Src family kinases are suppressed by a “tail bite” mechanism, in which the binding of a phosphorylated tyrosine in the C terminus of the protein to the Src homology (SH) 2 domain in the N-terminal half of the protein forces the catalytic domain into an inactive conformation stabilized by an additional SH3 interaction. In addition to this intramolecular suppressive function, the SH2 domain also mediates intermolecular interactions, which are crucial for T cell antigen receptor (TCR) signaling. To better understand the relative importance of these two opposite functions of the SH2 domain of the Src family kinase Lck in TCR signaling, we created three mutants of Lck in which the intramolecular binding of the C terminus to the SH2 domain was strengthened. The mutants differed from wild-type Lck only in one to three amino acid residues following the negative regulatory tyrosine 505, which was normally phosphorylated by Csk and dephosphorylated by CD45 in the mutants. In the Lck-negative JCaM1 cell line, the Lck mutants had a much reduced ability to transduce signals from the TCR in a manner that directly correlated with SH2-Tyr(P)505 affinity. The mutant with the strongest tail bite was completely unable to support any ZAP-70 phosphorylation, mitogen-activated protein kinase activation, or downstream gene activation in response to TCR ligation, whereas other mutants had intermediate abilities. Lipid raft targeting was not affected. We conclude that Lck is regulated by a weak tail bite to allow for its activation and service in TCR signaling, perhaps through a competitive SH2 engagement mechanism.

According to current models of TCR signaling, the Lck-mediated phosphorylation of the ITAM motifs in the CD3 and TCR chains is responsible for setting into motion the set of events that initiate T cell activation (1–5). However, the precise mechanism by which Lck is induced to carry out this phosphorylation upon TCR ligation has remained unclear.

Several mechanisms for TCR to Lck communication have been proposed, including mechanisms based on CD4/CD8 association (3), lipid raft-based concentration, and CD45-mediated dephosphorylation. However, none of these models can satisfactorily explain more than some of the existing data. There are many examples of TCR signaling without any participation of CD4 or CD8 (4, 6). Perhaps the most striking case is the Jurkat T cell leukemia line, in which the TCR machinery is remarkably efficient in the absence of CD4 or CD8 (4). Although this tumor cell line overexpresses Lck and lacks several protein tyrosine phosphatases, it still requires TCR ligation for Lck to phosphorylate ITAMs and other substrates and for initiation of the signaling cascades that characterize T cell activation (5). Lck-mediated TCR signaling also occurs in untransformed CD4–8 T cells (6), and T cells lacking these coreceptors can be activated by TCR stimulation in vitro. However, in vivo CD4 or CD8 are required because the affinity of the TCR for its ligand often is too low for a productive interaction of T cells with antigen-presenting cells. The association of Lck with these coreceptors may assist Lck function as well, but it is nevertheless clear that a mechanism must exist for the transmission of a signal from the TCR to Lck without the need for CD4 or CD8. Similarly, the TCR can activate the related kinase Fyn to carry out a set of phosphorylation events that are at least partly different (7). Fyn is also activated by an unknown molecular mechanism.

Lipid rafts play an important role as platforms for the assembly of signaling complexes, and much of Lck and Fyn is found in these dynamic structures. However, because the TCR is not raft-associated in resting T cells, and TCR ligation does not change the association of Lck and Fyn with rafts, it is difficult to imagine a mechanism by which TCR to Lck/Fyn communication would be mediated by rafts. Rather, rafts appear to provide the optimal environment for tyrosine phosphorylation of many Lck and Fyn substrates once these kinases have been activated. The role of CD45 is discussed under “Results” and “Discussion.”

Once phosphorylated by Lck, the ITAM motifs serve to recruit a second type of cytoplasmic PTK, ZAP-70 (8, 9), which is subsequently activated by direct phosphorylation at Tyr493 in its activation loop by Lck (10). Up to ten ZAP-70 molecules may cluster on each fully phosphorylated TCR complex. Once activated by Lck, these bound ZAP-70 molecules autophosphorylate, presumably in trans, and thereby create docking sites for...
SH2 domain-containing signaling proteins (11). ZAP-70 also directly phosphorylates a small set of proteins, including the adapter protein LAT (12), the dual-specific phosphatase VHR (13), and perhaps the adapter protein SLP-76 (14).

The Src family PTKs are also responsible for the recruitment and activation of another class of cytoplasmic PTKs, the Tec-related kinases Itk/Emt and Txk/Rlk (15, 16), which are involved in phosphorylation and activation of phospholipase Cγ1 (17, 18). In addition to ITAMs, ZAP-70, and Itk, Src family PTKs have numerous other substrates, including cytoskeletal proteins, regulators of small GTPases, phosphatases, and other signaling molecules, some of which may be independent of ITAM phosphorylation.

Given the central role of Src family PTKs, particularly Lck, in T cell activation, it is not surprising that these kinases are tightly regulated at all levels from transcription and translation (19) to multiple post-translational modifications and controlled subcellular location. Perhaps the best studied regulation is the phosphorylation of an inhibitory tyrosine in the C terminus of Lck, Tyr505 (reviewed in Refs. 3 and 20). Mutation of this residue results in a constitutively active form of Lck, which can transform fibroblasts (21, 22). In T cells, Tyr505 is phosphorylated by the Csk PTK (23) and dephosphorylated by the CD45 phosphatase (24–26). It has been estimated that ~50% of Tyr505 is phosphorylated under physiological conditions in resting T cells (27), with a relatively modest turnover (T½ = ~45 min (28)). In agreement with the notion that the balance between CD45 and Csk is important (29), most CD45-negative T cells fail to respond to TCR stimulation (30–32), whereas increased CD45 expression, e.g. in memory T cells (33), correlates with increased sensitivity to TCR ligation. Conversely, overexpression of Csk efficiently reduces TCR signaling (34, 35), whereas a dominant-negative Csk (36) or Csk knock-down by RNA interference (37) augments it. In addition, a physiological mechanism for immunosuppression by cAMP-inducing stimuli involves a 3-fold activation of Csk through cAMP-dependent protein kinase-mediated phosphorylation of Csk at Ser364 (35).

At the structural level, the phosphorylation of Src family PTKs at their negative regulatory tyrosine (Tyr505 in Lck) promotes the transition of the kinase into an inactive conformation, in which the phosphorylated tyrosine binds the SH2 domain, whereas the adjacent SH3 domain associates with a linker sequence between the SH2 and kinase domain (38). These two interactions force the catalytic domain into an inactive state, in which catalytic residues are displaced from their optimal configuration and the flexible activation loop collapses into the catalytic center of the enzyme to block substrate access.

In addition to this essential role in suppression of the kinase, the SH2 domain of Lck is also required for Lck participation in TCR signaling. Significantly, a Lck mutant with a nonfunctional SH2 domain had an elevated catalytic activity but was unable to phosphorylate cellular proteins in response to TCR triggering (39, 40). These findings suggest that Lck is recruited through its SH2 domain and/or that Lck needs to interact with downstream targets via the SH2 domain. A number of SH2 domain ligands for Lck have been reported, including ZAP-70 (41, 42), TSAd (43, 44), and phospholipase Cγ1 (45), but none of these can satisfactorily explain the need for the SH2 domain for the earliest tyrosine phosphorylation events triggered by the TCR, i.e. ITAM phosphorylation.

Here, we decided to skew the balance between these two opposite functions of the Lck SH2 domain in TCR signaling by strengthening the binding of Tyr(P)505 to the SH2 domain. To this end, we created three mutants of Lck in which one to three amino acid residues following Tyr505 were mutated to amino acids preferred by the SH2 domain. We report that the resulting sequences indeed had better affinity for the SH2 domain and that the mutations strongly affected the ability of Lck to transmit signals from the TCR.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The anti-Tyr(P) mAb 4G10 was from Upstate Biotechnology Inc. (Lake Placid, NY), and the anti-hemagglutinin epitope mAb 12CA5 was from Roche Applied Science (Indianapolis, IN). The hybridoma that produces the OKT3 anti-CD3ε mAb was from the American Type Cell Collection. TPCK-treated trypsin was from Worthington Biochemicals. The Lck expression plasmids were as before (13, 39). Recombinant Csk was as described earlier (46). The clonal Lck antibody (38.10) was a kind gift from Dr O. Acuto (Oxford, UK).

**Plasmids and Site-directed Mutagenesis**—Lck mutants were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). All of the constructs were verified by sequencing.

**Cells**—The Lck-negative subline of Jurkat JCaM1.6 (4) was kept at logarithmic growth in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, l-glutamine, and antibiotics. COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum.

**Transient Transfections**—20 × 10⁶ JCaM1 were transfected with a total of 10–20 μg of DNA by electroporation at 960 microfarads and 240 V. Empty vector was added to control samples to make a constant amount of DNA in each sample. The cells were harvested 2 days after electroporation. COS-1 cells were transfected by lipofection with 10 μg of DNA and grown for 48 h prior to the experiments.

**Immunoprecipitation**—These procedures were as reported before (47, 48). All of the steps were carried out at 0–4 °C. The cells were lysed in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA containing 1% Nonidet P-40, 1 mM Na₃VO₄, 10 μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 100 μg/ml soybean trypsin inhibitor and clarified by centrifugation at 13,000 × g for 10 min. The clarified lysates were preadsorbed on agarose-conjugated goat anti-rabbit IgG or protein G Sepharose. The lysates were then incubated with antibody for 2–4 h, followed by agarose-conjugated goat anti-rabbit IgG or protein G Sepharose. Immune complexes were washed three times in lysis buffer, once in lysis buffer with 0.5 mM NaCl, and again in lysis buffer and either suspended in SDS sample buffer or washed further for kinase assays.

**SDS-PAGE and Western Blotting**—The proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters. The antisera were used at 1:500–1:2000 dilution, and the blots were developed by the enhanced chemiluminescence tech-
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Tryptic Peptide Mapping—Lck was phosphorylated by recombinant Csk (23, 46) in the presence of $[^{32}P]ATP$. The proteins were resolved on 10% SDS gels, transferred onto a nitrocellulose filter, and exposed to film, and the correct band was excised. The filter piece was blocked and digested with TPCK-treated trypsin as described in detail by Luo et al. (49). The resulting phosphopeptides were separated by electrophoresis on cellulose thin layer plates at pH 1.9 for 27 min followed by ascending chromatography in n-butanol/pyridine/acetic acid/water (75:50:15:60) and exposed to film.

NFAT/AP1 Reporter Gene Assays—JCaM1 cells were transfected with NFAT/AP1-luc together with the Lck constructs or empty vector. 24 h post-transfection, the cells were either left unstimulated or treated with anti-CD3ε mAb for 7 h at 37 °C. The luciferase activity was normalized using a cotransfected Renilla luciferase.

In Vitro Kinase Assay—Lck immunoprecipitates were incubated in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MnCl₂, 10 μM ATP) containing 10 μCi of $[^{32}P]ATP$, with or without 2 μg of enolase (Sigma-Aldrich) for 20 min at 30 °C. The proteins were resolved on SDS-PAGE and visualized by autoradiography.

Binding Interactions Measured by Surface Plasmon Resonance—Surface plasmon resonance experiments and data analysis were performed using a BIACORE 2000 instrument and BIAEVALUATION software (BIACore AB, Uppsala, Sweden). All of the experiments were conducted at 25 °C in a running buffer containing 10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, and 0.005% Tween 20, pH 7.4. Immobilization of the different peptides on the CM5 sensor chip surface was performed using the amine-coupling kit according to the manufacturer’s instructions. The surface of the flow cell was activated with EDC/NHS for 15 min, followed by a 20-min injection of 2 mM concentration of each peptide. The chip was then deactivated by a 15-min injection of 1 mM ethanolamine. The reference flow cells were treated identically except that no peptide was added. In a typical experiment 30 μl of Lck-SH2-GST (Santa Cruz) at concentrations ranging between 0.5 and 20 nM, were injected over the sample and reference flow cells at a flow rate of 30 μl/min. The chip surface was regenerated with 0.2% SDS in 50 mM HCl. Each experiment was performed in triplicate. The net surface plasmon resonance signal was obtained by subtracting the signal of the reference flow cell from that in the sample flow cell.

Computer Modeling—A homology model of Lck was obtained using the SWISS-MODEL Server and the crystal structure of Y527-phosphorylated full-length Hck in the inactive closed conformation (50; Protein Data Bank code 2HCK) as template. The last five C-terminal residues (YQQPQ) were omitted from this first model. The obtained model structure, comprising Lck residues Gln⁵⁰₂–Gln⁵⁰⁴, aligned very well with the template structure (RMSD = 0.11). Next, the missing C-terminal tail was added by modeling one amino acid at a time starting with Tyr(P)⁵⁰⁵. After each step, the energy of a 11 Å radius subset was minimized, using Powell method with 0.5 kcal/(mol•Å) termination gradient and MMFF94 force field algorithm. The final Lck model accurately represents the nature of SH2 domain binding and was essentially identical to the crystal structure of Hck (50). Lck mutants were modeled using the same procedure.

RESULTS

Lck Tail Mutants with Improved Tyr(P)⁵⁰⁵-SH2 Domain Binding—The optimal sequence for a phosphopeptide binding the SH2 domain of Src family PTK is pYEEI (51), a sequence quite different from that present in their C-terminal tails (reviewed in 20), pYQQPQ (Lck), pYQQQ (Lyn and Hck), or pYQPG (c-Src, c-Yes, Fyn, Yrk, and c-Fgr). Only murine Blk has a better fit, pYELQ. As one would predict from these differences, the phosphorylated C terminus of c-Src binds to its SH2 domain with a ~100-fold lower affinity than the pYEEI peptide. In the context of full-length Src family PTKs, the affinity is probably improved by intramolecular constraints and the participation of the SH3 domain in the “tail bite” conformation. Nevertheless, this low affinity of the C-terminal phospho-site for the SH2 domain may allow for activation of Src family PTKs by competing ligands with higher affinity for the SH2 domain.

To better understand the structural basis for the interaction of Tyr(P)⁵⁰⁵ of Lck with its SH2 domain, we built a computer model of these parts of Lck using the crystal structure of Hck (50) as template (Fig. 1A). The model shows that residues 499–504 fit into a shallow groove with Thr⁴⁹⁹ and Gln⁵⁰⁴ interacting with a ridge formed by Arg¹⁵⁴ (yellow-orange in Fig. 1A). The phosphopeptide on Tyr⁵⁰⁵ fits into a large basic pocket formed by Arg¹³⁴, Arg¹⁵⁴, Ser¹⁵⁶, Ser¹⁶⁴, Ser¹⁶⁶, His¹⁸⁰, and Lys¹⁸², whereas the C-terminal proline residue lies in a shallow hydrophobic pocket (formed by Leu¹⁶⁵, Val¹⁶⁷, Tyr¹⁸², Ile¹⁸⁴, Ile¹⁹³, Leu²⁰⁵, Tyr²⁰⁹, Leu²¹⁶, and Leu²²⁰). The interactions between the C terminus of Lck and the SH2 domain are mostly mediated by Tyr(P)⁵⁰⁵, explaining the relatively low affinity of interaction.

To strengthen the tail bite of Lck, we created three mutants in which the three amino acids following Tyr⁵⁰⁵ were changed one at a time from the wild-type sequence YQQPQ toward the ideal sequence YEEI. The three mutants had the sequences YQPI, YQEI, and YEEI (Fig. 1B). A fourth mutant had Tyr⁵⁰⁵ changing to phenylalanine (Y505F; sequence FQQPQ). To first determine whether these sequences indeed generate intermediate affinities for the SH2 domain in the anticipated graded manner, we synthesized five 15-amino acid peptides corresponding to the wild-type C terminus (amino acid residues 498–509) and the four mutant versions. These peptides were immobilized on a CM5 chip for surface plasmon resonance measurements in a BIACore 2000 instrument with various concentrations of the SH2 domain. As shown in Fig. 1C, the phosphopeptides indeed bound to the Lck-SH2 with affinities varying from low nanomolar for the pYEEI peptide (average kb 12.7 nM from several measurements) to no binding at all for the pYQPI peptide and 0.032 μM for the pYQEI peptide, respectively. Thus, these sequences have the intended graded affinities.
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Structural Basis for Improved Affinity—To better understand the structural basis for the increased affinity of the mutated C termini to the SH2 domain of Lck, we modeled them using the same approach as for wild-type Lck. In the first mutant, Q508I (Lck-QPI) (Fig. 2A), the conformation of the tail is very similar to that in wild-type Lck (RMSD = 0.03). However, side chain of mutated Ile<sup>508</sup> makes additional hydrophobic interactions with Pro<sup>195</sup> and side chain methylene group of Ser<sup>194</sup>, which most likely accounts for slightly tighter binding of the mutant tail to the SH2 domain.

The second mutant, Q508I/P507E (Lck-QEI) (Fig. 2B), showed more drastic changes in tail conformation (RMSD = 0.46). The carboxylic group of the Glu<sup>507</sup> side chain forms a salt bridge with the guanidinium group of Arg<sup>196</sup> that forces the rest of the tail into a different position. Pro<sup>509</sup> now is no longer sitting in the shallow hydrophobic pocket but rather interacts with residues just outside of it. Moreover, the C-terminal carboxylic group is now in a position to form another salt bridge with the side chain of Arg<sup>196</sup>. Although salt bridges do not necessarily decrease the energy of a protein as a whole, the binding affinity of this mutated Lck tail to the SH2 domain becomes much higher because of energetically favorable Coulombic charge-charge interactions. As a result, the closed (inactive) conformation will be stabilized.

The third mutant (Lck-EEI) (Fig. 2C) with an additional Q506E mutation shows only a little difference in tail conformation compared with the Lck-QEI mutant (RMSD = 0.15), in which the Gln<sup>506</sup> side chain is involved in hydrogen bond interactions with the oxygen atom of side chain Tyr<sup>181</sup>. In the Lck-EEI mutant, the side chain of Glu<sup>506</sup> forms a similar hydrogen bond, which may be a bit stronger because of the charged nature of the carboxylic group. These models are in full agreement with the BIACore measurements and predict that the Lck tail mutants indeed should have strengthened intramolecular tail bite.

Function of Lck Tail Mutants in TCR-induced Activation of Signaling Molecules—To determine how the Lck tail mutants function in TCR signaling, we expressed them in the Lck-deficient JCaM1 cell line and measured the induction of ZAP-70 and Erk2 phosphorylation. As expected, wild-type Lck supported a substantial TCR-induced ZAP-70 activation (Fig. 3A). The three tail mutants showed a graded efficacy that correlated inversely with the strength of the tail bite: Lck-QPI was less efficient than wild-type Lck but was able to increase induced phosphorylation in response to TCR triggering (lanes 5 and 6), whereas Lck-QEI and Lck-EEI were unable to support any TCR-induced increase in ZAP-70 tyrosine phosphorylation (lanes 7–10). In contrast, the Lck-Y505F mutant was more potent than wild-type Lck and caused a higher level of phosphorylation, with a small additional increase upon TCR ligation. All of the lanes contained identical amounts of ZAP-70 (Fig. 3A, lower panel).

TCR-induced Erk2 mitogen-activated protein kinase activation was also affected in a very similar manner, as measured by either kinase assays of immunoprecipitated Erk2 (not shown) or by immunoblotting of lysates with anti-phospho-Erk antibodies (Fig. 3B, upper panel). All of the lanes had identical amounts of Erk2 (Fig. 3B, lower panel).

All of the Lck constructs were expressed at equal levels (Fig. 3C, lower panel). To demonstrate that these reconstituted JCaM1 cells indeed expressed only the mutated Lck molecules, we also immunoblotted the lysates with anti-Tyr(P)505, which showed good recognition of wild-type Lck and Lck-QPI, very faint recognition of Lck-QEI, and no recognition of Lck-EEI and Lck-Y505F (Fig. 3C, upper panel). It is important to note that the loss of reactivity was not due to a loss of Tyr<sup>505</sup> phosphorylation (see below).

These experiments were repeated many times and consistently showed that the Lck mutants had a reduced capacity to transmit signals from the TCR in a manner that correlated with the strength of the tail bite: the stronger the tail bite the weaker the signaling.

Effect of Lck Tail Mutations on Downstream TCR Signaling and Lck Kinase Activity—JCaM1 cells are defective in the trans-activation of the IL-2 gene in response to TCR ligation unless wild-type Lck is reintroduced by transfection (4). To determine the effect of the Lck tail mutants on TCR-induced gene activa-
tion, we transfected JCaM1 cells with an NFAT/AP-1 reporter gene (taken from the IL-2 promoter) and either wild-type Lck or the Lck tail mutants. As shown in Fig. 4A, the three tail mutants showed graded efficacy in inducing luciferase expression compared with wild-type Lck. Expression of all Lck constructs was comparable (Fig. 4A, lower panel). To determine that the observed effects were not the result of reduced Lck kinase activity, possibly resulting from the introduction of mutations in the protein, anti-Lck precipitates were used in an in vitro kinase assay. The tail mutations did not have any effect on Lck kinase activity (Fig. 4B, upper panel) as assessed by autophosphorylation of the Lck proteins as well as transphosphorylation of enolase, an exogenous substrate. All of the Lck proteins were expressed at the same level (Fig. 4B, lower panel).

C-terminal Phosphorylation of the Lck Tail Mutants—To begin to explore the mechanism(s) responsible for the reduced efficacy of the Lck tail mutants in TCR signaling, we measured a number of important aspects of Lck biology. First, we asked whether the tail mutations alter the recognition by the two enzymes that control the phosphorylation of Lck at Tyr505, namely Csk and CD45.

When the Lck mutants were expressed in COS cells and immunoblotted with the 4G10 anti-Tyr(P) mAb, it was clear that the mutants contained somewhat lower amounts of Tyr(P).
than wild-type Lck (Fig. 5A, top panel). They also showed a lower reactivity with a Tyr(P)394 antibody (Fig. 5A, middle panel), in a manner that paralleled the decrease in overall Tyr(P) levels. It therefore seems likely that the tail mutants are less phosphorylated at the activating Tyr394 in non-T cells, whereas Tyr505 phosphorylation appears to be largely unchanged. It should be emphasized that the phosphorylation status of Tyr505 could not be directly evaluated by a Tyr(P)505 antibody because the mutations alter the epitope recognized by this antibody.

To further test the notion that phosphorylation at Tyr505 by Csk is not compromised by the amino acid substitutions, we immunoprecipitated the mutant Lck proteins and compared their ability to be phosphorylated at Tyr505 by recombinant Csk in the presence of [γ-32P]ATP. In these experiments, Csk incorporated equal amounts of 32P into all Lck proteins (Fig. 5B). Analysis of the Lck bands by tryptic peptide mapping also showed that all mutants contained phosphate at Tyr505 (Fig. 5C). As a control for the migration of Tyr505, we included Lck-Y505F in these experiments. The missing Tyr505 spot is indicated with an arrow in the tryptic peptide map of this mutant (Fig. 5C).

Together, these experiments indicate that Csk can recognize the mutated tails and still phosphorylate Tyr505 as efficiently as in wild-type Lck. This result is in agreement with the TCR signaling results. A reduced recognition by Csk would be expected to lead to increased Lck activity rather than the reduced activity we observed.

Dephosphorylation by CD45 Is Not Compromised—Next, we treated the Csk-phosphorylated 32P-labeled Lck tail mutants with recombinant active CD45 to ask whether the strengthened tail bite prevented CD45 from dephosphorylating Tyr505. These experiments showed that CD45 could remove the phosphate as efficiently from all the mutant Lck molecules as from wild-type Lck (Fig. 6A). To ensure that this result was not due to any skewing of Tyr394/Tyr505 ratio or preferential Tyr394 dephos-
phorylation of the mutants, we analyzed the proteins by tryptic peptide mapping, which showed that both Tyr505 and Tyr394 were very rapidly dephosphorylated by CD45 (Fig. 6B). These experiments indicate that CD45 is not sensitive to the nature of the amino acid residues that follow Tyr505. Indeed, CD45 has been reported to dephosphorylate Lck Tyr394 (52) and TCR-ζ (53), which have little in common with the amino acid sequence surrounding Tyr505 in wild-type Lck but resemble more the sequence in the Lck-EEI mutant. Thus, the impaired function of the Lck tail mutants in TCR signaling is unlikely because of a reduced ability of CD45 to dephosphorylate Tyr505 in these mutants.

Because it has been suggested that the participation of Lck in TCR signaling is determined by the amount of already Tyr505-dephosphorylated Lck, we determined how the tail mutations affect the balance between phosphorylated and unphosphorylated Lck molecules using an immunodepletion protocol. Fig. 6C shows that ~50% of wild-type Lck was precipitated by anti-Tyr(P) antibodies from resting T cells (lower panel, lanes 2 and 6), whereas 50% remained in the supernatant after three consecutive anti-Tyr(P) immunoprecipitations. The latter fraction corresponds to the unphosphorylated form (i.e. no reactivity with anti-Tyr(P) antibodies was left; not shown). Upon TCR triggering, this balance changed to 65% (top panel, lanes 2 and 3). The Lck tail mutant behaved very similarly, although the balance between phosphorylated and unphosphorylated Lck was skewed a little toward the unphosphorylated form for Lck-EEI in resting cells. We conclude that the amounts of unphosphorylated Lck prior to TCR triggering was not decreased (but perhaps slightly increased) when the Lck tail mutants were expressed in JCaM1 cells. This is in agreement with the unchanged ability of CD45 to dephosphorylate the Lck tail mutants.

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DISCUSSION

Together, our results demonstrate that the amino acids that follow immediately after Tyr505 are critical for Lck function in TCR signaling and suggest that Lck requires a weak tail bite. The nonoptimal sequence YQPQP in Lck and similar sequences in other Src family PTKs have presumably been selected by evolutionary pressure because they bind the SH2 domain with an affinity that serves the physiological functions of these PTKs best. A weaker binding may allow for more dynamic regulation and for rapid displacement in the presence of other phospho-ligands for the SH2 domain or proteins binding to the tail, e.g., phosphatases like CD45. Indeed a recent study has shown that only a weak affinity for binding of the SH2 domain of Src to its C-terminal inhibitory tyrosine was required to inactivate the kinase. A strong intramolecular interaction could "lock" Src in an inactive form that would not be activated (54).

The simplest model to explain our finding that Lck mutants with an increased tail bite affinity fail to support TCR signaling would be that Lck molecules are recruited and activated by a competitive mechanism, in which a phosphoprotein ligand with a better affinity \( \left( i.e., K_d < 0.3 \mu M \right) \) for the SH2 domain displaces Tyr(\( \Phi \))\textsuperscript{505} and thereby opens up the kinase to activate it (Fig. 7A). Whether phosphate remains on the exposed tail is not important, but because the site is no longer bound by the SH2 domain, it may well be rapidly dephosphorylated as a consequence of SH2 domain occupancy, not as a prerequisite for it. We tested this model by determining the ability of the SH2 domain of the Lck tail mutants to bind exogenous ligands. The YSDP motif, containing Tyr(P)\textsuperscript{319}, of ZAP-70 is a well characterized interactor of the SH2 of Lck (42). We used immunoprecipitated ZAP-70 subjected to an in vitro kinase assay, to induce Tyr\textsuperscript{319} autophosphorylation (55) and performed pull-down experiments with lysates of JCaM1 cells transfected with either Lck-wt or Lck-EEI. The Lck-EEI mutant bound less efficiently to ZAP-70 when compared with the wt protein (data not shown), further supporting the hypothesis that impaired accessibility of the SH2 domain to exogenous interactors can result to reduced biological activity of Lck. A similar pattern was observed with Hck, where it was shown that, unlike the wt protein, a strong tail bite mutant of the kinase was no longer activated by SH2 domain ligands, presumably because an exogenous SH2 ligand could not compete with the intramolecular pYEEI motif (56).

An equally plausible version of our model (Fig. 7B) is that a protein that can serve as a ligand for the SH3 domain of Lck would activate Lck. Because both the SH2 and SH3 domains of Lck are involved in maintaining the suppressed tail bite conformation of Lck, it is possible that the strong tail bite in the Lck-EEI mutant prevents an SH3 domain ligand from opening the closed conformation (Fig. 7C). In either case, our model predicts that Lck can be activated without prior dephosphorylation of Tyr\textsuperscript{505}, as has been shown for Hck activation by the HIV Nef protein (57, 58).

Our experimental approach and conclusions could be compromised by several potential pitfalls, which we have tried to address. First we checked that the subcellular localization of the Lck mutants was not altered. It is well established that the ability of Lck to reside in lipid rafts is a key aspect of its biological function. Depending on the choice of detergent, between 20 and 80% of Lck is detected in the buoyant top layer after ultracentrifugation on a sucrose gradient. Our lipid raft isolation experiments consistently failed to detect any differences between wild-type Lck and the tail mutants (data not shown). Thus, the loss of TCR signaling in cells expressing Lck-QEI or Lck-EEI was not due to a loss of Lck targeting to lipid rafts.

Second, one could argue that Csk recognition of Tyr\textsuperscript{505} may be increased in the mutants leading to a higher level of suppression and therefore reduced TCR signaling. Our data are not

![Figure 7. Models of Lck activation. A, activation of wild-type Lck with a weak tail bite by a competing SH2 domain ligand (1) or by dephosphorylation of Tyr\textsuperscript{505} (2). B, alternatively, wild-type Lck with a weak tail bite could be activated by a competing SH3 domain ligand (1). C, in contrast, Lck-EEI with a strong tail bite cannot be activated by competing SH2 or SH3 ligands but can still be activated by CD45 and autophosphorylation.]
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compatible with this notion. We did not detect any signs of increased Csk action or Tyr505 phosphorylation stoichiometry in vitro or in cells. Based on what is known about Csk, it seems very unlikely that Csk would phosphorylate Tyr505 better if the sequence context would resemble the substrates preferred by Src family kinases, such as the YEEI sequence of our Lck-EEI mutant. We also find that the amount of unphosphorylated Lck is not decreased, perhaps even increased, in cells expressing Lck-EEI. Thus, increased Csk activation is a very unlikely explanation for our findings.

Third, one may ask whether reduced CD45 action is behind the inability of Lck tail mutants to support TCR signaling. We had expected that the Lck-YEEI mutant would be more phosphorylated at Tyr505 than wild-type Lck because we expected that the stronger tail bite would reduce the access of CD45 to Tyr505. This does not appear to be the case. Interestingly, the surface topology of the Lck SH2 domain with the Lck tail bound to it resembles a mirror image of the surface of the D1 domain of CD45 (59), suggesting that the two can come very close to each other without any steric hindrance or charge repulsion.

The CD45 literature is full of controversial data and opposing interpretations. An unresolved question is whether dephosphorylation of Lck-Tyr505 occurs acutely upon TCR triggering or needs to take place in a constitutive manner prior to TCR triggering. In support of the former, it was reported (60) that ligand-induced dimerization of an epidermal growth factor receptor-CD45 chimera expressed in CD45-deficient Jurkat T cells results in abrogation of TCR signaling within seconds. This was proposed to be due to suppression of the phosphatase activity of CD45 and therefore loss of Lck dephosphorylation at Tyr505, suggesting that the turnover of phosphate on Tyr505 is extremely rapid and that continuous Tyr505 dephosphorylation is crucial for ongoing TCR signaling. However, this model is not compatible with the well established notion that Csk leaves the lipid rafts in the immune synapse (36, 61) and with our measurements of Tyr505 phosphate turnover, which had a half-life of ~45 min (28). Even more importantly, many papers have reported that Lck is not acutely dephosphorylated at Tyr505, and some even see an increase in phosphate at this site (62, 63). Of course, these papers do not rule out the possibility that CD45 acutely dephosphorylates some minor pool of Lck, perhaps the 2–5% of Lck molecules involved in TCR signaling at any given moment. Indeed, localized decreases in Tyr505 phosphate in the center of the immune synapse have been reported (64). However, this could reflect exposure of Tyr505 by displacement from the SH2 domain, per our model, rather than a prerequisite for Tyr505 dephosphorylation within the immune synapse.

Finally, if, in fact, TCR ligation induced a rapid CD45-mediated dephosphorylation of Lck at Tyr505, it would raise the question of how the TCR stimulates this function of CD45 and whether indeed CD45 is upstream of Lck in TCR signaling. Evidence for this model is lacking.

Our model for Lck activation by SH2 or SH3 ligands (which is also applicable to Fyn and other Src family kinases) poses a number of questions that can be addressed experimentally. Which protein(s) serves as a link between the TCR and Lck? If the key mediator is an SH2 ligand, is it phosphorylated prior to TCR triggering? Or is it phosphorylated by Lck to provide a positive forward loop? Clearly, the known SH2 and SH3 ligands of Lck should be tested for their capacity to couple the TCR to Lck activation. Candidates include the adapter molecules TShAd (43, 44), HS1/LckBP1 (65), Unc19 (66), the Syk kinase (67), and G-proteins like Gα16 (68). Several of these candidates have already been suggested to play Lck-activating roles in T cells.

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