Lck Regulates the Tyrosine Phosphorylation of the T Cell Receptor Subunits and ZAP-70 in Murine Thymocytes

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Summary

The Src-family and Syk/ZAP-70 family of protein tyrosine kinases (PTK) are required for T cell receptor (TCR) functions. We provide evidence that the Src-family PTK Lck is responsible for regulating the constitutive tyrosine phosphorylation of the TCR ζ subunit in murine thymocytes. Moreover, ligation of the TCR expressed on thymocytes from Lck-deficient mice largely failed to induce the phosphorylation of TCR-ζ, CD3ε, or ZAP-70. In contrast, we find that the TCR-ζ subunit is weakly constitutively tyrosine phosphorylated in peripheral T cells isolated from Lck-null mice. These data suggest that Lck has a functional role in regulation of TCR signal transduction in thymocytes. In peripheral T cells, other Src-family PTKs such as Fyn may partially compensate for the absence of Lck.

Engagement of the T cell receptor (TCR)[1] leads to the activation of two families of protein tyrosine kinases (PTKs) that are essential for the induction of cellular responses (for review see references 1–3). Studies in T cell clones and lines have shown that the Src-family and the Syk/ZAP-70 family are required for TCR-mediated signal transduction processes (2, 3). After TCR engagement, the Src-family PTKs Lck or Fyn are proposed to initiate TCR signaling by phosphorylating tyrosine residues in the cytoplasmic portion of the CD3 and TCR-ζ subunits (4–6). This phosphorylation occurs on two tyrosine residues present in a common signaling motif, termed ITAM (immune receptor tyrosine-based activation motif), which is present as a single copy in CD3γ, -δ, -ε, and in three copies in TCR-ζ (7). The phosphorylation of tyrosines within an ITAM leads to the recruitment of a member of a second family of PTKs, the Syk/ZAP-70 family, to the TCR complex (8–11). This association is mediated by a high-affinity interaction between the tandem Src-homology 2 (SH2) domains of Syk/ZAP-70 and the two phosphotyrosine residues located in an ITAM (11–13).

The importance of ZAP-70 in TCR signaling was initially revealed with the characterization of a ZAP-70 deficiency in humans (14). Thus, peripheral $CD4^+$ T cells isolated from ZAP-70–deficient patients are unable to transduce intracellular signals after TCR engagement (15–17). More recently, ZAP-70 was shown to reconstitute B cell receptor (BCR) signaling in a Syk-deficient cell line (18). Lck and Fyn are also required for phosphorylating ZAP-70, resulting in an increase in the catalytic activity of ZAP-70 (12, 19–21). In fact, the tyrosine phosphorylation of ZAP-70 by Lck or Fyn is absolutely required for lymphocyte antigen receptor functions (20). The SH2 domain of Lck is also capable of binding to phospho-ZAP-70 and phospho-Syk (22, 23). Since Lck is also associated with the CD4 and CD8 coreceptor molecules, the binding of Lck to phospho-ZAP-70 or -Syk may help to coordinate the interaction between the activated TCR complex and the coreceptors, thereby promoting antigen recognition (24). Thus, studies with T cell lines have demonstrated important roles for Lck and/or Fyn in regulating TCR/CD3 subunit phosphorylation as well as in the activation of the Syk/ZAP-70 family of PTKs.

The regulation of TCR/CD3 subunit phosphorylation and Syk/ZAP-70 PTKs by the Src-family PTKs are less well defined in ex vivo thymocytes and peripheral T cells. Lck is expressed at all stages of thymocyte development and is essential for the clonal expansion and the maturation of thymocytes (for reviews see references 1, 25). The requirements for Lck in thymocyte development were firmly established by the targeted disruption of Lck, which led to a block in the expansion of $CD4^+CD8^+$ thymocytes (26). In addition, transgenic mice overexpressing a catalytically inactive form of Lck fail to generate $CD4^+CD8^+$ thymocytes (27). Overexpression of a constitutively active form of Lck can overcome the developmental defects seen in RAG-1 null mice, resulting in normal numbers of $CD4^+CD8^+$ thymocytes (28). As a consequence of its interaction with CD4 and CD8, Lck can also influence the positive and negative selection processes occurring at the $CD4^+CD8^+$

[1] *Abbreviations used in this paper:* BCR, B cell receptor; ITAM, immune receptor tyrosine-based activation motif; PTK, protein tyrosine kinase; PVDF, polyvinyl difluoride; SH2, Src-homology 2.

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stage of thymocyte development (1, 3, 25). Therefore, Lck has several functional roles at multiple stages of thymocyte development and is likely to function in a prominent role in TCR signal transduction in thymocytes.

In contrast to Lck, Fyn is expressed at levels 10-fold lower in immature thymocytes relative to mature T cells (29). Although thymocyte development proceeds normally in Fyn-deficient mice, Fyn appears to influence TCR-mediated signaling events in mature single-positive thymocytes (30, 31). Thus, TCR stimulation of mature thymocytes from Fyn-deficient mice results in diminished calcium and proliferative responses.

Like Lck, ZAP-70 also plays a critical role in T cell ontogeny. For instance, $CD8^+$ T cells fail to develop in ZAP-70–deficient patients, whereas the $CD4^+$ T cells that are detected in the periphery are defective in TCR signaling functions (14–17). Mice rendered deficient in ZAP-70 have normal numbers of $CD4^+CD8^+$ thymocytes but exhibit a complete block in positive and negative selection (32). Therefore, the two families of PTKs implicated in TCR-mediated signal transduction have important functions during thymic development.

In murine thymocytes, and unlike cultured T cell clones and lines, the TCR-ζ subunit is constitutively tyrosine phosphorylated (phospho-ζ) (33–35). In contrast to ζ, the other CD3 subunits are not constitutively phosphorylated, but TCR engagement induces their phosphorylation. Moreover, ZAP-70 is constitutively associated with phospho-ζ, yet TCR ligation is required for the induction of ZAP-70 phosphorylation (36). The constitutive interaction between ZAP-70 and phospho-ζ is not restricted to thymocytes as similar interactions are detected in murine LN T cells (36). The phospho-ζ detected in murine thymocytes and LN T cells migrates with an apparent molecular mass of 21 kD, similar to the form induced in T cell clones stimulated with altered peptide ligand–MHC complexes (37, 38). TCR ligation in thymocytes or agonist peptide stimulation of T cell clones both result in the induction of a second form of phospho-ζ (23 kD), as well as the phosphorylation of ZAP-70 and the CD3 subunits (36–38). These results suggest that the regulation of TCR/CD3 subunit phosphorylation and ZAP-70 activation are important control points for T cell activation and/or positive and negative selection (39).

To determine which PTK is responsible for regulating the constitutive phosphorylation of TCR-ζ and the inducible tyrosine phosphorylation of TCR-ζ, CD3ε, and ZAP-70 in murine thymocytes, we analyzed the phosphorylation status of these molecules in both Lck- and Fyn-deficient mice before and after TCR engagement. We report here that Lck but not Fyn is required for regulating the constitutive tyrosine phosphorylation of TCR-ζ and the inducible phosphorylation of TCR-ζ, CD3ε, and ZAP-70 in murine thymocytes.

Materials and Methods

Cell Lines and Animals. C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Animal Care Facility at the University of California (San Francisco, CA). The Lck- and Fyn-deficient mice were obtained from Drs. Tak Mak (Amgen and the Ontario Cancer Institute, Toronto, Ontario, Canada) and Roger Perlmutter (University of Washington, Seattle, WA), respectively. A full description of their phenotypes has been published (26, 31). The Lck- and Fyn-deficient mice were bred and maintained in microisolator cages at UCSF. Murine thymocytes, LN T cells, and erythrocyte-depleted spleen cells were isolated as previously described (36).

Antibodies and Antisera. The antibodies used for immunoprecipitation, immunoblotting, and FACScan™ analyses are as follows: 145-2C11, CD3ε (American Type Culture Collection [ATCC], Rockville, MD); 4G10, phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, NY); G3 and 6B10.2, TCR-ζ chain (40, 41); 1F6, Lck, kindly provided by Dr. J. Bolen (Bristol-Meyers Squibb, Princeton, NJ); anti-Lyn and anti-Syk polyclonal antisera were generously provided by Dr. Tony DeFranco (UCSF); anti-Fyn polyclonal antisera was kindly supplied by Dr. A. Veillette (McGill University, Montreal, Quebec, Canada); anti-Fyn mAbs and anti-Yes antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein-conjugated anti-CD8α and PE-conjugated anti-CD4 were purchased from Collaborative Biomedical Products, Bedford, MA. Tricolor anti-CD4 and PE–anti-CD8α were obtained from Caltag Laboratories (South San Francisco, CA). PE-conjugated anti-CD3ε (145-2C11) and fluorescein-conjugated anti-CD69 were obtained from PharMingen (San Diego, CA). Horse radish peroxidase–conjugated goat anti–mouse IgG and goat anti–rabbit IgG were obtained from Southern Biotechnology Associates (Birmingham, AL); alkaline phosphatase–conjugated goat anti–rabbit Ig and goat anti–mouse Ig were purchased from BioRad Laboratories (Hercules, CA). For Syk immunoblotting experiments, a polyclonal rabbit antisera generated against a peptide sequence (amino acids 314–339) of the human Syk PTK was used. For Western blots, this antibody was diluted 1:500 in combination with a 1:500 dilution of an antimurine Syk antisera provided by Dr. Tony DeFranco.

Stimulation, Precipitation, and Immunoblotting of Thymocytes and LN T Cells. Preparation of murine thymocytes and LN T cells, antibody-mediated TCR stimulation, as well as immunoprecipitation and Western blotting techniques, have all been described in detail elsewhere (36). For comparative immunoblotting, lysates from the different thymocyte, LN T cell, or spleen cell preparations were prepared, and the protein content of each preparation was subsequently determined with a protein assay kit (BioRad Laboratories). 150 μg of protein was resolved by SDS-PAGE and immunoblotted as described in the figure legends. Pervanadate stimulation of murine thymocytes was performed for 10 min at 37°C essentially as described using a final concentration of 100 μM sodium orthovanadate and 10 mM H_2O_2 (42).

Thymocyte Cultures. Thymocytes were cultured overnight at 37°C in plates coated with PBS or 10 μg/ml of anti-CD3ε (145-2C11). The cells were subsequently harvested and stained with a combination of antibodies to CD69, CD4, and CD8. The expression of CD69 on the $CD4^+CD8^+$ population of cells was determined by electronic gating with a FACScan® flow cytometer.

Results

Lck Regulates the Constitutive and Inducible Tyrosine Phosphorylation of the TCR-ζ Subunit. As previously reported, a proportion of TCR-ζ molecules are constitutively tyrosine phosphorylated and associated with ZAP-70 in murine

thymocytes and LN T cells (Fig. 1, lanes *1–4*) (36). Since the ZAP-70 PTK family member Syk is also expressed in thymocytes and LN T cells, we examined whether Syk was also constitutively associated with phospho-ζ. Fig. 1 demonstrates that, like ZAP-70, a population of Syk molecules are constitutively associated with phospho-ζ in thymocytes (Fig. 1, lanes *5* and *6*). TCR ligation led to the tyrosine phosphorylation of Syk as well as the coprecipitation of the 23-kD form of phospho-ζ (Fig. 1, lanes *5* and *6*) (36). However, consistent with our previous studies, Syk expression is very low in LN T cells, and its association with phospho-ζ was difficult to detect (Fig. 1, lanes 7 and *8*) (9). These findings extend our previous observations demonstrating that both ZAP-70 and Syk are constitutively associated with phospho-ζ in murine thymocytes, and TCR ligation is necessary to induce an appreciable level of ZAP-70 or Syk tyrosine phosphorylation. However, these experiments do not address which PTK is responsible for regulating TCR-ζ phosphorylation.

Since the Lck PTK has been implicated in the phosphorylation of the TCR/CD3 subunit in T cell lines, we examined its role in regulating TCR-ζ phosphorylation in thymocytes and LN T cells from mice lacking Lck. As previously reported, mice rendered deficient in the Lck PTK ($Lck^{-/-}$) exhibit a 10–20-fold reduction in overall thymic cellularity with a significant reduction in mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cell populations when compared with wild-type mice (shown in Fig. 2 for comparative purposes) (26, 28). The targeted disruption of Lck also results in an increased surface expression of the TCR in thymocytes (Fig. 2).

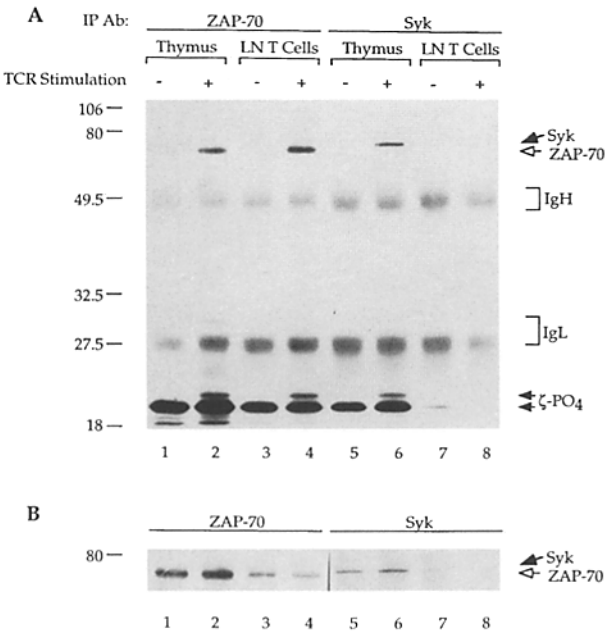

Figure 1. The ZAP-70 and Syk PTKs are constitutively associated with the tyrosine-phosphorylated TCR-ζ subunit. (*A*) Murine thymocytes (lanes *1*, *2*, *5*, and *6*) (2×10^8 cells/lane) or LN T cells (lanes *3*, *4*, *7*, and *8*) (4×10^7 cells/lane) were either unstimulated (lanes *1*, *3*, *5*, and *7*) or stimulated with anti-CD3ε mAbs for 3 min (lanes *2*, *4*, *6*, and *8*) and subsequently lysed in a 1% Triton X-100 containing lysis buffer. ZAP-70 or Syk were immunoprecipitated from the lysates with affinity-purified anti–ZAP-70 or anti-Syk polyclonal antisera. The precipitates were resolved in 12.5% SDS-PAGE, the gels were transferred to polyvinyl difluoride (PVDF) membranes, and immunoblotted with an antiphosphotyrosine mAb (4G10). (*B*) The blots were subsequently stripped and reprobed with anti–ZAP-70 (lanes *1–4*) or anti-Syk antisera (lanes *5–8*).

To directly address whether the constitutive and inducible tyrosine phosphorylation of TCR-ζ (phospho-ζ) in murine thymocytes is regulated by Lck, thymocytes from normal and Lck-deficient mice were lysed, and the TCR/CD3 complexes were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted with an antiphosphotyrosine mAb. In contrast to normal mice, in which the TCR-ζ subunit is constitutively phosphorylated, there was a markedly reduced level of phospho-ζ in thymocytes isolated from $Lck^{-/-}$ mice (Fig. 3 *A*, lane *3* vs. lane *1*). In fact, prolonged enhanced chemiluminescence exposures of 20–30 min were required in order to reveal a small degree of phospho-ζ in the $Lck^{-/-}$ thymocytes. Furthermore, there was no detectable induction of phospho-ζ or tyrosine-phosphorylated CD3ε in the $Lck^{-/-}$ thymocytes after TCR ligation, in contrast to the phosphorylation of CD3ε seen in wild-type mice (Fig. 3 *A*, lane *4* vs. lane *2*). The upper band, which migrates near 28 kD, may correspond to phospho-CD3-δ, although this band comigrates with the Ig light chain. The extremely low levels of phospho-ζ in $Lck^{-/-}$ mice were not attributable to decreases in the amounts of TCR-ζ coprecipitating with CD3ε, since similar levels of nonphosphorylated TCR-ζ (16 kD) were present in both the wild-type and $Lck^{-/-}$ thymocytes (Fig. 3 *B*, lanes *1–4*).

It is possible that thymocytes from Lck-null mice express some TCR complexes that maintain a weak biochemical association with the TCR-ζ subunit, similar to that described for the pre-TCR complex (41, 43–45). In fact, the $CD4^+CD8^+$ thymocytes from $Lck^{-/-}$ mice may include a population of cells expressing the pre-TCR (28, 45). Thus, it was conceivable that phospho-ζ was present in thymocytes from $Lck^{-/-}$ mice, but simply failed to coprecipitate with CD3ε. To examine this possibility, we precipitated the TCR-ζ subunit from the TCR/CD3–depleted lysates with a TCR-ζ–specific mAb. However, we were unable to detect any phospho-ζ in the $Lck^{-/-}$ thymocyte lysates, although some additional phospho-ζ was precipitated from the wild-type mice (Fig. 3 *A*, lanes 7 and *8* vs. *5* and *6*).

We also examined whether the ZAP-70 PTK could be inducibly tyrosine phosphorylated in thymocytes from the Lck-null mice. Lysates were prepared from unstimulated or anti-TCR–stimulated thymocytes. ZAP-70 was precipitated from the lysates with affinity-purified polyclonal antisera (Fig. 3 *C*). In thymocytes from normal mice, TCR ligation results in the tyrosine phosphorylation of ZAP-70 (Fig. 3 *C*, lane *2*). In contrast, we were unable to detect any inducible tyrosine phosphorylation of ZAP-70 in the thymocytes from $Lck^{-/-}$ mice (Fig. 3 *C*, lane *4* vs. lane *3*). However, it was apparent that a small degree of phospho-ζ was coprecipitated with ZAP-70 in both unstimulated and TCR-stimulated $Lck^{-/-}$ thymocytes. To assess whether a

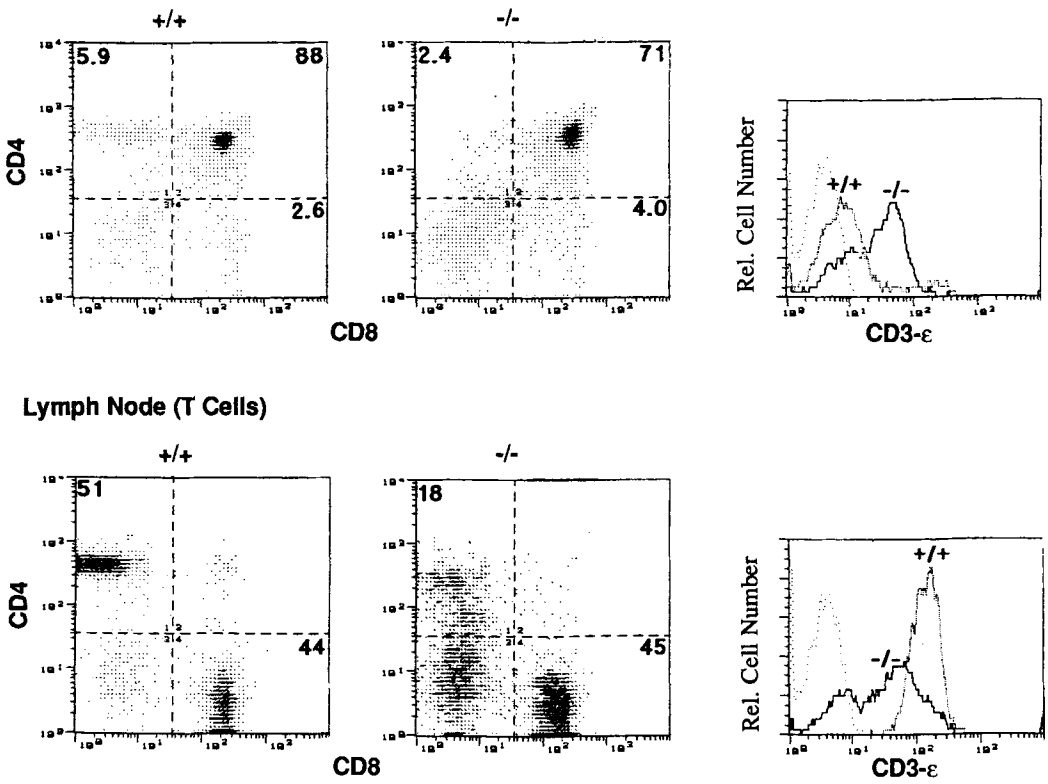

Figure 2. Flow cytometric analysis of thymocytes and LN T cells from wild-type and Lck-deficient mice. Thymocytes from normal C57BL ($^{+/+}$) or Lck-deficient C57Bl ($^{-/-}$) mice (4–6 wk of age) were stained with directly labeled mAbs for CD4, CD8, or TCR/CD3 and analyzed by two- or one-color flow cytometry. For two-color plots, the percentage of cells in each quadrant is listed. For one-color histograms, the dotted line represents staining with a control PE-conjugated mAb. LN were isolated from the same mice whose thymocyte profiles are displayed, depleted of B cells, and the remaining cells were stained as described for thymocytes. The thymic cellularity varied from 5×10^6 to 2×10^7 cells/thymus in $Lck^{-/-}$ mice versus $1–3 \times 10^8$ cells in wild-type mice. LN T cell yields typically varied from $1–2 \times 10^6$ cells in $Lck^{-/-}$ mice to $1–3 \times 10^7$ cells in normal mice.

significant level of phospho-ζ or tyrosine-phosphorylated CD3ϵ (phospho-ϵ) could be elicited in $Lck^{-/-}$ mice, thymocytes were stimulated with the protein tyrosine phosphatase inhibitor pervanadate. Pervanadate treatment of human peripheral blood lymphocytes has been shown to cause dramatic increases in protein tyrosine phosphorylation (42). After pervanadate treatment of thymocytes from normal and Lck-null mice, the cells were lysed, and the TCR complex was immunoprecipitated and blotted with antiphosphotyrosine mAbs. Both the CD3ϵ and TCR-ζ chains were extensively phosphorylated in normal and $Lck^{-/-}$ thymocytes after pervanadate-mediated activation (Fig. 3 *C*, lanes *6* and *8*). However, the levels of phospho-ζ and phospho-ϵ elicited with pervanadate were always two- to fourfold lower in the $Lck^{-/-}$ thymocytes. Therefore, based on these observations, it appears that Lck is the primary PTK responsible for regulating the constitutive and inducible tyrosine phosphorylation of the TCR subunits and the phosphorylation of ZAP-70 in murine thymocytes, although under some circumstances, other PTKs may be able to contribute to these effects.

The Constitutive Tyrosine Phosphorylation of TCR-ζ Occurs in the Absence of the Fyn PTK. Some mature T cells can develop in the thymuses of Lck-deficient mice (26). Moreover, we detected a small amount of phospho-ζ in Lck-deficient thymocytes, and a substantial increase was elicited upon treatment with pervanadate (Fig. 3 *C*). These observations suggested that additional Src-family PTKs or other PTKs may contribute to the regulation of TCR subunit phosphorylation. One Src-family PTK that may mediate these effects is Fyn (1). This is supported by the observation that thymocytes from Fyn-mutant mice exhibit impaired calcium mobilization and proliferative responses after TCR engagement (30, 31). To determine whether the Fyn PTK was also required for regulating TCR-ζ phosphorylation in thymocytes, we compared normal and Fyn-deficient mice for the expression of phospho-ζ. Thymocytes from both types of mice were lysed, and the TCR/CD3 complex was subsequently precipitated with an anti-CD3ϵ mAb. The precipitates were immunoblotted with an antiphoshotyrosine mAb and an anti–TCR-ζ mAb. As shown in Fig. 4, we were unable to detect significant differences in the levels of phosho-ζ or nonphosphorylated TCR-ζ coprecipitating with the TCR complex when comparing thymocytes from normal and Fyn-deficient mice (Fig. 4, *A* and *B*, lanes *1* and *2* vs. *3* and *4*).

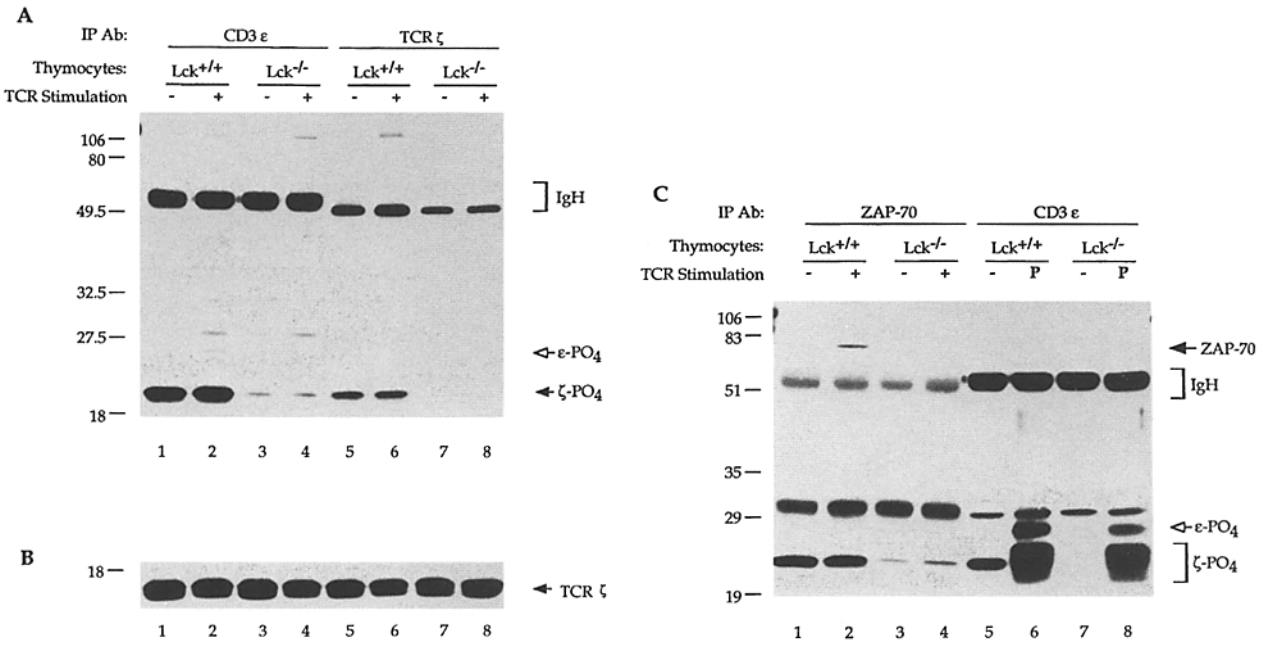

Figure 3. The Lck PTK regulates the constitutive and inducible tyrosine phosphorylation of the TCR-ζ subunit. (*A*) Thymocytes (4×10^7 cells/lane) from normal C57Bl/6J mice (lanes *1*, *2*, *5*, and *6*) or Lck-deficient mice (lanes *3*, *4*, *7*, and *8*) were left untreated (lanes *1*, *3*, *5*, and *7*) or stimulated with anti-CD3ε mAbs for 3 min (lanes *2*, *4*, *6*, and *8*), rapidly pelleted, and subsequently lysed in 0.5% Triton X-100 containing lysis buffers. The TCR/CD3 complex from either unstimulated or TCR-stimulated lysates was sequentially immunoprecipitated with anti-CD3ε mAbs (lanes *1–4*) followed by an anti-TCR-ζ mAb (lanes *5–8*). The precipitates were resolved on 12.5% SDS-PAGE, transferred to PVDF, and blotted with antiphosphotyrosine mAbs. (*B*) The region below 18 kD was blotted with anti-ζ–specific mAbs. The results are representative of four independent experiments. (*C*) Thymocytes from normal (lanes *1*, *2*, *5*, and *6*) or Lck-deficient mice (lanes *3*, *4*, *7*, and *8*) were left untreated (lanes *1*, *3*, *5*, and *7*), stimulated with anti-CD3ε mAb for 3 min (lanes *2* and *4*), or treated with pervanadate for 10 min (lanes *6* and *8*). Lysates were prepared from 7.5×10^7 cells (lanes *1–4*) or 1.5×10^7 cells (lanes *5–8*) and immunoprecipitated with affinity-purified anti-ZAP-70 antisera (lanes *1–4*) or an anti-CD3ε mAb (lanes *5–8*). The precipitates were resolved by SDS-PAGE and Western blotted with an antiphosphotyrosine mAb (4G10).

We also performed immunoblotting experiments to assess whether Fyn or other PTKs, normally implicated in TCR- or BCR-mediated signaling events, may be upregulated in the thymus of Lck-deficient mice. Equivalent amounts of protein from lysates prepared from wild-type and $Lck^{-/-}$ thymocytes, as well as wild-type spleen cells, were resolved by SDS-PAGE and immunoblotted with a number of different antibodies. Thymocytes from $Lck^{-/-}$ mice express no Lck and essentially undetectable levels of Lyn and Yes under the blotting conditions used (Fig. 5 *A*, lane *2*, and data not shown). However, Fyn was expressed at roughly equivalent amounts in both wild-type and Lck-null thymocytes, at levels that are substantially lower when compared with that seen in peripheral LN T cells (Fig. 5 *B*). Thymocytes from the $Lck^{-/-}$ mice also express slightly lower levels of ZAP-70 (Fig. 5 *A*, lane *2* vs. lane *1*). These results are all consistent with previously published findings that Fyn levels are substantially reduced in $CD4^+CD8^+$ thy-

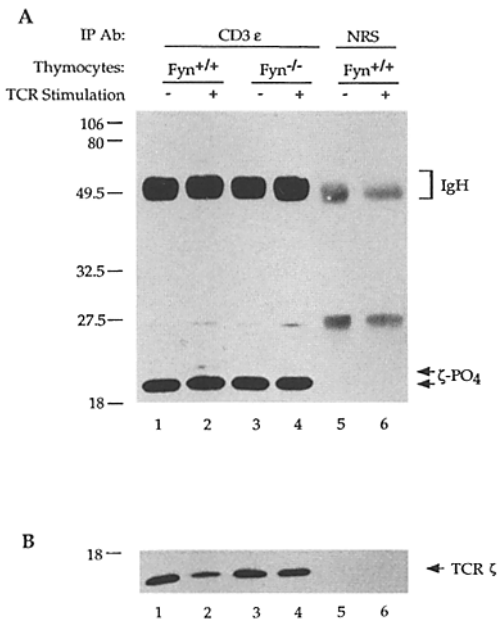

Figure 4. The Fyn PTK is not required for the constitutive tyrosine phosphorylation of the TCR-ζ subunit. Thymocytes (2.5×10^7 cells/lane) from normal C57Bl/6 mice (lanes *1*, *2*, *5*, and *6*) or Fyn-deficient mice (lanes *3* and *4*) were left untreated (lanes *1*, *3*, and *5*) or stimulated with anti-CD3ε mAbs for 3 min (lanes *2*, *4*, and *6*) and processed as described in Fig. 3. The precipitates were resolved on 12.5% SDS-PAGE, transferred to PVDF, and blotted with antiphosphotyrosine mAbs (*A*), whereas the region of the membrane below 18 kD was blotted with anti-ζ–specific mAbs (*B*). The results are representative of three independent experiments.

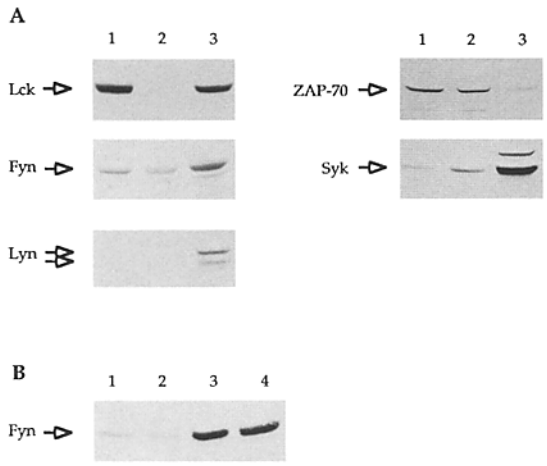

Figure 5. Analysis of Src-family and Syk/ZAP-70 family PTK expression in normal mice and mice lacking Lck. (*A*) Thymocyte lysates (150 μg) from wild-type (lane *1*) and Lck-deficient mice (lane *2*), and spleen cell lysates (150 μg) from wild-type mice (lane *3*), were prepared and resolved on 10% SDS-PAGE. The gels were transferred to nitrocellulose and subsequently immunoblotted with antibodies against the Src-family PTKs Lck, Fyn, and Lyn as well as the Syk/ZAP-70 family of PTKs. Spleen cell lysates (containing both T and B cells) were first depleted of erythrocytes and are included as positive controls for Lyn and Syk expression. (*B*) Thymocyte (lanes *1* and *2*) and LN T cell lysates (lanes *3* and *4*) were prepared from equivalent numbers of cells (5×10^6) from normal (lanes *1* and *3*) and Lck-deficient mice (lanes *2* and *4*). The lysates were separated on 10% SDS-PAGE and subsequently Western blotted with anti-Fyn polyclonal antisera.

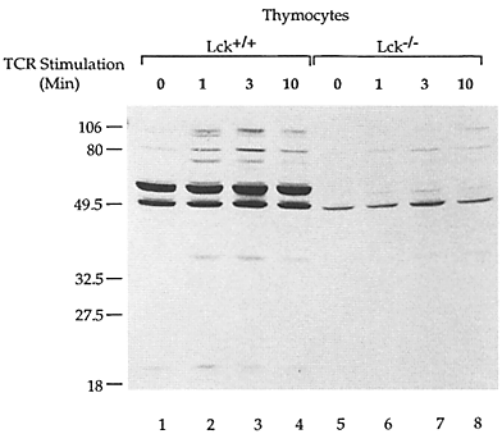

Figure 6. Tyrosine phosphorylation in thymocytes after TCR/CD3 engagement. Thymocytes (10^7 cells/lane) from normal and Lck-deficient mice were left untreated or stimulated with anti-CD3ε mAbs for 1, 3, or 10 min, rapidly sedimented, and whole-cell lysates were prepared. The lysates were loaded onto 12.5% SDS-PAGE gels, subsequently transferred to PVDF membranes, and immunoblotted with an antiphosphotyrosine mAb (4G10).

mocytes, and that the ZAP-70 PTK is expressed at slightly lower levels in immature versus mature T cells (9, 29). Interestingly, Syk was expressed at roughly 1.5–2-fold higher levels in $Lck^{-/-}$ thymocytes relative to unfractionated thymocytes from normal mice (Fig. 5 *A*). In summary, the results demonstrate that the Src-family PTK Lck, but not Fyn, has a specific role in regulating TCR-ζ phosphorylation in thymocytes. However, these results do not rule out the possibility that Fyn may contribute to the small levels of phospho-ζ seen in the Lck-deficient thymocytes. The molecular mechanism underlying the contribution of Fyn to TCR-mediated signaling events in thymocytes has yet to be resolved.

TCR-mediated Signaling in Lck-deficient Mice after TCR Ligation. Since the phosphorylation of TCR-ζ could not be induced in thymocytes from $Lck^{-/-}$ mice after TCR ligation, we were interested in determining whether any tyrosine-phosphorylated proteins could be detected in $Lck^{-/-}$ thymocytes when stimulated with mAbs against the TCR complex. TCR ligation of thymocytes from normal mice results in the tyrosine phosphorylation of a number of phosphoproteins with apparent molecular masses of 110, 95, 80, 70, and 36 kD (Fig. 6, lanes *1–4*). Surprisingly, several of these phosphoproteins are also induced in the $Lck^{-/-}$ thymocytes, although the degree of phosphorylation was less and kinetics of activation were somewhat delayed when compared to wild-type mice (Fig. 6, lanes *6–8* vs. *2–4*). Thus, phosphoproteins of 110, 95, 80, and 36 kD were detected in the $Lck^{-/-}$ cells after TCR ligation. In contrast, almost no constitutive or inducible phosphoproteins of 70, 56, or 21 kD were detected, these proteins likely corresponding to phospho–ZAP-70, phospho-Lck, and phospho-ζ, respectively. These results suggest that additional PTKs can mediate the phosphorylation of several substrates in Lck-deficient thymocytes. We also noted that TCR engagement resulted in the induction of CD69 expression in the $CD4^+CD8^+$ population of thymocytes from Lck-deficient mice, albeit at levels substantially less than wild-type mice (Fig. 7). Importantly, the critical components of TCR-mediated signal transduction processes including TCR-ζ, CD3ε, and ZAP-70 are, at best, only poorly phosphorylated in $Lck^{-/-}$ thymocytes.

LN T Cells from $Lck^{-/-}$ Mice Express a Constitutively Tyrosine-phosphorylated TCR-ζ Subunit. Because small numbers of T cells are also present in the peripheral lymphoid organs of Lck-deficient mice (26), we were interested in assessing their signaling functions. To perform these experiments, LN cells had to be pooled from a large number of $Lck^{-/-}$ mice and enriched for T cells by depleting murine B cells (Fig. 1). Interestingly, we detected a constitutively tyrosine-phosphorylated 21-kD phosphoprotein that comigrated with phospho-ζ in the wild-type mice (Fig. 8, lane *3* vs. lane *1*). By immunodepletion experiments, we have determined that this phosphoprotein is, in fact, tyrosine-phosphorylated TCR-ζ (data not shown). From three independent experiments, we noted that the levels of phospho-ζ in the LN T cells from the $Lck^{-/-}$ mice were always three- to four-fold lower when compared with wild-type mice. This may be a consequence of the lower cell surface TCR density seen in the $Lck^{-/-}$ LN T cells (Fig. 1). Stimulation of the LN T cells from wild-type mice results in the induction of many of the phosphoproteins detected in the stimulated thymocyte cell lysates (Fig. 8, lane *2*, and Fig. 5, lanes *2–4*).

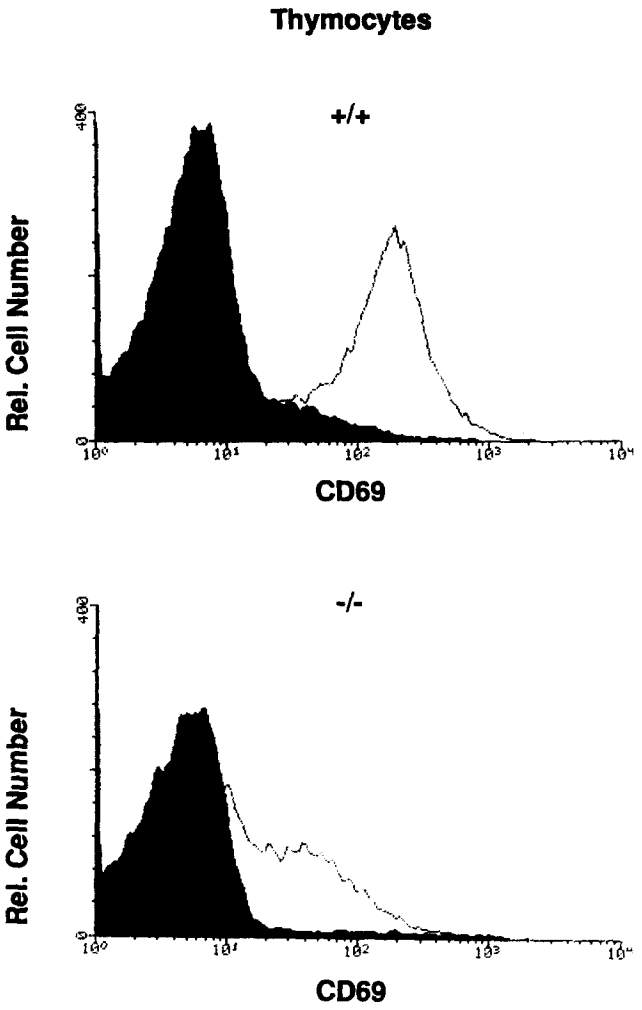

Figure 7. CD69 induction on $CD4^+CD8^+$ thymocytes. Thymocytes (2×10^6 cells/ml) from normal and Lck-deficient mice were incubated at 37°C in plates precoated with an anti-CD3ε mAb. After 20 h, the cells were harvested and stained with a combination of anti–CD69-FITC, anti–CD8α-PE, and anti–CD4-Tricolor. The cells were analyzed for CD69 expression by three-color flow cytometry using software gating on the $CD4^+CD8^+$ population.

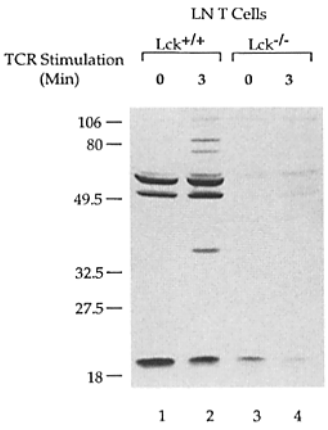

Figure 8. Tyrosine phosphorylation in peripheral T cells after TCR/CD3 engagement. LN T cells (10^7 cells/lane) from normal and Lck-deficient mice were left untreated or stimulated with anti-CD3ε mAbs for 3 min, rapidly pelleted, and whole-cell lysates were prepared. The lysates were loaded onto 12.5% SDS-PAGE gels, subsequently transferred to PVDF membranes, and immunoblotted with an anti-phosphotyrosine mAb (4G10).

In contrast, we were unable to induce any additional phosphoproteins in the $Lck^{-/-}$ LN T cells (Fig. 8, lane *4*). It is currently unclear which PTK is responsible for the constitutively phosphorylated TCR-ζ chain in the absence of Lck, but one likely candidate is Fyn, since Fyn is upregulated in the peripheral T cells relative to thymocytes (Fig. 4 *B*) (29).

Discussion

We have previously shown that ZAP-70 is constitutively associated with a 21-kD form of phospho-ζ in $CD4^+CD8^+$ thymocytes, unfractionated thymocytes, and peripheral LN T cells (36). Stimulation of T cell clones with antagonist peptide–MHC complexes also results in the generation of the 21-kD form of phospho-ζ, which is associated with ZAP-70 (38). In this report, we provide evidence that Lck regulates the tyrosine phosphorylation of the TCR-ζ subunit, the CD3 subunits, and the ZAP-70 PTK in murine thymocytes. Lck is required for regulating the constitutive tyrosine phosphorylation of the TCR-ζ subunit in murine thymocytes. The targeted disruption of Lck also prevents the inducible tyrosine phosphorylation of both the TCR-ζ and CD3ε subunits as well as the ZAP-70 PTK. In contrast to these findings, the constitutive tyrosine phosphorylation of TCR-ζ and the inducible phosphorylation of the TCR/CD3 subunits after TCR ligation appear normal in Fyn-deficient mice. These results demonstrate a specific role for Lck in regulating the tyrosine phosphorylation of the TCR/CD3 subunits and ZAP-70 during thymocyte development. Thus, Lck performs several regulatory roles in thymopoiesis (27, 28, 46, 47).

In the absence of Lck, the constitutive phosphorylation of TCR-ζ is substantially reduced in murine thymocytes, suggesting that TCR-ζ is a direct substrate for Lck. This interpretation is consistent with earlier studies with an Lck-deficient Jurkat T cell mutant and experiments with heterologous COS cell systems as well as in vitro assays (4, 12, 48). However, none of the experiments preclude the possibility that Lck functions upstream of another PTK that phosphorylates TCR-ζ. In fact, a direct coupling between the TCR and Lck has proven extremely difficult to observe, and the molecular mechanism resulting in the constitutive phosphorylation of TCR-ζ remains unclear. In any event, the expression of phospho-ζ leads to the association of ZAP-70 or Syk, which are themselves not tyrosine phosphorylated. In fact, both ZAP-70 and Syk may additionally protect phospho-ζ from protein tyrosine phosphatases. This is consistent with the observation that overexpression of the tandem SH2 domains of Syk or ZAP-70 results in a basal hyperphosphorylation of the ITAMs, which are constitutively associated with the tandem SH2 construct (49, 50). Mapping the sites of TCR-ζ phosphorylation will be important in determining the requirements for ZAP-70 association, phosphorylation, and, presumably, ZAP-70 activation. Although all six tyrosines in TCR-ζ can be phosphorylated by Lck in vitro, the selective phosphorylation of

certain tyrosines in vivo may result in the formation of the 21-kD form of phospho-ζ (51). This may promote ZAP-70 binding without its concomitant phosphorylation and activation.

For thymocytes and LN T cells, the constitutive association between ZAP-70 and phospho-ζ may poise a proportion of TCR complexes to respond to antigenic stimulation. TCR ligation would promote the activation or relocalization of Lck, resulting in the tyrosine phosphorylation of ZAP-70, the CD3 subunits, and an increase in the phosphorylation of TCR-ζ. Notably, in the absence of Lck, we were unable to detect any significant induction in the tyrosine phosphorylation of TCR-ζ, CD3ε, or ZAP-70 in murine thymocytes (Fig. 3). This is in agreement with previously published reports that the TCR/CD3 subunits and ZAP-70 are not tyrosine phosphorylated in an Lck-deficient Jurkat T cell line after TCR ligation (4, 12, 52).

It should be noted that some phospho-ζ is detected in thymocytes from $Lck^{-/-}$ mice, and ZAP-70 is constitutively associated with this small pool of phospho-ζ. In the absence of Lck, the regulation of this phospho-ζ may be attributable to Fyn, which is expressed, albeit weakly, in $CD4^+CD8^+$ thymocytes. Therefore, Fyn may promote the development of some mature T cells in $Lck^{-/-}$ mice by regulating TCR-ζ phosphorylation and, possibly, TCR signaling. Moreover, we detected substantial levels of phospho-ζ in peripheral LN T cells isolated from Lck-deficient mice. The increased expression of Fyn in the peripheral T cells relative to thymocytes is consistent with Fyn compensating for the lack of Lck. In support of this notion, LN T cells from Lck-deficient mice do exhibit TCR-mediated proliferative responses, although at levels four-fold lower than wild-type mice (26). The potential compensation by Fyn may be more definitively addressed with the analyses of mice deficient in both Fyn and Lck. It is also possible that other PTKs may be upregulated or activated in the peripheral LN T cells to compensate for the loss of Lck.

In spite of the presence of phospho-ζ in LN T cells isolated from Lck-null mice, we were unable to detect any phosphoproteins that are induced after TCR ligation. This result contrasts with the observation that several additional phosphoproteins are weakly induced in Lck-deficient thymocytes after TCR engagement. One potential explanation for these differences is the elevated expression of Syk in thymocytes relative to peripheral T cells (9). In fact, Syk expression can promote some TCR-mediated signaling events in ZAP-70–deficient human thymocytes (53). Moreover, Syk can reconstitute some TCR-mediated signals in Lck-deficient cell lines (Chu, D., and A. Weiss, submitted for publication).

In summary, Lck performs several important functions influencing TCR signaling in thymocytes. Lck regulates the constitutive phosphorylation of TCR-ζ and the inducible phosphorylation of the TCR/CD3 subunits as well as Syk/ZAP-70. These functions appear specific to Lck as other Src-family PTKs are unable to compensate fully in the absence of Lck.

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