The Lck SH3 Domain Negatively Regulates Localization to Lipid Rafts through an Interaction with c-Cbl*

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Lck is a member of the Src family of protein-tyrosine kinases and is essential for T cell development and function. Lck is localized to the inner surface of the plasma membrane and partitions into lipid rafts via dual acylation on its N terminus. We have tested the role of Lck binding domains in regulating Lck localization to lipid rafts. A form of Lck containing a point mutation inactivating the SH3 domain (W97ALck) was preferentially localized to lipid rafts compared with wild type or SH2 domain-inactive (R154K) Lck when expressed in Lck-deficient J.CaM1 cells. W97ALck incorporated more of the radioiodinated version of palmitic acid, 16-[12]iodohexadecanoic acid. Overexpression of c-Cbl, a ligand of the Lck SH3 domain, depleted Lck from lipid rafts in Jurkat cells. Additionally, Lck localization to lipid rafts was enhanced in c-Cbl-deficient T cells. The association of Lck with c-Cbl in vivo required a functional SH3 domain. These results suggest a model whereby the SH3 domain negatively regulates basal localization of Lck to lipid rafts via association with c-Cbl.

Members of the Src family of tyrosine kinases such as Lck play an important role in transducing signals from the extracellular environment into the cell interior in many different cell types (1). Lck is expressed predominantly in T lymphocytes and is essential for T cell antigen receptor (TCR) signaling and T cell development (2, 3). Lck is localized to the plasma membrane because of its irreversible myristoylation on Gly-2 and reversible palmitoylation on Cys-3 and -5 (4–6). In addition, palmitoylation is necessary for targeting Lck to lipid rafts (5, 7, 8). Lipid rafts are subdomains within cellular membranes that are enriched in glycosphingolipids, cholesterol, and lipid-modified proteins (9). The unique lipid raft environment favors the partitioning of proteins carrying saturated acyl chains, such as myristate and palmitate, and glycosylphosphatidylinositol linkages (10, 11). Indeed, the first 10 amino acids of Lck containing the dual acylation site is sufficient for targeting cytosolic proteins to lipid rafts, indicating that this short motif is a lipid raft-targeting signal (12–14). Certain palmitoylated proteins are found constitutively enriched within lipid rafts in T cells such as Lck and the transmembrane adaptor protein LAT, although a significant portion of Lck remains excluded from rafts (5, 15). Other proteins are reported to be constitutively excluded from lipid rafts in T cells including c-Cbl and CD45 (4, 15). Upon TCR cross-linking, non-lipid-modified proteins such as the c chain of the TCR and the cytoplasmic proteins ZAP-70, phospholipase C-γ, Vav, Grb2, phosphatidylinositol 3-kinase, and SLP-76 are recruited to lipid rafts (15, 16) suggesting that protein-protein interactions are directing the localization of these proteins to rafts. Importantly, the localization of Lck and LAT to rafts is needed for effective TCR signaling (5, 15, 17). Moreover, disruption of lipid rafts by pharmacological agents abrogates TCR signaling (18). These observations suggest that lipid rafts play a regulatory role in TCR activation by selectively concentrating certain molecules while excluding others, thereby forming a platform for coordinating signal transduction events (4, 19–21).

Similar to other members of the Src family, Lck has an N-terminal membrane targeting motif (SH4 domain), single SH3 and SH2 domains followed by a C-terminal tyrosine kinase or SH1 domain. SH3 domains bind to proline-rich sequences, whereas SH2 domains bind to phosphotyrosine-containing sequences. The Lck SH3 domain has been reported to bind to a number of cellular proteins including phosphatidylinositol 3-kinase, Ras-GAP, HSP70, SLP-76, CD2, Cdc2, mitogen-activated protein kinase, and Sam-68 (22–29). In addition, Lck binds c-Cbl in vitro (30), and the Lck SH3 domain binds c-Cbl in vitro (30–34). The Lck SH3 domain is required in TCR downstream signaling events including activation of the mitogen-activated protein kinase pathway (32) and in T cell activation by the costimulatory receptors CD28 and CD48 (31, 35, 36). However, the molecular mechanisms by which the Lck SH3 domain functions in T cell signaling are not yet clear.

One potential binding protein for the Lck SH3 domain is c-Cbl. The proto-oncogene product c-Cbl is a multifunctional protein that binds to numerous signaling molecules and participates in a number of tyrosine kinase signaling pathways (37–39). c-Cbl is a 120-kDa protein that consists of several domains including a proline-rich C-terminal region that binds constitutively to the SH3 domains of several members of Src family kinases. Biochemical and genetic analyses have indicated that c-Cbl negatively regulates tyrosine kinases, including the Src family kinases, in part by promoting their ubiquitination and subsequent degradation. c-Cbl becomes highly...
phosphorylated upon TCR stimulation. Additionally, TCR signaling is negatively regulated by c-Cbl as evidenced by inhibition of the induction of the transcription factors AP1 and NFAT upon c-Cbl overexpression (40) and by enhanced activation of ZAP-70 upon loss of c-Cbl (41, 42). More recently, c-Cbl was shown to promote ubiquitination of the TCR β chain (43) as well as ubiquitination and degradation of the Lck homologue Fyn (44). Consistently, T cells from mice lacking c-Cbl exhibited elevated expression of TCR, CD3, CD4, CD5 and CD69, and enhanced activation upon TCR stimulation (41, 42). On the other hand, positive signaling roles for c-Cbl also have been reported (45–48).

Although the palmitoylation of Lck is essential for its localization to lipid rafts, it is not clear whether protein-protein interactions also contribute to the regulation of Lck localization. We have examined the role of the Lck binding domains in its localization by expressing domain-inactive mutants of Lck in J.CaM1 T cells. The Lck SH3 domain-inactive mutant was preferentially localized to lipid rafts and incorporated more of the radioiodinated version of palmitic acid, 16-[^125]Iiodohexadecanoic acid. Furthermore, the localization of Lck to lipid rafts was diminished in c-Cbl overexpressing Jurkat cells and was enhanced in c-Cbl knockout T cells. Lck binding to c-Cbl in vivo was inhibited by inactivation of the Lck SH3 domain. Our results suggest that the interaction of c-Cbl with the SH3 domain of Lck is important for regulation of the basal localization of Lck to lipid rafts.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**—The human leukemia T cell line Jurkat (clone E6.1) and the Lck-deficient mutant of Jurkat (J.CaM1) were from American Type Culture Collection. c-Cbl-deficient (206/−−) and the corresponding c-Cbl-expressing (230/−−) murine T cell lines (44) were generously provided by Dr. Hamid Band (Harvard Medical School, Boston). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 μg 2-mercaptoethanol, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. The Lyn-deficient DT-40 chicken B cells were grown and maintained as described (49). The following antibodies were used: monoclonal anti-Lck, polyclonal anti-CD48, and polyclonal anti-ERK1/2 were from Santa Cruz Biotechnology; polyclonal anti-LAT was from Upstate Biotechnology, Inc.; monoclonal anti-c-Cbl and monoclonal anti-CD4 were from Sigma. Monovalent antibodies to protein A-Sepharose (Sigma). Immunoprecipitates were washed 3 times with RIPA buffer. Samples were dissolved in SDS sample buffer and separated by SDS-PAGE. Gels were dried, and radiolabeled proteins were detected by autoradiography. Lck palmitoylation levels were quantified using a ChemiImager 5500 (Alpha Innotech Corp.).

**Lipid Raft Patching and Fluorescence Microscopy**—For all fluorescence microscopy experiments, cells were washed in serum-free RPMI and attached to coverslips precoated with polylysine (100 μg/ml) by incubation for 10 min at room temperature in a concentration of 5 × 10⁶ cells/coverslip. For fixation, cells were treated with 3.7% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. Lipid raft or GM1 patching was performed as described previously (50). Briefly, transfected J.CaM1 cells (1 × 10⁶) were incubated for 30 min on ice with 100 μg of rhodamine-conjugated cholera toxin B-subunit (CT-B) at 10 μg/ml in phosphate-buffered saline containing 0.1% bovine serum albumin. Lipid raft patching was induced by incubating the cells with anti–CT-B antibody (1:250 in phosphate-buffered saline with 0.1% bovine serum albumin) for 30 min on ice and then 15 min at 37°C. Samples were examined by fluorescence microscopy as described previously (51).

**Lytic Cytotoxicity Assay**—The human leukemia T cell line (clone E6.1) and the Lck-deficient mutant of Jurkat (J.CaM1) were from American Type Culture Collection. c-Cbl-deficient (206/−−) and the corresponding c-Cbl-expressing (230/−−) murine T cell lines (44) were generously provided by Dr. Hamid Band (Harvard Medical School, Boston). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 50 μg 2-mercaptoethanol, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. The Lyn-deficient DT-40 chicken B cells were grown and maintained as described (49). The following antibodies were used: monoclonal anti-Lck, polyclonal anti-CD48, and polyclonal anti-ERK1/2 were from Santa Cruz Biotechnology; polyclonal anti-LAT was from Upstate Biotechnology, Inc.; monoclonal anti-c-Cbl and monoclonal anti-CD4 were from Sigma. Monovalent antibodies to protein A-Sepharose (Sigma). Immunoprecipitates were washed 3 times with RIPA buffer. Samples were dissolved in SDS sample buffer and separated by SDS-PAGE. Gels were dried, and radiolabeled proteins were detected by autoradiography. Lck palmitoylation levels were quantified using a ChemiImager 5500 (Alpha Innotech Corp.).

**RESULTS**

**Role for the Sh3 Domain in Regulating Localization of Lck to Lipid Rafts**—Lipid rafts in T cells differentially concentrate signaling proteins. Initially, we tested the distribution of Lck in lipid rafts from resting Jurkat cells. Cells were washed, lysed,
and subjected to sucrose density gradient centrifugation to isolate lipid raft and Triton X-100-soluble fractions. Fractions 1–12 were subjected to SDS-PAGE and probed to detect Lck, LAT, and c-Cbl or probed with horse-radish peroxidase-conjugated cholera toxin to detect the ganglioside GM1. B, Lck-deficient J.CaM1 cells were transiently transfected with empty vector (Mock) or transfected with expression constructs encoding wild type Lck (WT), SH2-inactive Lck (R154K), or SH3-inactive Lck (W97A). Transfected cells (5 × 10⁶) were lysed in 1% Triton X-100 lysis buffer at 4 °C and were subjected to sucrose gradient ultracentrifugation to purify lipid rafts. Fractions 1–12 from cells transfected with Lck variants were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Lck antibody. C, the pooled lipid raft fractions (fractions 2 and 3) and the pooled Triton X-100-soluble fractions (fractions 10 and 11) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Lck antibody or with anti-c-Cbl antibody as a control for equal loading and lipid raft purity. To assess the expression levels of Lck constructs, transfected cells (2 × 10⁶) were lysed in 1% Triton X-100, and lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Lck antibody and with anti-c-Cbl antibody as a loading control. The results shown are representative of five experiments.

The partitioning of Lck between lipid raft and Triton X-100-soluble fractions is potentially regulated through posttranslational modifications (e.g. palmitoylation or phosphorylation), protein-protein interactions, or a combination of both. The palmitoylation of Lck is essential for targeting Lck to the plasma membrane (5, 7), but it is unknown whether protein-protein interactions play a role in the regulation of Lck localization within the plasma membrane. We used the Lck-deficient T cell line, J.CaM1, derived from Jurkat cells, to examine the contribution of the Lck SH2 and SH3 domains to the localization of Lck. Constructs encoding the wild type (WT Lck), SH3 domain-inactive (W97ALck) (32), or SH2 domain-inactive (R154KLck) (53) forms of Lck were transiently expressed and analyzed for localization to lipid rafts. As shown in Fig. 1B, W97ALck was enriched in lipid rafts as compared with WT Lck and R154KLck. To compare further the distribution of Lck variants, we pooled fractions containing lipid rafts (fractions 2 and 3) and fractions representing Triton X-100-soluble lysates (fractions 10 and 11). W97ALck was preferentially localized to lipid rafts compared with WT Lck and R154KLck (Fig. 1C, left panel). Densitometric analysis indicated that W97ALck was 2–3-fold enriched in rafts compared with WT Lck and R154KLck (Fig. 1C, middle panel). Immunoblotting of whole cell lysates verified equal expression levels of wild type and mutant Lck (Fig. 1C, right panel). These results showed that inactivation of the SH3 domain resulted in enhanced Lck partitioning to lipid rafts suggesting that the SH3 domain negatively contributes to the localization of Lck within lipid rafts. In contrast, the SH2 domain does not appear to play a dominant role in this localization.
W97ALckGFP Preferentially Colocalizes with Lipid Raft Patches—We next investigated the role of the SH3 domain in the subcellular localization of Lck in intact cells. To accomplish this, we generated expression constructs with the cDNA encoding W97ALck and R154KLck fused upstream from the cDNA for green fluorescent protein (GFP). It was demonstrated that GFP fused to the C terminus of Lck (WT LckGFP) does not affect Lck function and subcellular localization (50). Constructs encoding WT, R154K, or W97A LckGFP were transiently expressed in J.CaM1 cells and examined by fluorescence microscopy. Cells were attached to polylysine-coated coverslips, fixed, and examined by fluorescence microscopy. J.CaM1 cells transiently transfected with WT LckGFP or W97ALckGFP were incubated with rhodamine-conjugated cholera toxin B-subunit (CT-B) and treated by cross-linking with anti-CT-B antibody. Cells were attached to polylysine-coated coverslips, fixed, and examined by fluorescence microscopy. J.CaM1 cells transfected with WT LckGFP or W97ALckGFP were treated as in B. The percentage of cells showing overlap (complete colocalization) between LckGFP and rhodamine CT-B signals was determined. Between 40 and 100 transfected cells showing lipid raft patching were counted from five independent experiments. The results shown are representative of eight experiments.

WT LckGFP, we used the Lyn-deficient DT40 B cell line. As shown in Fig. 2A, right panel, W97ALckGFP exhibited a similar intracellular localization as in J.CaM1 cells. Analysis by confocal microscopy confirmed the above results and showed that both WT LckGFP and W97ALckGFP were not localized inside the nucleus of J.CaM1 cells (data not shown). These results indicate that inactivation of the SH3 domain did not affect the localization of Lck to the plasma membrane but appeared to minimize its localization to late endocytic vesicles.

We next compared the extent of colocalization of W97ALckGFP and WT LckGFP with lipid rafts using rhodamine-conjugated cholera toxin B-subunit (CT-B), which binds with high affinity to the lipid raft constituent GM1 (54). J.CaM1 cells transiently expressing WT LckGFP or W97ALckGFP were incubated with CT-B followed by cross-linking with anti-CT-B antibody. Cells were washed, fixed onto coverslips, and examined by fluorescence microscopy. Signifi-
cantly, W97ALckGFP showed enhanced colocalization with lipid raft patches as compared with WT LckGFP (Fig. 2B, merged images, right panel). We observed that increasing the time of treatment with the anti-CT-B antibody leads to increased colocalization of WT LckGFP with lipid raft patches (data not shown). We quantified the fraction of transfected cells that showed complete co-clustering of the LckGFP proteins and lipid raft patches. As shown in Fig. 2C, 94% of W97ALckGFP coclustered with lipid raft patches as compared with 13% of WT LckGFP. These results further support the conclusion that the SH3 domain negatively regulates Lck localization to lipid rafts.

Inactivation of the SH3 Domain Increased the Level of Incorporation of 16-\[^{125}\text{I}\]iodohexadecanoic acid into Lck—Several palmitoylated proteins are preferentially enriched in lipid rafts (10). Because W97ALck is predominantly localized to lipid rafts, we tested whether it would exhibit a different degree of palmitoylation. Therefore, we compared the incorporation of 16-\[^{125}\text{I}\]iodohexadecanoic acid into WT Lck and W97ALck. This radiiodinated version of palmitic acid has been used previously to study palmitoylation and offers considerable advantage in exposure time over commercially available \[^{3}\text{H}\text{palmitic acid (55).}^2\] J.CaM1 cells were transiently transfected with control vector, WT Lck, or W97ALck. Transfectants were incubated with 16-\[^{125}\text{I}\]iodohexadecanoic acid, and Lck was immunoprecipitated and detected by autoradiography. As can be seen in Fig. 3, elevated levels of the radioactive probe were incorporated into W97ALck compared with WT Lck. Densitometric analysis indicated that W97ALck incorporated ~10-fold more of the radioactive probe compared with WT Lck. The specificity of the immunoprecipitation was confirmed using preimmune rabbit serum as a control. Immunoblot analysis of the immunoprecipitates confirmed equal levels of WT Lck and W97ALck (Fig. 3, lower panel).

Overexpression of c-Cbl, a Reported Ligand of the Lck SH3 Domain, Results in Depletion of Lck from Lipid Rafts—The enhanced localization of W97ALck to lipid rafts suggested that binding partners of the SH3 domain of Lck contributed to the regulation of Lck localization. The Lck SH3 domain has been reported to bind to several proteins including c-Cbl (30–34). Because c-Cbl was found exclusively outside lipid rafts in our preparations from Jurkat cells, it was a potential candidate for keeping Lck in the non-raft region of the plasma membrane. To test this possibility, we transfected an expression construct encoding HA-tagged c-Cbl into Jurkat cells and examined the localization of Lck as described above. As shown in Fig. 4A (upper panel), the localization of Lck to lipid rafts was decreased in HA-c-Cbl-transfected cells as compared with control cells. This depletion of Lck from lipid rafts was accompanied by a slight increase in the amount of Lck in Triton X-100-soluble fractions in HA-c-Cbl-transfected cells as compared with control cells (Fig. 4A, lower panel). Immunoblotting for the glycosylphosphatidylinositol-anchored protein CD48 confirmed that c-Cbl overexpression had no effect on the localization of CD48 in rafts (Fig. 4A, upper panel). Immunoblotting for ERK1/2 confirmed equal gel loading of the pooled Triton X-100-soluble fractions (Fig. 4A, lower panel). As can be seen in Fig. 4B, immunoblotting of whole cell lysates confirmed that equal amounts of Lck were present in the transfected cells and verified the expression of HA-c-Cbl. These results suggest that Lck interaction with c-Cbl can regulate Lck localization to lipid rafts.

Lck Localization to Lipid Rafts Is Enhanced in c-Cbl\(^{-/-}\) T Cells—To investigate further the role of c-Cbl in regulating the localization of Lck to lipid rafts, we used the c-Cbl-deficient T cell line (206\(^{-/-}\)), derived from c-Cbl knockout mice, and a corresponding c-Cbl containing control T cell line (230\(^{-/-}\)) (44). First we verified that similar levels of Lck were present in the two cell lines (Fig. 5B, right panel), because it had been reported that the 206\(^{-/-}\) cells contain more of the Src family member Lyn (44). To determine the localization of Lck in the two cell lines, lysates from 206\(^{-/-}\) and 230\(^{-/-}\) cells were subjected to sucrose density gradient centrifugation. The distribution of Lck in fractions 1–12 and in pooled fractions was determined as described above. Significantly, in c-Cbl\(^{-/-}\) cells, the presence of Lck in lipid rafts was enhanced 2-fold compared with c-Cbl-expressing cells (Fig. 5, A and B). We also observed a 1.6-fold decrease in the amount of Lck in the Triton X-100-soluble fraction in 206\(^{-/-}\) cells compared with 230\(^{-/-}\) cells. Immunoblotting for CD48 and ERK1/2 demonstrated that the distribution of these molecules between lipid rafts and the non-lipid raft region of the membrane was not altered by the absence of c-Cbl (Fig. 5B, left and middle panel). Immunoblot analysis of whole cell lysates confirmed the absence of c-Cbl in 206\(^{-/-}\) cells (Fig. 5B, right panel). The enhanced localization of Lck to lipid rafts in 206\(^{-/-}\) cells further supports the conclusion that the Lck-c-Cbl interaction negatively regulates Lck localization to lipid rafts.

SH3 Domain Is Required for the Association of Lck with c-Cbl—c-Cbl has been reported to bind the SH3 domain of Src family kinases. We therefore tested the requirement of the SH3 domain of Lck for association with c-Cbl in vivo. To accomplish this, J.CaM1 cells were transiently transfected with control vec-
Fig. 4. c-Cbl overexpression decreased Lck localization to lipid rafts. Jurkat cells were transfected with empty vector (Mock) or transfected with an HA-c-Cbl expression construct. After 20 h, cells were harvested. A, transfected cells (5 × 10⁵) were lysed in 1% Triton X-100 lysis buffer at 4 °C and were subjected to sucrose gradient ultracentrifugation to purify lipid rafts. The pooled lipid raft fractions (fractions 2 and 3) and the pooled Triton X-100 soluble fractions (fractions 10 and 11) were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Lck antibody, anti-CD48, or anti-ERK1/2 antibodies. B, transfected cells (2 × 10⁶) were lysed in 1% Triton X-100, and lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Lck and anti-c-Cbl antibodies or with anti-ERK1/2 antibody as a loading control. The results shown are representative of three experiments.

Fig. 5. Lck is preferentially localized to lipid rafts in c-Cbl⁻⁻⁻⁻ T cells. c-Cbl⁻⁻⁻⁻ T cells (206⁻⁻⁻⁻) (6.5 × 10⁷) and corresponding c-Cbl-expressing T cells (230⁻⁻⁻⁻) (10 × 10⁷) were lysed in 1% Triton X-100 lysis buffer at 4 °C, and lysates were subjected to sucrose gradient ultracentrifugation and SDS-PAGE analysis. A, fractions 1–12 were analyzed by probing for GM1 or Lck. B, the pooled lipid raft fractions (fractions 2 and 3) were analyzed by immunoblotting for Lck and CD48, and the pooled Triton X-100-soluble fractions (fractions 10 and 11) were analyzed by immunoblotting for Lck and ERK 1/2. To assess protein levels, equal volumes from 206⁻⁻⁻⁻ and 230⁻⁻⁻⁻ lysates were directly analyzed by immunoblotting with anti-Lck and anti-c-Cbl antibodies or with anti-ERK as a loading control (B, right panel). This experiment was performed twice with similar results.
We have identified a role for the SH3 domain in the regulation of Lck localization to lipid rafts, whereby this domain mediates Lck binding to the lipid raft-excluded protein c-Cbl. This conclusion is based on the following observations: (i) the SH3 domain-inactive W97ALck preferentially localizes to lipid rafts as compared to wild type Lck; (ii) W97ALck incorporates more of 16-[125I]iodohexadecanoic acid (125I) palmitic acid) in comparison with wild type Lck; (iii) overexpression of the Lck SH3 domain ligand c-Cbl depletes Lck from lipid rafts; (iv) Lck shows enhanced localization to lipid rafts in c-Cbl-deficient T cells; and (v) Lck associates with c-Cbl in an SH3 domain-dependent manner.

The observation that W97ALck is enriched in lipid rafts (Figs. 1 and 2) suggests that the SH3 domain negatively regulates the localization of Lck to lipid rafts in unstimulated Jurkat T cells by binding to a protein found outside lipid rafts. Inactivation of the SH2 domain does not affect basal localization of Lck to lipid rafts (Fig. 1), which is consistent with the role of the SH2 domain in mediating interactions following T cell activation. W97ALck has slightly elevated catalytic activity consistent with the autoinhibitory role of the SH3 domains in regulating Src kinase activity (32). Therefore, the possibility exists that the enhanced lipid raft localization of W97ALck is due to increased tyrosine kinase activity. However, catalytically activated Lck (Y505FLck) and kinase-inactive Lck (K273RLck) both distribute to lipid rafts similar to wild type Lck suggesting that the kinase activity of Lck does not exert a major influence in the regulation of Lck basal localization to lipid rafts (7). In apparent contrast to the results presented here, Patel et al. (31) recently reported a similar distribution of an SH3 Lck mutant and wild type Lck between rafts and non-raft regions of the membrane. The basis of this discrepancy is not known, although it may reflect the use of different cell lines, different detergents for the preparation of lipid rafts, and different lipid rafts. We have identified a role for the SH3 domain in regulating Src kinase activity (32).

Interestingly, inactivation of the SH3 domain leads to the enhanced incorporation of 16-[125I]iodohexadecanoic acid into Lck (Fig. 3). This indicates that either W97ALck has a higher rate of turnover of palmitate than wild type Lck or that W97ALck is more extensively palmitoylated than wild type Lck, or both. The half-life of [3H]palmitate on Lck in Jurkat cells is 1–2 h, well within the 3-h labeling period used in the experiments reported here. Based on the results of Jackson et al. and the 10-fold increase in the level of 16-[125I]iodohexadecanoic acid incorporated into the W97ALck mutant in the 3-h incubation period (Fig. 3), it is likely that the turnover rate of palmitate on W97ALck is higher than that for wild type Lck. It is also possible that more of the W97ALck is dually palmitoylated than wild type Lck. However, this by itself could not account for the high level of incorporation of 16-[125I]iodohexadecanoic acid into W97ALck relative to wild type Lck because W97ALck was only 2–3-fold enriched in rafts.

The increased turnover rate of palmitate on W97ALck raises intriguing questions regarding the mechanism of palmitoylation and depalmitoylation of Lck. Our data do not distinguish between whether the higher rate is due to its preferential localization into lipid rafts or whether the higher rate contributes to its preferential localization into lipid rafts, two possibilities that are not mutually exclusive. A very recent study by Dunphy et al. (56) reports that protein acyltransferase activity, catalyzing the enzymatic palmitoylation of Gα-subunits, is

required for an optimal constitutive interaction between Lck and c-Cbl. Lck was not detected in HA immunoprecipitates from cells transfected with Lck alone (Fig. 6A), and HA also was not detected in Lck immunoprecipitates from cells transfected with HA-c-Cbl alone (Fig. 6B), verifying the specificity of the communoprecipitation. Immunoblot analysis showed comparable levels of expression of WT Lck and W97ALck in the transfected cells (Fig. 6A). Likewise, comparable levels of expression of HA-c-Cbl were detected (Fig. 6B). These results support the conclusion that the SH3 domain of Lck negatively regulates Lck localization to lipid rafts through an interaction with c-Cbl.

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**FIG. 6.** SH3-dependent association of Lck with c-Cbl. JCaM1 cells were transfected with empty vector (control (−)) or transfected with expression constructs encoding WT Lck or W97ALck with (+) an HA-c-Cbl expression construct. After 20 h, cells were lysed. A, lysates (2 mg) were incubated with anti-HA antibody, and immunoprecipitates were analyzed by immunoblotting for HA (upper panel) or Lck (middle panel). To assess Lck expression levels, 20 μg of cell lysates was directly analyzed by immunoblotting for Lck (lower panel). B, lysates (2 mg) were incubated with anti-Lck antibody, and immunoprecipitates were analyzed by immunoblotting for Lck (upper panel) or HA (middle panel). To assess HA-c-Cbl expression levels, 20 μg of cell lysates was directly analyzed by immunoblotting for HA (lower panel). This experiment was performed twice with similar results. IP, immunoprecipitation.

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3 C. S. Jackson, S. C. Ley, and A. I. Magee, personal communication.
preferentially localized to lipid rafts. In light of these results, we think it is likely that the increase in the rate of palmitoyl turnover in the W97A Lck mutant is due to its increased presence in lipid rafts. Palmitoylation is a reversible process, and the preferential colocalization of W97A Lck and protein acyltransferase would facilitate the rapid repalmitoylation of W97A Lck relative to wild type Lck. The subcellular location of the thioesterase responsible for the removal of palmitate from Lck is not known, so it is not clear if depalmitoylation of Lck occurs within the rafts or in the non-raft regions of the plasma membrane. Either way, our data are consistent with increased repalmitoylation of the SH3 domain mutant of Lck within membrane rafts and the subsequent preference of rapidly repalmitoylated W97A Lck for the lipid raft environment. Alternatively, it is possible that the palmitoylation of Lck is nonenzymatic, and examples of nonenzymatic palmitoylation in vitro have been reported (57, 58).

Several mechanisms are possible for the increased localization of the Lck SH3 domain mutant to lipid rafts. As discussed above, the rapid repalmitoylation of the mutant is a factor. Because the SH3 domain of Lck is known to bind to proline residues within its own linker region, an interaction that contributes to an inactive conformation, the altered conformation of W97A Lck may contribute to its subdomain membrane localization and/or rapid repalmitoylation. Conformational changes affecting the susceptibility to protein acylthioesterases and/or protein acyltransferases have been suggested for the heterotrimeric G protein subunits (59–61).

Our results indicate that c-Cbl plays a role in keeping Lck from entering membrane rafts. c-Cbl is primarily cytosolic in resting Jurkat cells, although a readily detectable fraction is found in the particulate (membrane) fraction when cells are homogenized in the absence of detergent (62). Because Lck is exclusively membrane-associated, it is presumably this membrane-associated c-Cbl that is influencing the localization of Lck. Although the association of c-Cbl with some Src family members is readily demonstrated in vivo (37–39), its association with Lck appears to be relatively weak (39). In resting cells the SH3 domains of the Src kinases mediate the association between Src family members and c-Cbl, and in this regard the SH3 domain of Lck readily associated with c-Cbl in vitro (30–34). Our results suggest that one consequence of the interaction between the SH3 domain of Lck and c-Cbl is to sequester Lck away from the lipid raft-associated protein acyltransferase. It is of interest to note that although W97A Lck mediates the initiation of T cell receptor signaling, it fails to support activation of the mitogen-activated protein kinase pathway (32), through what appears to be a failure to interact with CD28 (35). It should be noted that a population of Lck is found outside of lipid rafts in c-Cbl-deficient T cells, suggesting that mechanisms other than binding to c-Cbl contribute to regulating the basal localization of Lck.

In conclusion, we provide evidence that the SH3 domain negatively regulates Lck basal localization to lipid rafts and that association with c-Cbl contributes to this process. Further understanding of the functional significance of the SH3 domain in the signaling functions of Lck and other Src kinases will be important.

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