Exclusion of CD45 Inhibits Activity of p56^{lck} Associated with Glycolipid-enriched Membrane Domains

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Abstract. p56^{lck} (Lck) is a lymphoid-specific Src family tyrosine kinase that is critical for T-cell development and activation. Lck is also a membrane protein, and approximately half of the membrane-associated Lck is associated with a glycolipid-enriched membrane (GEM) fraction that is resistant to solubilization by Triton X-100 (TX-100). To compare the membrane-associated Lck present in the GEM and TX-100–soluble fractions of Jurkat cells, Lck from each fraction was immunoblotted with antibody to phosphotyrosine. Lck in the GEM fraction was found to be hyperphosphorylated on tyrosine, and this correlated with a lower kinase specific activity relative to the TX-100–soluble Lck. Peptide mapping and phosphatase digests showed that the hyperphosphorylation and lower kinase activity of GEM-associated Lck was due to phosphorylation of the regulatory COOH-terminal Tyr^{505}. In addition, we determined that the membrane-bound tyrosine phosphatase CD45 was absent from the GEM fraction. Cells lacking CD45 showed identical phosphorylation of Lck in GEM and TX-100–soluble membranes. We propose that the GEM fraction represents a specific membrane domain present in T-cells, and that the hyperphosphorylation of tyrosine and lower kinase activity of GEM-associated Lck is due to exclusion of CD45 from these domains. Lck associated with the GEM domains may therefore constitute a reservoir of enzyme that can be readily activated.

p56^{lck} (Lck) is a lymphoid-specific Src family kinase that is required for T cell development and stimulation (29, 43, 62). In addition, Lck is a peripheral membrane protein that requires both NH$_2$-terminal myristoylation and palmitoylation for membrane binding (65). Lck also binds to a low density, nonionic detergent-resistant membrane fraction that is present in detergent lysates of cells (8, 13, 49, 57, 61). We refer to this fraction as a glycolipid-enriched membrane (GEM)[1] fraction (49). Besides glycolipids, GEM fractions also contain sphingolipids, cholesterol, glycosylphosphatidylinositol (GPI)–anchored proteins, and a wide variety of signal transducing molecules (5, 8, 9, 12, 13, 18, 19, 23, 35, 48, 49, 53, 58, 60, 61). In cells expressing caveolin, the GEM fraction contains caveolae (12, 53, 54).

It has been proposed that the GEM fraction represents glycolipid-enriched membrane domains that are present in cells and are resistant to solubilization by nonionic detergents. Experiments with model membranes show that the poor solubilization of GEM domains in nonionic detergents could be due to their glycolipid content. For example, lipid vesicles with a composition similar to the lipid content of the GEM fraction are also resistant to solubilization by nonionic detergents (55). Experiments with cells labeled with fluorescein-conjugated CD59, a GPI-anchored protein, have provided evidence that the GEM fraction represents domains in animal cell membranes (6). In this report, it was shown that inclusion of CD59 into large patches in the outer membrane coincides with protein incorporation in the GEM fraction. Others, however, have argued that the GEM fraction is an artifact of detergent extraction (40).

The enrichment of signal transducing molecules in the GEM fraction suggests that glycolipid domains function in signal transduction. The possibility that GEM domains function in signal transduction is also suggested by the observed stimulation of T cells after antibody cross-linking of GPI-anchored protein (16, 31, 47). If glycolipid domains function in signal transduction, then one might expect selective regulation of proteins associated with them.

We report here experiments using the human T cell lymphoma Jurkat cell line to study the regulation of Lck associated with the GEM fraction of T cells. We have determined that Lck in the GEM fraction is selectively hyperphosphorylated on tyrosine, and this coincides with a kinase activity that is lower than the activity of the remaining membrane-associated Lck. Furthermore, the tyrosine phosphatase

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[1] *Abbreviations used in this paper*: GEM, glycolipid-enriched domain; GPI, glycosylphosphatidylinositol; PAP, potato acid phosphatase; TX-100, Triton X-100.

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CD45 is absent from the GEM fraction. In cell lines that lack CD45 expression, the GEM-associated Lck is not selectively hyperphosphorylated. We suggest a model in which association of Lck with GEM domains results in selective regulation of Lck by sequestration away from the phosphatase activity of CD45.

Materials and Methods

Cells and Antibodies

Jurkat (clone E6-1), JCaM1, and J45.01 cells were purchased from American Type Culture Collection (Rockville, MD). Monoclonal antibodies to phosphotyrosine (4G10), CD45 (clone HI30), and Lck were purchased from Upstate Biotechnology Inc. (Lake Placid, NY), Caltag Laboratories (South San Francisco, CA), and Transduction Laboratories (Lexington, KY), respectively. Rabbit polyclonal antiserum to Lck was described previously (56).

Protein Expression

Transient expression was done by transfection of HeLa cells infected with the recombinant vaccinia virus vTF7-3 encoding the T7 polymerase (21). HeLa cells were infected at a multiplicity of infection of 20, followed by transfection with 5 μg of DNA using cationic liposomes as the carrier (51). The infection and transfection were performed in DME. At 3 h after transfection, the media was replaced with DME supplemented with 10% FCS. At 7 h after transfection, the cells were harvested for equilibrium centrifugation.

Cell Lysis and Equilibrium Centrifugation

10^7 cells were washed twice with chilled PBS containing 0.4 mM $NaVO_4$ to inhibit phosphatases. For lysis of the cells with Triton X-100 (TX-100), the cells were suspended in 1.0 ml of 1% TX-100, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM $NaVO_4$, and 75 U of aprotinin, incubated in the detergent solution for 20 min, and mechanically disrupted by Dounce homogenization (10 strokes). For lysis in hypotonic media, the cells were suspended in 0.5 ml of 10 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 1 mM $NaVO_4$, and 75 U aprotinin, incubated for 20 min, and Dounce homogenized (25 strokes). All lysates were centrifuged for 5 min at 1,300 g to remove nuclei and large cellular debris. All steps were done at 0–4°C. TX-100 lysis of transfected HeLa cells was done the same as described above, except that the lysate was collected with a rubber policeman before Dounce homogenization.

For equilibrium centrifugation of the TX-100 lysates, the clarified lysate was diluted with an equal volume of 85% wt/vol sucrose in TNEV (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1 mM $NaVO_4$). In an SW 41 centrifuge tube, the diluted lysate was overlaid with 6 ml of 30% wt/vol and 3.5 ml 5% wt/vol sucrose solutions in TNEV. The samples were centrifuged for 16–18 h at 200,000 g at 4°C. The same protocol was followed for equilibrium centrifugation of cells lysed by Dounce homogenization in hypotonic media, except that the lysate was diluted with 1.5 ml of 85% wt/vol sucrose in TNEV and overlaid with 6.0 and 3.5 ml of 60% and 5% wt/vol sucrose in TNEV, respectively.

Immunoprecipitations

After equilibrium centrifugation, the gradients were fractionated from the top. Each fraction was diluted with an equal volume of 1% TX-100 in TNEV, and 1.0 μl of rabbit antiserum to Lck was added to each fraction. Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA) was used as the solid phase for the immunoprecipitation. All immunoprecipitates were washed twice with 1% TX-100 in TNEV before elution in SDS-PAGE sample buffer (33).

Sedimentation of the GEM Fraction

Cells were lysed with TX-100, and the GEM fraction was separated by equilibrium centrifugation. The gradient fractions containing the GEM fraction (fractions 3–5 in Fig. 1) were collected and pooled. The sample was then diluted with 5 vol of chilled PBS and centrifuged at 200,000 g for 90 min at 4°C. The supernatant was removed and the pellet was washed once with chilled PBS. After the wash, the pellet was suspended in SDS-PAGE sample buffer.

In Vivo Labeling and CNBr Mapping

2×10^7 Jurkat cells were labeled in 5 ml of phosphate-free RPMI containing 5% dialyzed FCS and 5 mCi [^{32}P]orthophosphate (carrier free) (Amersham Corp., Arlington Heights, IL) for 2.5 h at 37°C. Lck immunoprecipitated from the GEM fraction after equilibrium centrifugation was separated by SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). The Lck bands were located by immunoblotting and autoradiography, and they were excised from the nitrocellulose. The CNBr digest was performed using 100 mg/ml CNBr in 70% formic acid. The samples were incubated in the CNBr for 1.5 h at room temperature, followed by a wash with 70% formic acid. The digest was lyophilized and washed twice with water (Millipore Corp., Milford, MA) before suspension in SDS-PAGE sample buffer. The peptide fragments were separated by SDS-PAGE (20% acrylamide). To generate standards, unlabeled Jurkat cells were lysed with 1% NP-40, 0.4% deoxycholate, 66 mM EDTA, and 10 mM Tris-HCl (pH 7.4), followed by immunoprecipitation of Lck and in vitro labeling. For this experiment, the in vitro labeling was performed for 15 min at 24°C. All remaining steps were done in parallel with the in vivo–labeled Lck.

In Vitro Kinase Assays

GEM and TX-100 Lck immunoprecipitates were washed once with 60 mM β-octylglucoside in TNE (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA). This was followed by a second wash with 25 mM Hepes, pH 7.4, 3 mM $MnCl_2$, 3 mM $MgCl_2$, 100 μM $NaVO_4$, 0.1% TX-100 (kinase buffer). The immunoprecipitates were then suspended in 25 μl of kinase buffer containing 5 μg of acid-denatured enolase and 10 μCi [γ-^{32}P]ATP and incubated at 24°C for 1 min. All steps before the reaction were done at 4°C. The acid-denatured enolase was prepared by diluting the enolase in an equal volume of 100 mM acetic acid. The reactions were stopped by addition of an equal volume of SDS-PAGE sample buffer and immediately placed in boiling water for 5 min. The enolase and Lck were separated by SDS-PAGE and detected with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The amount of $^{32}PO_4$ labeling of enolase was measured with the Phosphorimager. The relative amount of Lck present in each sample was measured by immunoblotting.

Potato Acid Phosphatase Digests

Potato acid phosphatase (PAP) (Sigma Chemical Co., St. Louis, MO) was prepared as described by Cooper and King (14a). Lck immunoprecipitated from the GEM fraction after equilibrium centrifugation was washed once with 1% TX-100 in TNE, and twice with 40 mM Pipes, pH 6.0, 1 mM DTT, 20 μg/ml aprotinin, and 20 μM leupeptin (phosphatase buffer). The sample was then suspended in phosphatase buffer containing 330 μg/ml PAP and incubated for 1 h. After the digest, the sample was either washed with kinase buffer and used for an in vitro kinase assay, or suspended in SDS-PAGE sample buffer for immunoblotting. All steps before the kinase assay were done at 4°C.

Immunoblotting

Protein samples were separated by SDS-PAGE (10% acrylamide) in reducing conditions for Lck and phosphotyrosine blotting, and SDS-PAGE (8% acrylamide) in nonreducing conditions for CD45 blotting. The proteins were transferred to polyvinylidene difluoride (Immobilon-P; Millipore Corp.) using a wet transfer system (Hoefer, San Francisco, CA). The transfer buffer was 20 mM Tris-HCl, 150 mM glycine, and 20% methanol, and the membrane was pretreated as instructed by the manufacturer. After transfer, the membrane was blocked for 1 h in 1% wt/vol BSA in TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). Next, the membrane was incubated with the respective primary antibody for 1 h, followed by a biotinylated secondary antibody for 1 h and avidin-conjugated HRP for 1 h. All antibody dilutions for blotting and intermediate washes were with TTBS. The detection was done using enhanced chemiluminescence (ECL; Amersham Corp.) on preflashed film (X-OMAT; Eastman Kodak Co., Rochester, NY). For quantitation, the exposed films were scanned with a gel scanner (Molecular Dynamics), and quantitation was done using the manufacturer's software.

Results

Approximately Half of Membrane-associated Lck Is Present in the GEM Fraction

To separate the Lck in the GEM fraction from the TX-100–soluble Lck of Jurkat cells, equilibrium centrifugation was performed on cell lysates generated by TX-100 (Fig. 1 *A*). The lysates were diluted with an equal volume of an 85% sucrose solution, placed at the bottom of a centrifuge tube, and overlaid with 5% and 30% sucrose layers. Equilibrium centrifugation of the sample resulted in banding of the low density GEM fraction at the interface between the top two sucrose layers. This coincides with gradient fractions 3–5. The TX-100–soluble proteins, which are not in membrane sheets and vesicles and therefore have a much higher density than proteins in the GEM fraction, remain at the bottom of the gradient in fractions 9 and 10. In Fig. 1 *A*, the amount of Lck in the GEM fraction was 47% of the total Lck. An equal amount of Lck was recovered by either sedimentation or immunoprecipitation of the GEM fraction after equilibrium centrifugation, indicating that losses were not encountered during immunoprecipitation (data not shown).

Since Lck is myristoylated and palmitoylated on its NH_2 terminus, it is strongly associated with cell membranes (65). To verify this membrane association, Jurkat cells were lysed by Dounce homogenization in hypotonic buffer. Equilibrium centrifugation of the lysate was done to separate the low density membrane fraction from the cytosolic fraction (Fig. 1 *B*). The membrane fraction was present in gradient fractions 3–5, and the cytosolic fraction was present in gradient fractions 9 and 10. All of the Lck in Jurkat cells was in the membrane fraction.

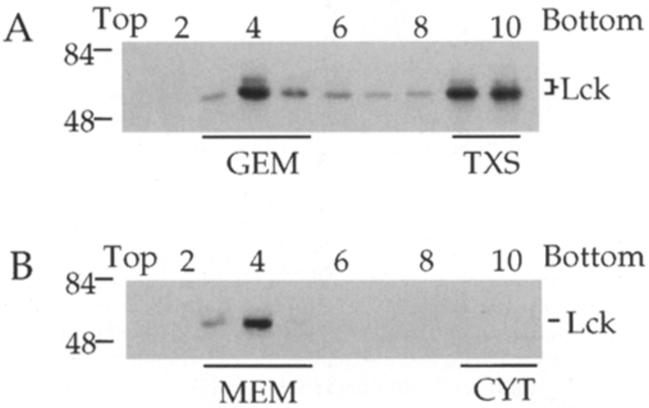

Figure 1. Association of Lck with the GEM and total membrane fractions of Jurkat cells. (*A*) Jurkat cells were lysed with TX-100, and the GEM and TX-100–soluble fractions were separated by equilibrium centrifugation. After centrifugation, the gradient was fractionated, and each gradient fraction was immunoprecipitated with antiserum to Lck. Lck was detected by immunoblotting with an mAb. Gradient fractions 3–5 and 9 and 10 correspond to the GEM and TX-100–soluble (*TXS*) membrane fractions, respectively. Based on the amount of Lck in gradient fractions 3–5 and 9 and 10, it was determined that the GEM-associated Lck represents 47% of the total Lck. The amount of Lck in the pellet of the low speed spin done during sample preparation and in the pellet of the sucrose gradient represented ~10% of the total Lck of each step. (*B*) Jurkat cells were lysed by Dounce homogenization in hypotonic buffer. The membrane fraction was separated by equilibrium centrifugation using a sucrose step gradient similar to that used in *A*. Gradient fractions 3–5 and 9 and 10 correspond to the membrane (*MEM*) and cytosolic (*CYT*) fractions, respectively. Molecular weights (in thousands) are indicated at left.

Lck Associated with the GEM Fraction Is Hyperphosphorylated on Tyrosine

The phosphotyrosine content of the Lck in the GEM and TX-100–soluble membrane fractions was measured since this is indicative of Lck activation and inactivation. For example, phosphorylation of Tyr^{394} is correlated with Lck activation (1, 25). Conversely, phosphorylation of Tyr^{505} results in Lck inactivation (2, 39). The tyrosine phosphorylation state of the Lck in each membrane fraction was measured by immunoblotting with antibody to phosphotyrosine. The relative amount of Lck in each membrane fraction was determined by immunoblotting with antibody to Lck. Fig. 2 *A* shows that GEM-associated Lck is hyperphosphorylated on tyrosine relative to the TX-100–soluble Lck.

Fig. 2 *B* shows the average of three experiments where the phosphotyrosine content of the Lck in the GEM and TX-100–soluble fractions was measured. The phosphotyrosine content of the Lck in each sample is represented by the ratio of the phosphotyrosine signal divided by the Lck signal. On average, the GEM-associated Lck had a 3.8-fold greater phosphotyrosine content compared with that of the TX-100–soluble Lck.

Lck Associated with the GEM Fraction Has Reduced Kinase Activity

To determine if the greater phosphotyrosine content of the Lck in the GEM fraction affected its kinase activity, we compared the kinase activity of Lck present in each membrane fraction. In vitro kinase assays were conducted using Lck immunoprecipitated from the GEM and TX-100–soluble fractions after equilibrium centrifugation (Fig. 3 *A*). To solubilize the lipids in the GEM fraction before the assay, the immunoprecipitates were washed with 60 mM β-octylglucoside (9). This prevents any differences in activity arising from incomplete solubilization of the membranes. The kinase specific activity was measured by $^{32}PO_4$ labeling of enolase, and the amount of Lck present in each assay was measured by immunoblotting. For the experiment shown in Fig. 3 *A*, the specific activity of the TX-100–soluble Lck was threefold greater than the specific activity of the GEM-associated Lck. The activity of the GEM-associated Lck was also less than the TX-100–soluble Lck when the samples were washed with TX-100 rather than β-octylglucoside before the assay (data not shown).

To determine if kinases other than Lck contributed to a background in the assay, an in vitro kinase assay was done using an immunoprecipitate from the Jurkat JCaM1 cell line. JCaM1 cells express a truncated Lck molecule that lacks kinase activity (62), so any measurable enolase phosphorylation must arise from kinases other than Lck. The samples were prepared for the assay by immunoprecipitating the TX-100–soluble fraction of Jurkat and JCaM1 cells with antibody to Lck. Fig. 3 *B* shows that only a trace amount of enolase phosphorylation (6% of Jurkat sample)

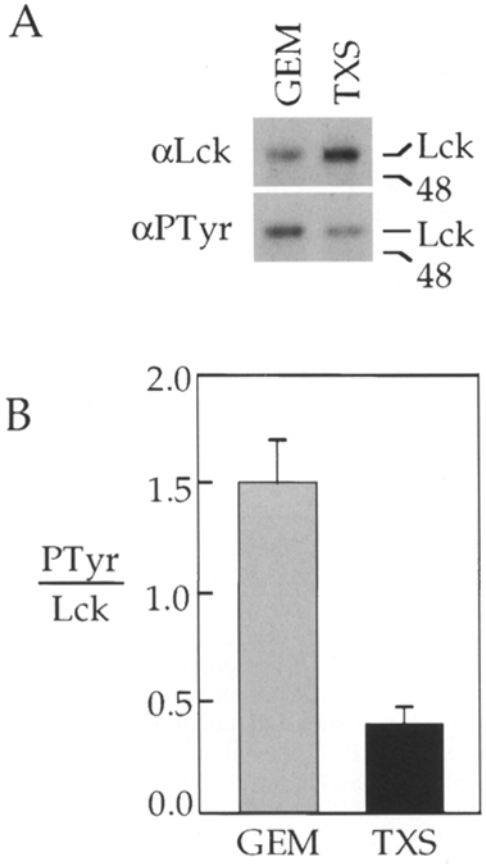

Figure 2. Phosphotyrosine content of Lck in the GEM and TX-100–soluble fractions. (*A*) Jurkat cells were lysed with TX-100, and the GEM and TX-100–soluble membrane fractions were separated by equilibrium centrifugation. The gradient fractions containing the GEM and TX-100–soluble fractions (3–5 and 9 and 10 in Fig. 1 *A*, respectively) were immunoprecipitated with antiserum to Lck and immunoblotted with mAbs to Lck (*top*) and phosphotyrosine (*PTyr*; *bottom*). Molecular weights (in thousands) are indicated at right. (*B*) The graph shows the average of three experiments where the phosphotyrosine content of Lck in each membrane fraction was measured. Each of the experiments was done as described in Fig. 2 *A*, and all of the samples were immunoblotted together. The y axis represents the phosphotyrosine content of the Lck in each membrane fraction, and it was calculated by dividing the phosphotyrosine and Lck signals measured in the respective immunoblots.

can be detected in the JCaM1 sample. The faster migrating Lck band in the immunoblot present in Fig. 3 *B* represents the truncated Lck expressed in the JCaM1 cells.

Fig. 3 *C* presents quantitation from four separate experiments where both the phosphotyrosine content and kinase activities of Lck in the GEM and TX-100–soluble fractions were measured. Hyperphosphorylation of tyrosine in the GEM-associated Lck fraction is represented by the ratio of the phosphotyrosine content of Lck from each membrane fraction (*left axis*). The relative inactivation of the Lck in the GEM fraction is represented by the ratio of the specific activities (*right axis*). The GEM-associated Lck had a threefold greater phosphotyrosine content than the TX-100–soluble Lck and a specific activity that was one-third of that measured for the TX-100–soluble Lck.

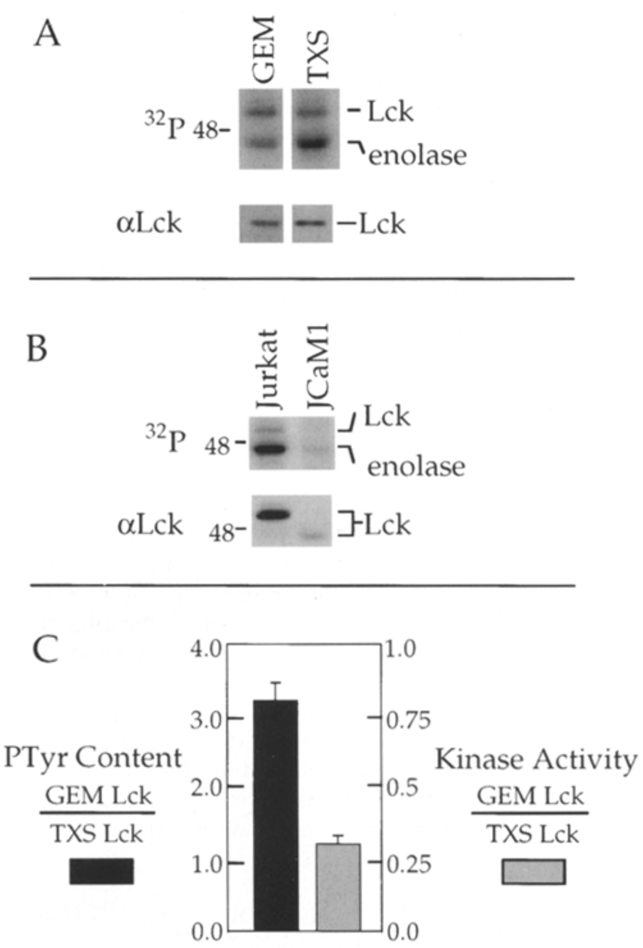

Figure 3. In vitro kinase assays of Lck from the GEM and TX-100–soluble fractions. (*A*) The GEM and TX-100–soluble membrane fractions were separated and immunoprecipitated as described in Fig. 2. In vitro kinase assays were performed using Lck from each membrane fraction (*top*). Enolase was used as an exogenous substrate, and the amount of $^{32}PO_4$ labeling of enolase was measured using a Phosphorimager. The amount of Lck in each fraction was measured by immunoblotting. The specific activity of the Lck in the GEM fraction was determined to be one-third of the specific activity of the Lck in the TX-100–soluble fraction. (*B*) A kinase assay using the TX-100–soluble fraction of Jurkat and JCaM1 cells after immunoprecipitation with antibody to Lck. The kinase assay and Lck detection were done as described in (*A*). (*C*) The graph shows the average of four experiments where the phosphotyrosine content and kinase activity of GEM-associated and TX-100–soluble Lck was measured. In each experiment, the Lck in the GEM and TX-100–soluble fractions was separated and immunoprecipitated as described in Fig. 2. The phosphotyrosine content and kinase activity of the Lck in each membrane fraction were measured and calculated as described in Figs. 2 and 3 *A*. (*Bar, left*) Ratio of the phosphotyrosine content of Lck in the GEM and TX-100–soluble fractions. (*Bar, right*) Ratio of specific activity of the Lck in the TX-100–soluble and GEM fractions.

Although the activity of Lck toward an exogenous substrate was very different in the two fractions, the autophosphorylation of Lck in each membrane fraction was similar. Variability in the activity of Lck has also been noted previously when using different exogenous substrates (37). We attribute the apparent discrepancy in Lck activity measured by the enolase and autophosphorylation reactions to the different nature of these two reactions. The autophosphorylation reaction is apparently less sensitive to inactivation of Lck. The phosphorylation of other proteins by Lck in vivo is probably represented best by the phosphorylation of exogenous substrates such as enolase.

Inactivation of Lck in the GEM Fraction Is Due to Hyperphosphorylation of Tyr^{505}

Hyperphosphorylation on Tyr^{505} of Lck in the GEM fraction could account for its relative inactivity. Characterization of Lck from different T cell lines has shown that Tyr^{505} is frequently the predominant site of tyrosine phosphorylation in resting T cells, which is what we used in our experiments (25, 26, 41, 64). However, some T cell lines have a substantial amount of phosphorylation of Tyr^{394} in the resting state, and the proportion of Tyr^{505} and Tyr^{394} phosphorylation varies with cell type (26).

To determine the site of tyrosine phosphorylation in the GEM-associated Lck, peptide mapping by CNBr cleavage was done using Lck from Jurkat cells that were metabolically labeled with [^{32}P]orthophosphate (see Fig. 5 *A*, *left*). CNBr cleavage of Lck produces three main fragments (36), and these are shown in Fig. 4 *A* with a digest of Lck labeled in vitro as markers: F1, F2, and F3 (Fig. 4 *A*, *right*). The F1 fragment corresponds to the NH$_2$-terminal domain of Lck, and it is phosphorylated principally on serine residues. F2 represents a series of fragments between 10 and 14 kD in size, and these contain Tyr^{394}. The F3 fragment represents the COOH-terminal domain of Lck and contains Tyr^{505}. In GEM-associated Lck, 75% of the signal from [^{32}P]orthophosphate labeling is in the F3 fragment, with no labeling of the F2 fragment.

If Tyr^{505} phosphorylation were responsible for inactivation of Lck in the GEM fraction, then tyrosine dephosphorylation should result in kinase activation. To determine if Lck in the GEM fraction could be activated by dephosphorylation, a sample was treated with PAP before an in vitro kinase assay (Fig. 4 *B*). PAP treatment of GEM-associated Lck resulted in a 70% decrease in its phosphotyrosine content and a fivefold increase in its specific activity.

Hyperphosphorylation of Lck in the GEM Fraction Correlates with CD45 Expression and Exclusion from the GEM Fraction

Lck is activated in vivo by dephosphorylation of Tyr^{505}, and the transmembrane protein tyrosine phosphatase CD45 is responsible for this dephosphorylation (41, 44, 46, 63). In addition, the transmembrane domain of proteins causes them to be excluded from the GEM fraction (4, 49). This was shown by expressing constructs of GPI-anchored proteins where the GPI anchor was replaced with a transmembrane domain.

To determine if CD45 were present in the GEM fraction of Jurkat cells, the GEM fraction was sedimented and immunoblotted using antibody to CD45 (Fig. 5). Lck was used as a marker for the GEM fraction, and it was also detected by immunoblotting. The level of CD45 expression occurring with Lck expression was measured by immunoblotting a whole cell lysate with antibody to each protein. Based on the amount of Lck and CD45 coexpression in Jurkat cells and the amount of Lck detected in the GEM fraction after sedimentation, CD45, if present, should have been detected. However, no CD45 was detected in the GEM fraction.

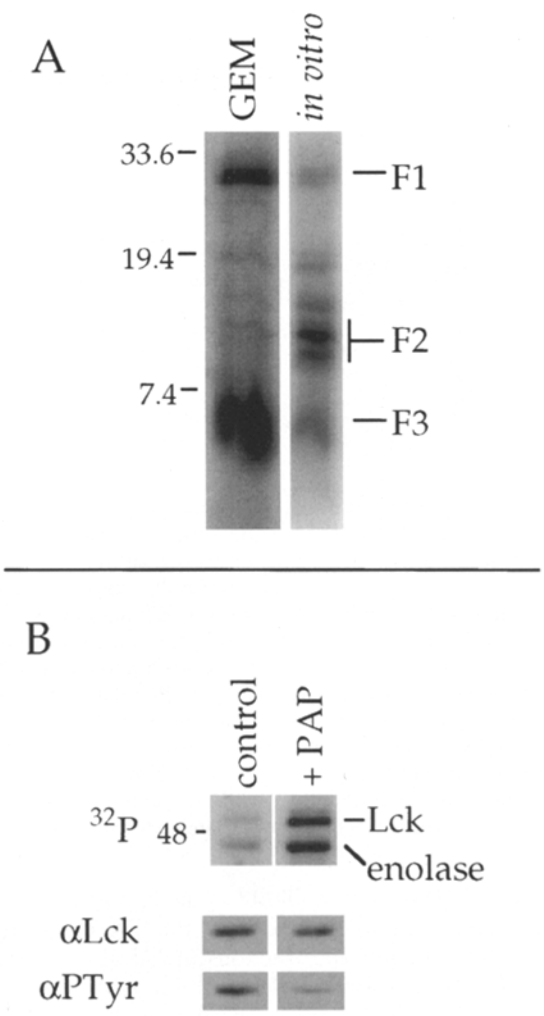

Figure 4. Mapping of phosphotyrosine in GEM-associated Lck. (*A*) CNBr mapping of the phosphotyrosine site of Lck in the GEM fraction. Jurkat cells were labeled in vivo with [^{32}P]orthophosphate, and the cells were lysed with TX-100. The GEM-associated Lck was separated by equilibrium centrifugation, immunoprecipitated with rabbit antiserum, and digested with CNBr. The resulting fragments were separated by SDS-PAGE (20% acrylamide) (*left*). As a standard, Lck from unlabeled cells was labeled in an in vitro reaction (*right*). (*B*) Activation of GEM-associated Lck by PAP treatment. Jurkat cells were lysed with TX-100, and the GEM-associated Lck was separated by equilibrium centrifugation and immunoprecipitated with rabbit antiserum. One-half of the sample was incubated with PAP in phosphatase buffer, and the second half of the sample was incubated in the phosphatase buffer without PAP as a control. The activity of the Lck in each sample was measured by $^{32}PO_4$ labeling of enolase during an in vitro kinase reaction as described in Fig. 3. The amount of phosphotyrosine and Lck in each sample was measured by immunoblotting. The kinase activity increased by fivefold, and the phosphotyrosine content decreased by 70% upon treatment with PAP. Molecular weights (in thousands) are indicated at left.

Exclusion of CD45 from the GEM fraction could have been responsible for the observed hyperphosphorylation

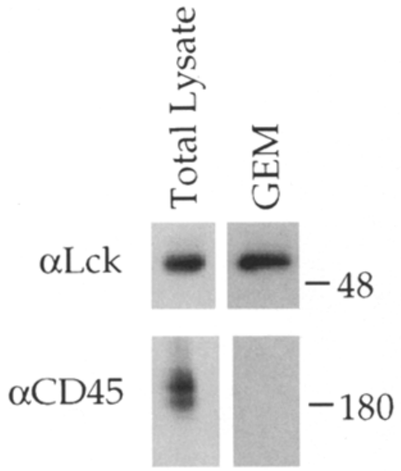

Figure 5. Immunoblotting of the GEM fraction from Jurkat cells with antibody to CD45. (*Right*) 5 × 10^6 Jurkat cells were lysed with 1% TX-100, and the GEM fraction was separated by equilibrium centrifugation and sedimented as described in the Materials and Methods. After sedimentation, the GEM fraction was suspended in SDS-PAGE sample buffer, and 25% of the sample was used for immunoblotting with antibody to each protein. (*Left*) The amount of CD45 expression in Jurkat cells relative to Lck expression was determined by immunoblotting a total cell lysate (*left*) prepared by suspending 10^6 cells in 100 μl of sample buffer, followed by boiling and clarifying (16,000 *g* for 5 min). 5% of the sample was used for immunoblotting with antibody to each protein. Molecular weights (in thousands) are indicated at right.

of Lck in the GEM fraction. To test this hypothesis, the phosphotyrosine content of Lck from the TX-100–resistant and -soluble membrane fractions of J45.01 cells was compared. J45.01 cells are a Jurkat cell line that does not express CD45 (30). Fig. 6 *A* shows that Lck in the TX-100–soluble fraction of J45.01 cells has a greater phosphotyrosine content than the Lck in the TX-100–soluble fraction of Jurkat cells. Consequently, the GEM-associated and TX-100–soluble Lck of J45.01 cells have a nearly identical phosphotyrosine content.

CD45 is expressed exclusively in hemopoetic cells (63). To determine if the results from the J45.01 cells would be similar in other cells that lack CD45, Lck was expressed in HeLa cells. The Lck was transiently expressed using a recombinant vaccinia expression system (21). Fig. 6 *B* shows that GEM-associated and TX-100–soluble Lck of transfected HeLa cells also have a similar phosphotyrosine content.

Fig. 6 *C* shows the average of three separate experiments measuring the relative enrichment of phosphotyrosine of Lck in the GEM fraction of Jurkat, J45.01, and transfected HeLa cells. Similar to what was shown in Figs. 2 *B* and 3 *C*, Lck in the GEM fraction of Jurkat cells is hyperphosphorylated on tyrosine. However, in the J45.01 and transfected HeLa cells, the phosphotyrosine content of GEM-associated and TX-100–soluble Lck is nearly identical.

Discussion

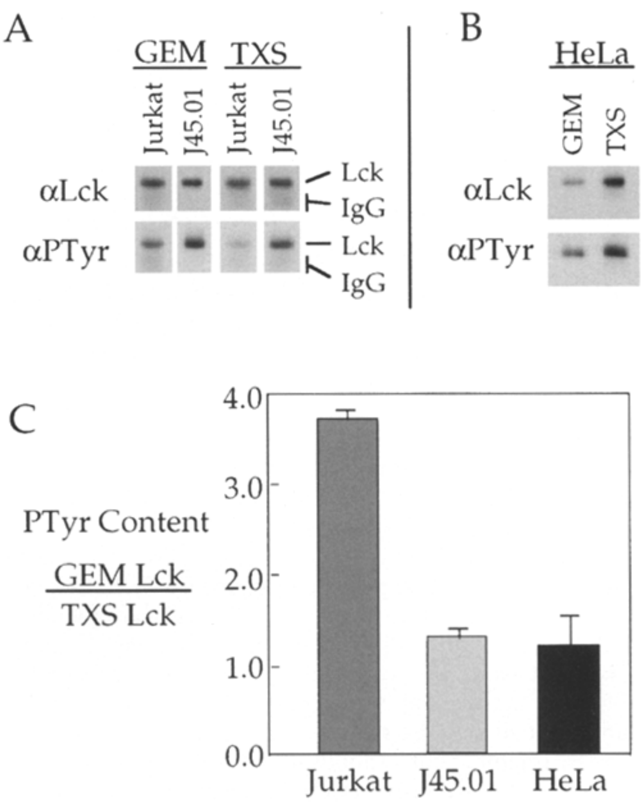

Figure 6. Phosphotyrosine content of Lck in the GEM or TX-100–soluble fractions of Jurkat, J45.01, and transfected HeLa cells. (*A*) The Lck in the GEM and TX-100–soluble membrane fractions of Jurkat and J45.01 cells were separated and immunoprecipitated as described in Fig. 2. The Lck in each membrane fraction was immunoblotted with antibody to Lck (*top*) and phosphotyrosine (*PTyr; bottom*). (*B*) HeLa cells were transfected with DNA encoding Lck (Materials and Methods). At 7 h after transfection, the cells were lysed with TX-100, and the GEM and TX-100–soluble fractions were separated by equilibrium centrifugation. The Lck in each fraction was immunoprecipitated and immunoblotted as in (*A*). (*C*) The relative phosphotyrosine content of Lck in the GEM and TX-100–soluble fractions of Jurkat, J45.01, and HeLa cells transfected with DNA encoding Lck was measured in three experiments, and the average was calculated. The Lck in each membrane fraction was prepared and immunoblotted as described in Fig. 6, *A* and *B*. The phosphotyrosine content of Lck in each membrane fraction was measured as described in Fig. 2. The y axis in the graph represents the ratio of the phosphotyrosine content of the GEM-associated and TX-100–soluble Lck.

We report here experiments showing that Lck present in the GEM fraction of Jurkat cells is less active than the remaining membrane-associated Lck because of hyperphosphorylation on Tyr^{505}. Two lines of evidence suggest that the mechanism of GEM-associated Lck down-regulation involves sequestration into a membrane fraction lacking the tyrosine phosphatase CD45. First, CD45 was not present in the membrane fraction containing inactivated Lck, and, second, cell lines lacking CD45 showed no difference in phosphorylation of GEM-associated and non–GEM-associated Lck.

The mechanism we propose for selective regulation of Lck is illustrated in Fig. 7. In this model, CD45 is excluded from the GEM fraction. As a result of exclusion, dephosphorylation of Lck on Tyr^{505} is prevented. Conversely, the

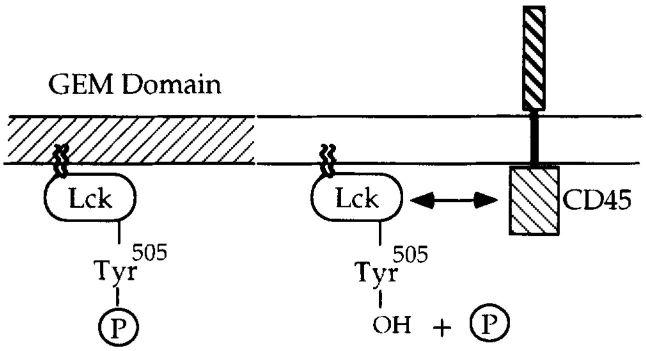

Figure 7. A model for the mechanism of selective regulation of Lck in the GEM fraction. The GEM domains consist of glycolipid-enriched domains that are poorly solubilized by nonionic detergents. CD45 does not associate with the GEM domains (Fig. 6). Consequently, GEM-associated Lck is sequestered from CD45. This results in hyperphosphorylation of Tyr^{505} and a kinase activity that is lower than the activity of Lck in the TX-100–soluble fraction.

TX-100–soluble Lck is in the same membrane fraction as CD45, and it can be dephosphorylated by CD45. The resulting hyperphosphorylation of Lck present in the GEM fraction produces its diminished kinase activity.

An important conclusion from this model is that the GEM fraction represents membrane domains that are present in the intact cell and not an artifact of detergent extraction. Interactions among lipids of the GEM domain may serve to form a "scaffolding" with which proteins and other lipids may associate, thereby forming a stable membrane structure. Based on experiments with model membranes (55), we surmise that the resistance of the GEM domain to solubilization by nonionic detergents arises from its glycolipid and cholesterol content. In addition, glycolipids and cholesterol are required for forming the GEM fraction in cell membranes. For example, when cholesterol synthesis was inhibited in a SPB-1 CHO cell line defective in sphingolipid synthesis, the solubility of the GPI-anchored protein placental alkaline phosphatase in nonionic detergents was increased by fivefold (24).

Other data also provide evidence that the GEM fraction represents membrane domains. For example, experiments using U937 cells labeled with fluorescently conjugated CD59 show that enrichment of CD59 into large domains in the outer membrane coincides with its association with the GEM fraction (6). In other experiments using fluorescence recovery after photobleaching, GPI-anchored proteins frequently have mobile fractions <50% (27, 28, 66). This is consistent with a large fraction of the protein being trapped in membrane domains. More recent work reported by Simson et al. (59) used single particle tracking to show that the GPI-anchored proteins Thy-1 and NCAM 125 are limited in their mobility to regions of ~300 nm, and this may reflect the average size of the GEM domains.

Mayor and Maxfield (40) have suggested that the detergent-insoluble fraction from cells does not represent a true membrane domain, but it is actually an artifact generated by differential detergent extraction of lipids and proteins by TX-100. However, the biochemical differences between the Lck in the GEM and TX-100–soluble fractions support the model that the GEM fraction represents a true domain of the cell membrane. The alternative, that hyperphosphorylation of Lck leads to concentration in the GEM fraction after lysis, is unlikely since Lck that is hyperphosphorylated on tyrosine is present in the TX-100–soluble fraction of J45.01 cells.

The experiments described here require ~24 h from the initial lysis of the Jurkat cells to the final suspension of the immunoprecipitates in sample buffer. This raises the possibility that the differences we observed between the Lck in the GEM and TX-100–soluble fractions represent an artifact arising from prolonged exposure of only the TX-100–soluble Lck to CD45 during the experiment. To address this issue, the GEM and TX-100–soluble fractions were separated by sedimenting the GEM fraction immediately after lysis of the cells. Immunoblotting showed that Lck in the GEM fraction had a fourfold greater phosphotyrosine content than Lck in the TX-100–soluble fraction (data not shown). Since the entire experiment was completed in <2 h, we conclude that the differences in phosphotyrosine content of the Lck in the GEM and TX-100–soluble fractions are not due to dephosphorylation of Lck during the experiment.

Selective phosphorylation by a separate tyrosine kinase present in the GEM domain is another possible explanation for hyperphosphorylation of Lck. Since Lck is inactivated by phosphorylation on Tyr^{505} by Csk (7), the GEM fraction from Jurkat cells was immunoblotted with antibody to Csk. However, no Csk was detected in the GEM fraction (data not shown). This result is not surprising since Csk does not contain any modifications or domains for binding to the membrane, and it is predominantly in the cytoplasmic fraction of cells (45, 52). We surmise that phosphorylation of Lck on Tyr^{505} is due to transient interaction with Csk and auto- or transphosphorylation by Lck. Whatever the mechanism of phosphorylation of Tyr^{505} that exists, it may be the same for Lck present in the GEM and TX-100–soluble fractions since cells lacking CD45 show similar Lck phosphorylation in both fractions.

Previous investigators have examined the localization of Lck in Jurkat cells. Immunofluorescence microscopy of resting cells shows that Lck is localized principally in the plasma membrane (34, 38). Our own data showing that the entire cellular Lck is associated with the membrane fraction corroborate the immunofluorescence data.

Immunoblotting showed that the level of Lck expression in J45.01 cells was up to twofold greater than that of the Jurkat cells. In addition, most of the overexpressed Lck in the J45.01 cells was associated with the GEM fraction. Overexpression of Lck in J45.01 cells is analogous to Syk overexpression in thymocytes that lack ZAP-70 expression (22). The proportionately greater binding of Lck to the GEM fraction in the J45.01 cells may be explained if Lck association with the TX-100–soluble fraction occurs because of binding to other proteins in this fraction, such as CD4 or CD45. If the binding of Lck to other molecules in the TX-100–soluble fraction is saturated, then additional expression of Lck could result in its association with GEM domains.

The ubiquitous presence of GEM domains in animal cell membranes suggests that these domains have important functional roles. In cells that express caveolin, the GEM fraction includes both the smooth membrane invaginations representing caveolae and the membrane surrounding the caveolae (54). Caveolae function in uptake of molecules from the extracellular environment through endocytosis and transcytosis (3). The protein and lipid components of the GEM fraction may assist in these processes.

Jurkat cells lack caveolin (20), but they do contain noncoated surface invaginations that may be analogous to caveolae (17). However, the relationship between these surface invaginations and the GEM domains remains unclear. In any event, we have found that the GEM domains maintain a large pool of Lck that is selectively regulated by exclusion of CD45. In turn, Lck repartitioning from the GEM domains could lead to activation through interaction with CD45. One possible example of activation of Lck by repartitioning is the observed activation of Lck after Jurkat cell stimulation by antibody cross-linking of the CD3 component of the T cell receptor (10, 15). Lck repartitioning may occur since CD3 is present only in the TX-100–soluble fraction (13), and CD3 cross-linking causes CD4–Lck complexes to bind to the T cell receptor complex (14, 32, 42, 50). Consequently, CD4–Lck complexes in the GEM fraction may repartition into the TX-100 fraction for interaction with the T cell receptor. A similar repartitioning of Lck from GEM domains may occur during antigen binding to the T cell receptor.

Kinase-independent roles of Lck may also be affected by its association with GEM domains. For example, Lck is proposed to bind to the phosphotyrosine residues of other proteins through its SH2 domain (11). However, Lck in the GEM domains may not be able to bind to other proteins as a result of occupation of its own SH2 domain by phosphorylated Tyr^{505}. Alternatively, phosphorylated Tyr^{505} may bind preferentially to SH2 domains in other proteins. Thus, association of Lck with GEM domains may affect its interaction with other proteins due to hyperphosphorylation of Tyr^{505}.

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