPAAQVDVLY
201  SRVCKPKRRD PGPTTDPLDP KGQGAILALA GDLAYOTLPL
241  RALDVIDSGPL ENVYESIREL GDPAGRSSTC GAGTPPSSC
281  PSLGRGWRPL PASLP
    
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Figure 1. Predicted amino acid sequence of human LIME. The putative transmembrane region is boxed, and the potential palmitoylation sequence and tyrosine motifs are in bold and underlined.

LIME transcripts were detected by PCR in cDNA prepared from PBLs, various lymphoid tissues, and liver but not other tissues (Fig. 2, A and B). In Western blotting experiments, both polyclonal and monoclonal antibodies directed to COOH-terminal fragments of LIME recognized a zone of the appropriate size in peripheral blood T cells, which strongly increased in intensity after overexpression of LIME (Fig. 2 C). Strong expression of LIME was also observed in purified CD56⁺ cells (mixture of NK cells and NK T cells). However, when further separated NK cells (CD3-negative) were found to express only little LIME compared with NK T cells (unpublished data). Similarly,

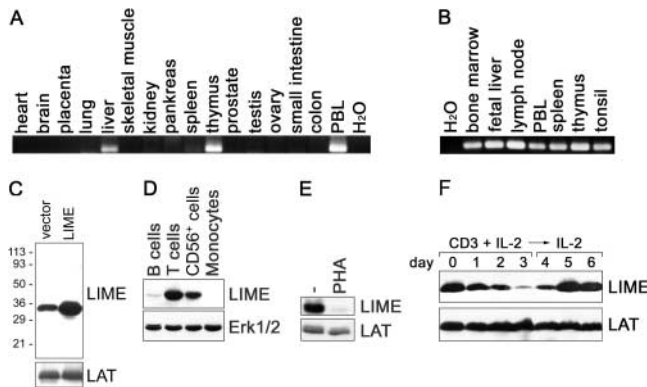


Figure 2. Expression of LIME. (A) Presence of LIME mRNA in various tissues as detected by PCR from the Multiple Tissue cDNA Panels I and II (CLONTECH Laboratories, Inc.) and (B) the Immune System Panel (CLONTECH Laboratories, Inc.). (C) Western blotting of vector- and LIME-transfected peripheral blood T cells (immunostaining for LIME and LAT; the latter was used as a loading control). (D) Western blotting of indicated subpopulations of human peripheral blood cells (immunostaining for LIME and Erk1/2; the latter was used as a loading control). (E) Western blotting of resting and PHA-activated peripheral blood T cells (immunostaining for LIME and LAT; the latter was used as a control). (F) Kinetics of LIME downmodulation in peripheral blood T cells stimulated by immobilized anti-CD3 mAb and LIME reexpression after removal of the cells from anti-CD3-coated wells (after day 3) and further culturing in the presence of IL-2. Cell lysates were analyzed by Western blotting for the presence of LIME and LAT. The same results were obtained when IL-2 was not added to the culture medium (not depicted).

purified blood B lymphocytes express only low amounts of LIME, and the molecule seems to be absent in monocytes (Fig. 2 D). In addition, all thymocyte subsets (double negative, double positive, single positive) contained comparable amounts of LIME, and no marked differences in the level of LIME expression could be detected in purified subsets of peripheral blood T cells (CD4⁺, CD8⁺, CD45RA⁺, CD45RO⁺, CD4⁺CD25⁺, CD4⁺CD25⁻, $\gamma\delta$ T cells; not depicted). Among the T cell lines tested (Jurkat, HPB ALL, HUT-78, SupT1, Molt-4, and CEM), only Jurkat cells are essentially negative for LIME protein expression (not depicted). Expression of LIME strongly decreased when peripheral blood T cells were activated *in vitro* for several days using PHA or plastic-immobilized CD3 mAb (Fig. 2, E and F). When the stimulated T cells were transferred in new culture dishes and cultivated further in the absence of external stimuli, LIME became rapidly and strongly reexpressed (Fig. 2 F). These data suggest that expression of LIME in peripheral blood T cells is controlled by external stimuli mediated via the TCR (or other PHA-responsive cell surface receptors).

As shown in Fig. 3, LIME is mostly present in buoyant lipid rafts (Fig. 3 A) and can be biosynthetically labeled by ³H-palmitate (Fig. 3 B), indicating that the membrane-proximal putative palmitoylation motif is used for targeting the protein into lipid rafts. Moreover, LIME clearly localizes to the plasma membrane as judged from confocal microscopy of Jurkat T cells stably overexpressing the molecule (Fig. 3C). Collectively, these data suggest that LIME represents an integral membrane protein associated with lipid rafts.

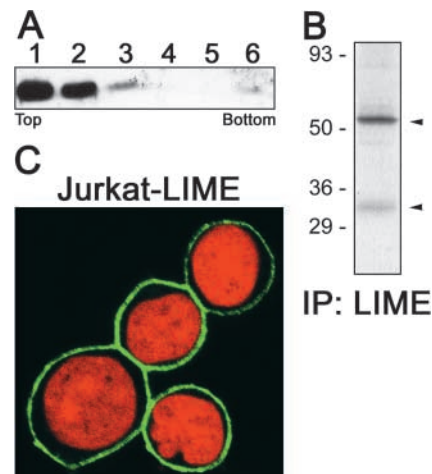
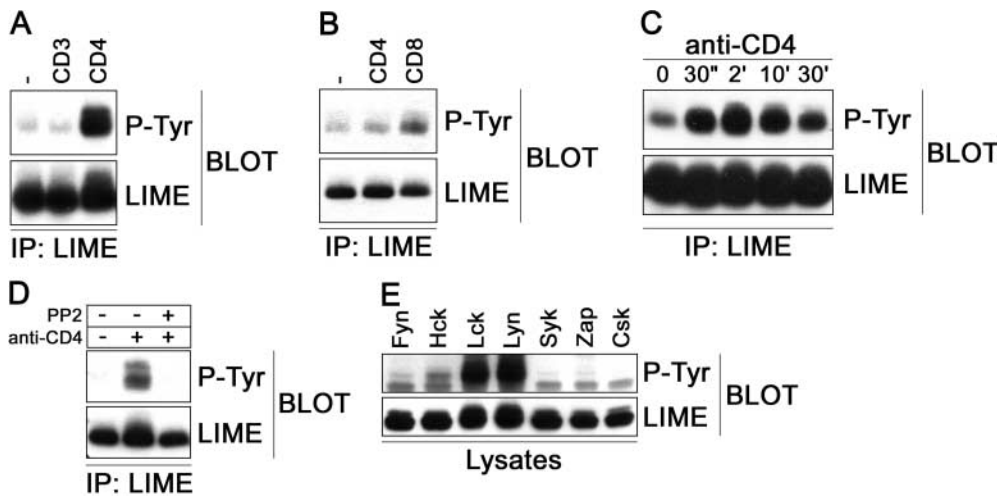


Figure 3. Subcellular localization of LIME. (A) Localization of LIME in buoyant detergent-resistant microdomains (lipid rafts). Peripheral blood T cells were solubilized in the presence of 3% nonionic detergent Brij-58 and subjected to sucrose density gradient ultracentrifugation; the fractions (numbered from top to bottom) were analyzed by Western blotting. (B) Biosynthetic labeling of LIME with ³H-palmitate; LIME immunoprecipitate was analyzed by SDS-PAGE followed by fluorography of the gel (the 56-kD zone corresponds probably to associated Lck). (C) Plasma membrane localization of LIME (green) as determined by confocal microscopy in J77-LIME.



or LIME (bottom). (D) Inhibition of anti-CD4-induced LIME phosphorylation in T cells by the inhibitor of Src kinases PP2. LIME immunoprecipitates were analyzed by Western blotting for P-Tyr and LIME. (E) Kinases phosphorylating LIME were examined by cotransfection of constructs encoding LIME, and the indicated kinases in 293T cells; total cell lysates were analyzed by Western blotting using antibodies to P-Tyr (top) or LIME (bottom). Expression of all the kinases was verified separately (not depicted).

Engagement of the CD4 or CD8 Coreceptors Induces Tyrosine Phosphorylation of LIME and Its Association with Protein Tyrosine Kinases Lck and Csk. The overall similarity between LIME, LAT, PAG/Cbp, and NTAL/LAB indicated that LIME might be inducibly tyrosine phosphorylated after ligation of some cell surface receptor(s). To identify such receptors, a broad screening involving antibody-mediated cross-linking of multiple lymphocyte surface molecules was performed. This approach revealed that LIME becomes tyrosine-phosphorylated exclusively after antibody-mediated engagement of the CD4 or CD8 coreceptors (Fig. 4) but not after triggering CD2, CD3, CD5, CD7, LFA-1, CD43, CD45, or MHC class I (not depicted). Cocross-linking of CD4 and CD3 consistently produced lower levels of LIME tyrosine phosphorylation than CD4 cross-linking alone (not depicted).

As shown in Fig. 4 C, after cross-linking of CD4 phosphorylation of LIME was maximal after 2 min and slowly declined thereafter. LIME became also tyrosine phosphorylated after cross-linking of CD4 on the surface of anti-CD3 activated, IL-2-propagated T cell blasts (not depicted).

To determine which protein tyrosine kinases (PTKs) might be responsible for phosphorylation of LIME, we treated human peripheral blood T cells with the Src family PTK inhibitor PP2 before CD4 cross-linking. As shown in Fig. 4 D, pretreatment with PP2 strongly suppressed the CD4-mediated tyrosine phosphorylation of LIME. In agreement with this result, coexpression of LIME with the protein tyrosine kinases Fyn, Lck, Lyn, Hck, Csk, Syk, or ZAP-70 in 293T-cells revealed that LIME represents a substrate for PTKs of the Src family, in particular Lck or Lyn (Fig. 4 E).

The next series of experiments was performed to assess molecular interactions between LIME and other signaling molecules expressed in T cells. These experiments indicated that the Src family kinase Lck and the negative regu-

lator of Src kinases, the Csk kinase, specifically associate with phosphorylated LIME in CD4-stimulated T lymphocytes (Fig. 5 A). Analysis of the LIME immunoprecipitates using phosphospecific antibodies further suggested that LIME-associated Lck was phosphorylated on both the COOH-terminal inhibitory and the activating tyrosine residues (Y⁵⁰⁵ and Y³⁹⁴, respectively; Fig. 5 A). The inducible association of LIME with PTK activity was also demonstrated by an *in vitro* kinase assay using LIME immunoprecipitates prepared from unstimulated or anti-CD4-stimulated T cells (Fig. 5 B).

Strikingly, when stably expressed in the CD4-positive Jurkat T cell line (J77), LIME displayed constitutive strong tyrosine phosphorylation and bound Lck, Csk, and also Fyn (Fig. 6) even without CD4 cross-linking; constitutive tyrosine phosphorylation of LIME was observed also in all

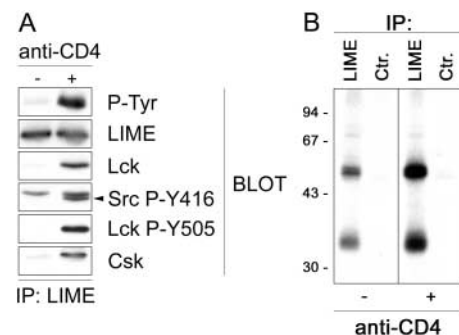


Figure 5. Proteins associated with phosphorylated LIME. (A) LIME immunoprecipitates prepared from unstimulated (left) or anti-CD4-stimulated (right) T cells (solubilized under raft-dissociating conditions) were analyzed by Western blotting using antibodies to the indicated molecules. (B) *In vitro* kinase assay of the LIME immunoprecipitates obtained from unstimulated and anti-CD4-stimulated T cells; as a negative control (Ctr.) an irrelevant antibody (anti-CD20) was used for the immunoprecipitation.

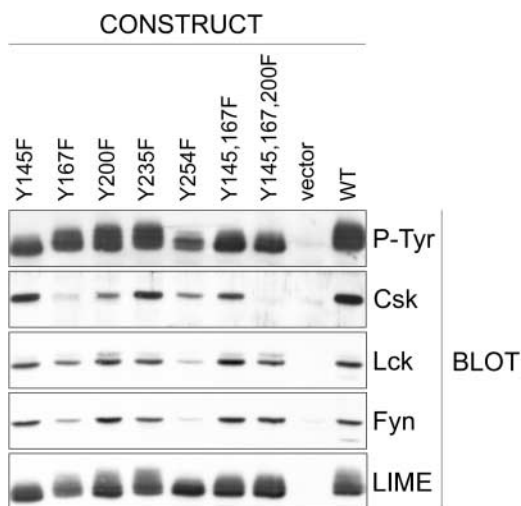


Figure 6. Identification of the P-Tyrs involved in binding of Lck, Fyn, and Csk. LIME immunoprecipitates from lysates of J77 cells stably transfected with the indicated Tyr to Phe mutants of LIME were analyzed by Western blotting for the presence of the indicated molecules.

T cell lines expressing LIME naturally [unpublished data]). Moreover, the level of LIME phosphorylation and its association with Lck and Csk, respectively, did not change significantly in these transfectants after CD4 cross-linking. However, brief pretreatment of these transfectants with the Src family PTK inhibitor PP2 led to a substantial decrease of the constitutive phosphorylation of LIME; in such pretreated cells, LIME rephosphorylation could be achieved by cross-linking of CD4 by antibodies or recombinant HIV-derived gp120 (not depicted). The reason for the constitutive tyrosine phosphorylation of LIME in J77 is unclear at present but might be due to a deregulation of the

membrane-proximal signaling pathways in the transformed cells (see Discussion).

To assess which tyrosine residues of LIME mediate its interaction with Lck and Csk, respectively, a series of stable J77 transfectants were generated expressing tyrosine mutants of LIME. It could be demonstrated that Y²⁵⁴ represents the binding site for Lck (and Fyn) (Fig. 6). Indeed, this site corresponds to a high stringency binding site for Lck and Fyn as predicted by the scansite program (<http://scansite.mit.edu>) (19). In contrast, phosphorylated Y¹⁶⁷ and potentially also Y²⁰⁰ appear to mediate the interaction between LIME and Csk. These tyrosine-based signaling motifs readily fit the consensus sequence that is found in various Cbps and the consensus sequence selected from peptide libraries (Table I).

Further analysis of the J77-LIME transfectant indicated that the amounts of lipid rafts-associated Lck and Csk are either slightly (in the case of Lck) or strongly increased (in the case of Csk) in these cells compared with WT J77 cells (Fig. 7 C). These data confirm that tyrosine-phosphorylated LIME is capable of targeting Lck and Csk to lipid rafts.

The data shown in Fig. 7 A demonstrate that in J77-LIME transfectants the LIME-associated fraction of Lck is more strongly phosphorylated on the inhibitory tyrosine Y⁵⁰⁵ compared with the total pool of Lck. In addition, an *in vitro* kinase assay of Lck immunoprecipitates prepared from J77-LIME cells results in a much stronger phosphorylation of Lck than in WT J77 cells (Fig. 7 B). Furthermore, LIME-associated Lck was even stronger phosphorylated than Lck immunoprecipitated from the total pool of Lck in the J77-LIME cells (Fig. 7 B). This could be either due to a higher enzymatic activity of the LIME-associated Lck (resulting in a higher level of autophosphorylation) or due to an increased phosphorylation of Lck by LIME-associated Csk.

Table I. Comparison of Published Csk Binding Sequences

Protein name and binding site position	Csk binding sequence	Reference
LIME Y ¹⁶⁷	V A E Y <u>A</u> R <u>V</u> <u>Q</u> <u>K</u> R K	This paper
LIME Y ²⁰⁰	D V <u>L</u> Y <u>S</u> R <u>V</u> <u>C</u> <u>K</u> P K	This paper
Paxillin Y ¹¹⁸	E H <u>V</u> Y <u>S</u> F P <u>N</u> <u>K</u> Q K	39
Caveolin Y ¹⁴	G H <u>L</u> Y <u>T</u> V P I <u>R</u> E Q	40
PTP-HSCF Y ³⁵⁴	A D T Y <u>A</u> V <u>V</u> <u>Q</u> <u>K</u> R G	41
PTP-HSCF Y ³⁸¹	T P I Y <u>S</u> Q <u>V</u> A P R A	41
IGF-IR Y ⁹⁴³	G V <u>L</u> Y <u>A</u> S <u>V</u> <u>N</u> P E Y	42
IGF-IR Y ¹³¹⁶	R Q P Y <u>A</u> H <u>M</u> <u>N</u> G G R	42
InsulinR Y ¹³²²	H I P Y <u>T</u> H <u>M</u> <u>N</u> G G K	42
p62dok Y ⁴⁴⁹	S A <u>L</u> Y <u>S</u> Q <u>V</u> <u>Q</u> <u>K</u> S G	43
PAG Y ³¹⁷	S A <u>M</u> Y <u>S</u> S <u>V</u> <u>N</u> <u>K</u> P G	9
SIT Y ¹⁶⁹	P E <u>L</u> Y <u>A</u> S <u>V</u> C A Q T	44
Hic-5 Y ⁶⁰	D H <u>L</u> Y <u>S</u> T <u>V</u> <u>C</u> <u>K</u> P R	45
Peptide library	Y [T/A/S][K/R/Q/N][M/I/V/R]	46

Critical tyrosines are bolded, other amino acids participating in the putative consensus motif are underlined.

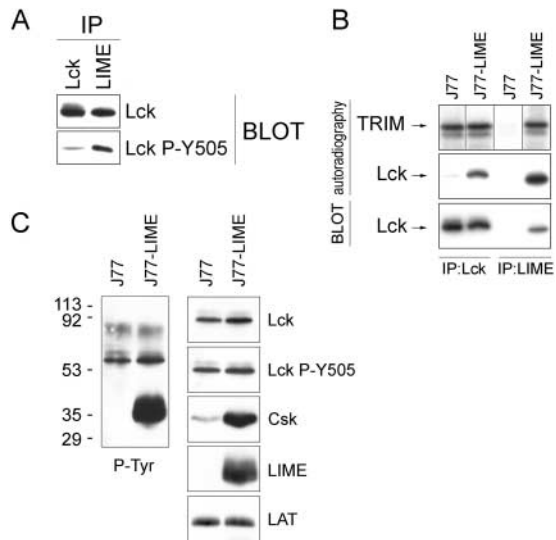


Figure 7. Effects of LIME on Lck and Csk in J77-LIME stable transfectants. (A) The concentration of directly immunoprecipitated Lck was adjusted to equate on the Western blot to the amount of Lck coprecipitated with LIME (top), and the blot was then stained with antibody specifically recognizing the phosphorylated Tyr⁵⁰⁵. (B) In vitro kinase assay was performed using purified cytoplasmic domain of TRIM protein as an exogenous substrate for Lck immunoprecipitated from J77-LIME transfectants or from cells transfected with an empty vector. The phosphorylated proteins in immunoprecipitates were also resolved to check content (immunoblotting) and phosphorylation status (autoradiography) of Lck. The dividing lines in the TRIM panel indicate that these lanes were not adjacent in the original gel, and the far right-hand lane is from a different gel, which was processed simultaneously. (C) Localization of Lck and Csk in lipid rafts of J77-LIME transfectants or cells transfected with an empty vector. Cells were solubilized in the presence of 3% nonionic detergent Brij-58 and subjected to sucrose density gradient ultracentrifugation; the fractions (numbered from top to bottom) were analyzed by Western blotting.

To distinguish between these possibilities, we assessed the capability of Lck to phosphorylate an exogenous substrate (the cytoplasmic domain of the transmembrane adaptor protein TRIM, which has been shown to be an excellent substrate for Lck in vivo [20]). As shown in Fig. 7 B (top), the ability of Lck to phosphorylate TRIM appears to be the same in J77-LIME cells and in J77 cells transfected with an empty vector. These data collectively support the hypothesis that the higher levels of tyrosine phosphorylation of LIME-associated Lck primarily result from phosphorylation of Y⁵⁰⁵ by the LIME-associated Csk. Moreover, they suggest that expression of LIME in Jurkat T cells leads to increased phosphorylation of Lck by Csk without dramatically altering the enzymatic activity of Lck. This finding can be best explained by the fact that the Src homology (SH)2 domain of Lck is not available for interaction with phospho-Y⁵⁰⁵ because it is engaged by phosphorylated LIME (Y²⁵⁴), and therefore, the closed inactive conformation of Lck cannot be formed.

LIME Increases Ca²⁺ Response After Stimulation of J77 Cells. Transient expression of LIME in J77 cell leads to a significantly higher Ca²⁺ response to anti-CD3 stimulation compared with cells transfected with an empty vector, whereas another raft-associated transmembrane adaptor

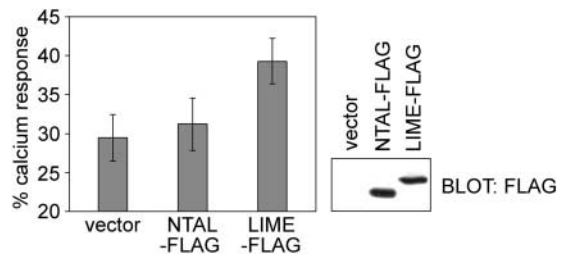


Figure 8. Effects of LIME on anti-CD3-induced Ca²⁺ response. J77 cells transiently transfected with the indicated constructs were stimulated by cross-linking of CD3. The results are presented as a proportion of the peak anti-CD3 response to the maximum response induced by Ca²⁺ ionophore ionomycin after correction for basal level. The results are representative of three independent experiments and show the mean and SD of four measurements. The observed increase is statistically significant at the 5% level (Student's *t* test). The right panel shows the comparable level of LIME and NTAL expression as determined by Western blotting and immunostaining of FLAG tag.

protein NTAL, expressed at the same level, does not change this response significantly (Fig. 8).

Discussion

Transmembrane adaptor proteins (TRAPs) are emerging as important molecules involved in coupling immunoreceptors to intracellular signaling pathways (1–3). Two types of TRAPs can be distinguished, namely those that are excluded from the rafts (TRIM [20], SHP2-interacting transmembrane adaptor [21], and linker for activation of X [LAX] [22]) and those that are targeted to rafts via juxtamembrane palmitoylation of a di-cysteine motif CxxC (LAT [7, 8], PAG/Cbp [9, 10], NTAL/LAB [11, 12], and LIME). The increasing number of the raft-associated TRAPs is in agreement with the currently recognized importance of membrane rafts in receptor signaling and other cellular processes (1–3). LAT and NTAL/LAB are involved in signaling initiated by immunoreceptors (TCR and BCR or Fc receptors, respectively [11, 12, 23]), whereas PAG/Cbp participates more generally in negative regulation of Src family kinases and thus indirectly controls immunoreceptor signaling (9, 10, 24–26).

LIME, the novel TRAP described herein, appears not to be a direct target of immunoreceptor-mediated signaling events. Rather, its phosphorylation and association with intracellular signaling and effector molecules (Lck and Csk) seems to be controlled in primary T cells by the coreceptors CD4 and CD8. Csk and Lck (plus Fyn) also associate with LIME when the molecule is expressed in the J77 Jurkat T cell line; notably, in these transfectants LIME is constitutively tyrosine phosphorylated, and its phosphorylation status apparently does not change upon CD4 (nor CD3) cross-linking. Importantly, the Lck molecules that associate with phosphorylated LIME are phosphorylated both on the COOH-terminal negative regulatory Y⁵⁰⁵ and on the positive regulatory Y³⁹⁴ and exhibit somewhat paradoxically an increased autophosphorylation activity. It seems likely that the inhibitory phospho-Y⁵⁰⁵ is unable to exert its inhibitory

function, since the Lck SH2 domain is bound to the phospho-LIME (see further discussion of this point below). Interestingly, despite the presence of two putative immunoreceptor tyrosine-based inhibition motifs in the cytoplasmic tail of LIME, so far we could not detect any SH2 domain containing phosphatase (SHP-1, SHP-2, or SHIP) in LIME immunoprecipitates prepared from activated (even pervanadate-treated) cells.

What are possible biological functions of this novel raft-associated adaptor? Induction of tyrosine phosphorylation and association with Lck and Csk after CD4 or CD8 cross-linking strongly indicates that LIME plays a role in regulation or propagation of coreceptor signaling.

The roles of the coreceptors (so far studied mainly for CD4) may be twofold, depending on whether the coreceptor is coengaged with the TCR or cross-linked separately. Cocross-linking of CD4 with the TCR generally potentiates TCR-mediated signaling events, obviously due to bringing the CD4-associated fraction of Lck into the proximity of the cytoplasmic tails of the CD3 complex and the ζ chains (27, 28). LIME might play a potentiating role in this physiological signaling. This view is supported by our observation that transient overexpression of LIME in J77 Jurkat T cells increases Ca^{2+} responses after CD3 stimulation. Moreover, when stably expressed in J77 cells LIME is expressed as a constitutively phosphorylated protein. This could correspond to the situation that occurs in primary T cells after coreceptor engagement.

In contrast to coengagement with the TCR, separate cross-linking of CD4 either by antibodies to suitable CD4 epitopes or by HIV gp120 is known to inhibit or markedly modify the outcome of the subsequent signaling induced by TCR engagement (29, 30). Moreover, in rodent models selective ligation of CD4 *in vivo* by certain antibodies results in remarkably robust alloantigen-specific immunosuppression and induction of specific transplantation tolerance, which is apparently mediated by regulatory T cells secreting suppressive cytokines (31, 32). Finally, CD4 signaling may also modify LFA-1-dependent adhesivity (33). Little is known about the molecular mechanisms underlying these inhibitory functions of CD4. However, CD4 cross-linking alone can induce signaling pathways similar to those triggered by TCR (activation of Lck and Fyn, initiation of the Ras-Raf-MAPK, and phospholipase $\text{C}\gamma$ -PKC- Ca^{2+} pathways, resulting in activation of the NFAT, NF κ B, and AP-1 transcription factors) (34, 35). The final result of signaling mediated solely via CD4 may be either induction of apoptosis or presensitization of T cells to CD3-mediated apoptosis (34, 36, 37).

It is tempting to speculate that LIME is involved in these inhibitory aspects of CD4 signaling: it may, for example, compete with CD4 for Lck and at the same time it may bring more Csk to membrane rafts (Fig. 7 C). This could eventually result in inhibition of Src family kinases, which are needed for TCR signaling. This model of LIME function is, however, not very well compatible with our observation that Lck isolated from J77-LIME transfectants and

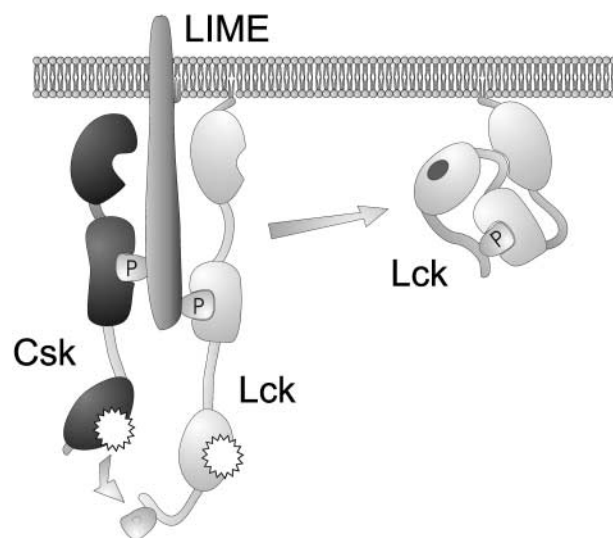


Figure 9. Hypothetical mechanism of LIME involvement in regulation of Lck activity.

LIME-associated Lck (which is more phosphorylated on the COOH-terminal inhibitory tyrosine) has a kinase activity comparable to Lck from control cells. Nevertheless, it is possible to propose the following model for LIME function (Fig. 9): cross-linking of CD4 induces aggregation or reorganization of lipid rafts containing LIME, CD4-Lck, and free Lck. By this, Lck (free or CD4-associated) is brought into proximity of LIME and phosphorylates the adaptor. Now, cytoplasmic Csk and free Lck bind to tyrosine-phosphorylated LIME via their SH2 domains. In the resulting complex, Csk phosphorylates Lck on Y⁵⁰⁵ (either on the same LIME adaptor or on a different LIME molecule in close proximity). However, the Lck phosphorylated on Y⁵⁰⁵ remains enzymatically active because its SH2 domain is bound to phospho-LIME and thus cannot be engaged by phospho-Y⁵⁰⁵ (with the consequence that the inactivating “closed” conformation of Lck cannot be formed). LIME-associated, “open” Lck could phosphorylate additional molecules thus propagating the CD4-mediated signal. In addition (or alternatively), phospho-Y⁵⁰⁵ of LIME-bound Lck could serve as a docking site for additional signaling proteins (including Lck or Fyn). Recruitment of these molecules to the LIME-Csk-Lck complex could further amplify the CD4-mediated signal. Subsequently, the phosphorylated Lck may dissociate from LIME, assume the “closed” conformation, and remain inactive in the lipid raft. A model in which LIME-associated Lck recruits and activates lipid raft-associated Fyn might explain the recent report that the activation of Fyn within the lipid microdomains is preceded (and requires) translocation of Lck to lipid rafts (38).

A remarkable feature of LIME is its downmodulation in T cells after stimulation with PHA or immobilized anti-CD3 antibodies and its reappearance after removal of the stimulating agents. A plausible explanation could be that down-regulation of LIME serves to limit CD3/TCR-

mediated activation processes by blunting coreceptor signaling. Such a mechanism could help to prevent hyperactivation of T cells that have already seen antigen and start to differentiate into effector T-lymphocytes.

In summary, we suggest that the newly described lipid raft-associated transmembrane adaptor protein LIME may have regulatory function(s) in T cells. At the beginning of an immune response where coreceptor signaling is required to activate T cells, LIME rapidly becomes tyrosine-phosphorylated, transiently increases the activity of Lck by blocking formation of its inactive conformation, and thus amplifies TCR-mediated signals. Upon prolonged stimulation, LIME helps to accumulate inhibited Lck in lipid rafts, thereby preventing hyperactivation of T cells. In the case of antigen persistence, T cells down-regulate expression of LIME. This may be a mechanism to prevent premature termination of the immune response by blunting membrane recruitment of enzymatically active Lck. Furthermore, one may speculate that strongly positive nonphosphorylated LIME may possibly interact with membrane phospholipids or other negatively charged cytoplasmic molecules (e.g., phosphoproteins) and thereby modify their interactions with other signaling molecules. To fully understand the role of LIME during T cell activation, it will be necessary to explore its possible functional interactions with PAG/Cbp, another raft-associated transmembrane adaptor that regulates the activity of Src kinases by recruiting Csk (10, 11). Moreover, more definitive answers regarding the physiological role of LIME in T cell activation should be provided by LIME knock-out mice, currently under construction.

We would like to thank Drs. R. Abraham, O. Acuto, S. Hobbs, W. Kolanus, G. Langsley, L. Samelson, A. da Silva, and S. Watson for providing us with cells and constructs as specified in Materials and Methods.

This work was supported from the project Center of Molecular and Cellular Immunology (LN00A026) of the Czech Ministry of Education, Youth and Sports, grant J1116W24Z from the Wellcome Trust to V. Hořejší, and grant SCH 533/6-1 from Deutsche Forschungsgemeinschaft to B. Schraven.

Submitted: 28 August 2003

Accepted: 10 September 2003

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