Regulation of T Cell Receptor Expression in Immature CD4+CD8+ Thymocytes by p56lck Tyrosine Kinase: Basis for Differential Signaling by CD4 and CD8 in Immature Thymocytes Expressing Both Coreceptor Molecules

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Summary

Signals transduced through the T cell antigen receptor (TCR) are modulated by the *src* family tyrosine kinase p56lck (lck), which associates in mature T cells with the coreceptor molecules CD4 and CD8. Here we describe a novel function of lck in immature CD4+CD8+ thymocytes, that of regulating TCR expression. Activation of lck in immature CD4+CD8+ thymocytes by intrathymic engagement of CD4 maintains low TCR expression by causing most TCR components to be retained and degraded within the endoplasmic reticulum. Importantly, activation of lck in immature CD4+CD8+ thymocytes results from engagement of surface CD4 molecules, but not surface CD8 molecules, despite the nearly fourfold greater surface expression of CD8 than CD4. The competence of CD4 to activate lck in CD4+CD8+ thymocytes relates to the fact that a relatively large fraction of surface CD4 molecules (25–50%) are associated with intracellular lck molecules, whereas only 2% of surface CD8 molecules are associated with lck. The amount of lck associated with CD4 in CD4+CD8+ thymocytes is diminished by chronic CD4 engagement in the thymus, as activated lck molecules subsequently dissociate from CD4. Indeed, the amount of lck associated with CD4 in CD4+CD8+ thymocytes is markedly increased in major histocompatibility complex (MHC) class II− mice that lack the intrathymic ligand for CD4 and in which surface CD4 molecules are consequently not engaged. Thus, the present study demonstrates that (*a*) activation of lck in CD4+CD8+ thymocytes regulates distribution and expression of TCR components; (*b*) unlike CD4 molecules, CD8 molecules on CD4+CD8+ thymocytes cannot efficiently activate lck despite their significantly greater surface expression; and (*c*) the amount of lck associated with CD4 in the CD4+CD8+ thymocytes is inversely related to the extent of CD4 engagement by MHC class II molecules in the thymus.

The ability of immature precursor cells in the thymus to differentiate into mature T cells is dependent on the specificity of the TCR molecules that individual precursor cells express. Indeed, the TCR repertoire expressed by mature CD4+ and CD8+ T cells is selected from a broader TCR repertoire expressed by immature CD4+CD8+ thymocytes (1–3). Because the selection of immature CD4+CD8+ thymocytes for differentiation into functional maturity is based on the specificity of their surface TCR complexes, it is paradoxical that most CD4+CD8+ thymocytes express few surface TCR complexes that are only marginally competent to mobilize intracellular calcium (4–6). We have previously demonstrated that the number of surface TCR complexes expressed by CD4+CD8+ thymocytes is regulated, at least in part, by CD4 inhibitory signals generated in vivo (6–8). The molecular mechanism by which CD4 signals regulate TCR expression in immature CD4+CD8+ thymocytes is not known, but it is known that such signals result in retention and degradation of most newly synthesized TCR chains within a pre-Golgi compartment (9).

Because the intracytoplasmic tails of surface CD4 molecules on both mature T cells and immature thymocytes are associated with the *src* family tyrosine kinase $p56^{lck}$ (lck)[1] (10–13), we performed this study to determine if CD4-mediated regulation of TCR expression in immature $CD4^+$ $CD8^+$ thymocytes is transduced by activation of CD4-associated lck molecules. Although modulation of TCR function by CD4- associated lck molecules is well established (14, 15), regulating intracellular distribution and surface expression of TCR molecules by lck would be a previously unappreciated function. The results of this study reveal that in $CD4^+CD8^+$ thymocytes (*a*) CD4-associated lck does indeed regulate the subcellular distribution and abundance of TCR, and (*b*) the status of CD4-associated lck molecules reflects chronic lck activation resulting from interaction of $CD4^+$ $CD8^+$ thymocytes with MHC class II molecules in vivo.

Materials and Methods

Animals. Young adult C57BL/6 (B6) mice were obtained from the National Cancer Institute (Frederick, MD). H-2^b mice lacking MHC class II were generated by targeted disruption of the A^b_β gene in embryonic stem cells as described (16).

Cell Preparation and Culture. $CD4^+CD8^+$ thymocytes were isolated by adherence to plastic plates coated with anti-CD8 mAb (83-12-5) and were >95% $CD4^+CD8^+$ (17). Splenic T cells were enriched by negative selection on plastic plates coated with rabbit anti-mouse IgG (Organon Teknika, Durham, NC). Induction cultures of $CD4^+CD8^+$ thymocytes were cultured at 5×10^6/ml (24-well plates) in RPMI containing 10% FCS for 14 h as indicated. Where specified, cultures contained either anti-CD4 (RL172.4) (18) or anti-CD8 (3.155) (19) mAb culture supernatants at a final concentration of 25%. Where indicated, $CD4^+CD8^+$ thymocytes were cultured with the tyrosine kinase inhibitors herbimycin A (HA) (0.5 μM) or geldanamycin (GDA) (0.05 μM), which were the kind gifts of Dr. Paul Booth (GIBCO BRL, Gaithersburg, MD), or the metal chelator *o*-phenanthroline (OP) (5 μM) (Sigma Chemical Co., St. Louis, MO), all of which were dissolved in DMSO. Cultures not containing drugs were treated with the DMSO vehicle control. Cultures treated with antibodies, drugs, or the two in combination were preincubated at 4°C for 1 h before transfer to 37°C. At the doses used, there was no significant effect of drug treatment on cell viability.

Short-term antibody stimulation of cells to evaluate kinase activity was performed on cells cultured for 14 h at 37°C. $CD4^+CD8^+$ thymocytes were stimulated at 5×10^6/ml in a solution of 25% mAb supernatant in complete medium for the times indicated (2–180 min), after which the reaction was quenched with ice-cold HBSS containing 5% FCS and phosphatase inhibitors (1 mM Na_3VO_4 and 2 mM EDTA).

Immunoelectron Microscopy. Ultrastructural localization of CD3-ϵ in purified $CD4^+CD8^+$ thymocytes and splenic T cells was performed using preembedding immunoperoxidase electron microscopy as described (20). Briefly, cultured cells were fixed, permeabilized, and incubated with anti-CD3-ϵ (500.A2) (4), followed by biotinylated goat anti–hamster Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 1:150 and horseradish peroxidase–conjugated avidin (Enzo-diagnostics, Farmingdale, NY). Bound horseradish peroxidase was visualized by reaction with diaminobenzidine HCl (Fisher Scientific, Fairlawn, NJ) as described (21).

Flow Cytometry. For quantitative immunofluorescence of $CD4^+CD8^+$ thymocytes, 10^6 cells were washed in staining buffer (0.2% BSA and 0.1% NaN_3 in HBSS lacking phenol red) and incubated at 4°C for 8 h with unlabeled Ab at concentrations empirically determined to be saturating (~0.5 $\mu g/10^6$ cells for each Ab used). After extensive washing, bound Ab was visualized with FITC-conjugated goat anti–rat Ab, which was also used at saturation. Cells were analyzed on a FACStar® Plus (Becton Dickinson & Co., San Jose, CA) using four-decade logarithmic amplification.

Surface Labeling. $CD4^+CD8^+$ thymocytes were washed twice with ice-cold PBS and incubated at 4°C for 30 min at 50×106 cells/ml in PBS containing 0.1 mg/ml water-soluble Bolton Hunter reagent (Pierce, Rockford, IL). The reaction was quenched by addition of HBSS containing 5% FCS and 0.1 mg/ml lysine. Cells were washed twice in PBS and iodinated using lactoperoxidase as described (22).

Immunoprecipitations and Immune Complex Kinase Assays. Cells were lysed at 100×10^6 cells/ml in lysis buffer (50 mM Tris, pH 7.4, 0.3M NaCl, 1 mM Na_3VO_4, 50 mM NaF, 10 mM $NaH_2P_2O_7$, 20 μg/ml aprotinin, and 10 μg/ml leupeptin) containing either digitonin (Wako Bioproducts, Richmond, VA) or Triton X-100 (Research Products International, Mt. Prospect, IL) at 1%. After 20 min on ice, lysates were clarified in a microfuge for 10 min at 4°C. Clarified postnuclear supernatants were agitated at 4°C for 1 to 2 h with Ab preadsorbed to beads (20 μl packed resin). Anti-CD4 (GK1.5) (23) and anti-CD8 (53-6-72) (24) were adsorbed to protein G–Sepharose (Sigma Chemical Co.); anti-CD3-ϵ (145-2C11) (25) and rabbit anti-lck (26) were adsorbed to protein A–Sepharose. After incubation, the beads were washed three times in lysis buffer lacking EDTA and incubated for 3 min at room temperature in kinase buffer (20 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 2 μM NaATP, and 15 μCi γ-[^{32}P]ATP per reaction; 7,000 Ci/mMol, ICN Biomedicals, Costa Mesa, CA). Kinase reactions were quenched with 50 μl 3X SDS–PAGE sample buffer and resolved on 8% gels. To remove free ^{32}P, gels were equilibrated against five to six changes of 10% methanol/10% acetic acid for a total of 6 h, dried, and autoradiographed at −80°C.

Immunoblotting. Samples intended for immunoblotting were boiled for 3 min in SDS sample buffer and resolved by SDS–PAGE. After electrophoresis, gels were blotted onto nitrocellulose (0.45 μm; Schleicher and Schuell, Keene, NH) at 4°C 50 V for 3 h in a transphor apparatus (model TE52; Hoefer Scientific Instruments, San Francisco, CA) in transfer buffer containing 50 mM Tris, pH 8.3, 0.384 M glycine, 20% methanol, and 0.01% SDS. Alternatively, samples to be blotted with anti-TCR-α (H28-710) (27) were electrophoresed nonreduced on minigels and transferred to immobilon PVDF (Millipore Corp., Bedford, MA) for 30 min at 150 mA using a transfer system (Polyblot; American Bionetics, Hayward, CA). After transfer, blots were blocked for 1 h at room temperature in 5% milk protein/PBS and then probed for 2 h with primary Ab diluted in 5% milk protein (1:200 for anti-lck, 1:4 for anti-TCR-α, and 1:400 for anti-TCR-ζ (28). The blots were washed three times (total, 10 min) with PBS/0.1% Triton X-100 and then incubated 1 h with ^{125}I-protein A (60 μCi/μg, ICN Biomedicals) at 1 μCi/ml in 5% milk protein/PBS, after which they were washed as above (five times for a total of 45 min). Antiphosphotyrosine blots were performed as above except that the blot was blocked in 50 mM Tris-buffered saline, pH 7.4, containing 3% BSA. Also,

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; FU, fluorescence units; GDA, geldanamycin; HA, herbimycin A; lck, $p56^{lck}$; NE, nuclear envelope; OP, o-phenanthroline.

the antiphosphotyrosine mAb (4G10, 1 μg/ml) (UBI, Lake Placid, NY) and ^{125}I-protein A were diluted in Tris-saline containing 0.5% BSA, and the blots were washed in the same buffer containing 0.2% NP-40.

Enzyme Digestions. Alkaline phosphatase digestions (24 U/reaction, Boehringer Mannheim, Indianapolis, IN) were performed on immune complexes bound to beads for 12 h at 37°C in 50 μl of 50 mM Tris, pH 8.5, containing 0.1 mM EDTA.

Results

CD4-mediated Regulation of TCR Expression in Immature CD4$^+$CD8$^+$ Thymocytes Requires Tyrosine Kinase Activity. The number of surface TCR complexes expressed by immature CD4$^+$CD8$^+$ thymocytes is regulated by CD4 signals generated in vivo (6, 7). Immature CD4$^+$CD8$^+$ thymocytes can be experimentally "released" from intrathymic CD4 signaling by physical removal from the thymus and placement in in vitro suspension cultures where CD4 is disengaged. In fact, CD4$^+$CD8$^+$ thymocytes spontaneously increase surface TCR expression by three- to fivefold within hours of being placed in short-term 37°C single-cell suspension cultures, referred to as induction cultures (6, 9). Release of CD4$^+$CD8$^+$ thymocytes from in vivo–generated CD4 signaling did not occur in 4°C suspension cultures, such that 4°C-cultured CD4$^+$CD8$^+$ thymocytes remained uninduced and resembled freshly isolated CD4$^+$CD8$^+$ thymocytes.

To identify the cellular compartment in which most TCR components are retained in CD4$^+$CD8$^+$ thymocytes, we performed immunoelectron microscopy with an anti-CD3ε mAb (Fig. 1). Whereas CD3ε chains were predominantly visualized on the surface of mature spleen T cells (Fig. 1 *B*), most CD3ε chains in uninduced CD4$^+$CD8$^+$ thymocytes were localized to either the endoplasmic reticulum (*ER*) or the nuclear envelope (*NE*), the outer membrane of which

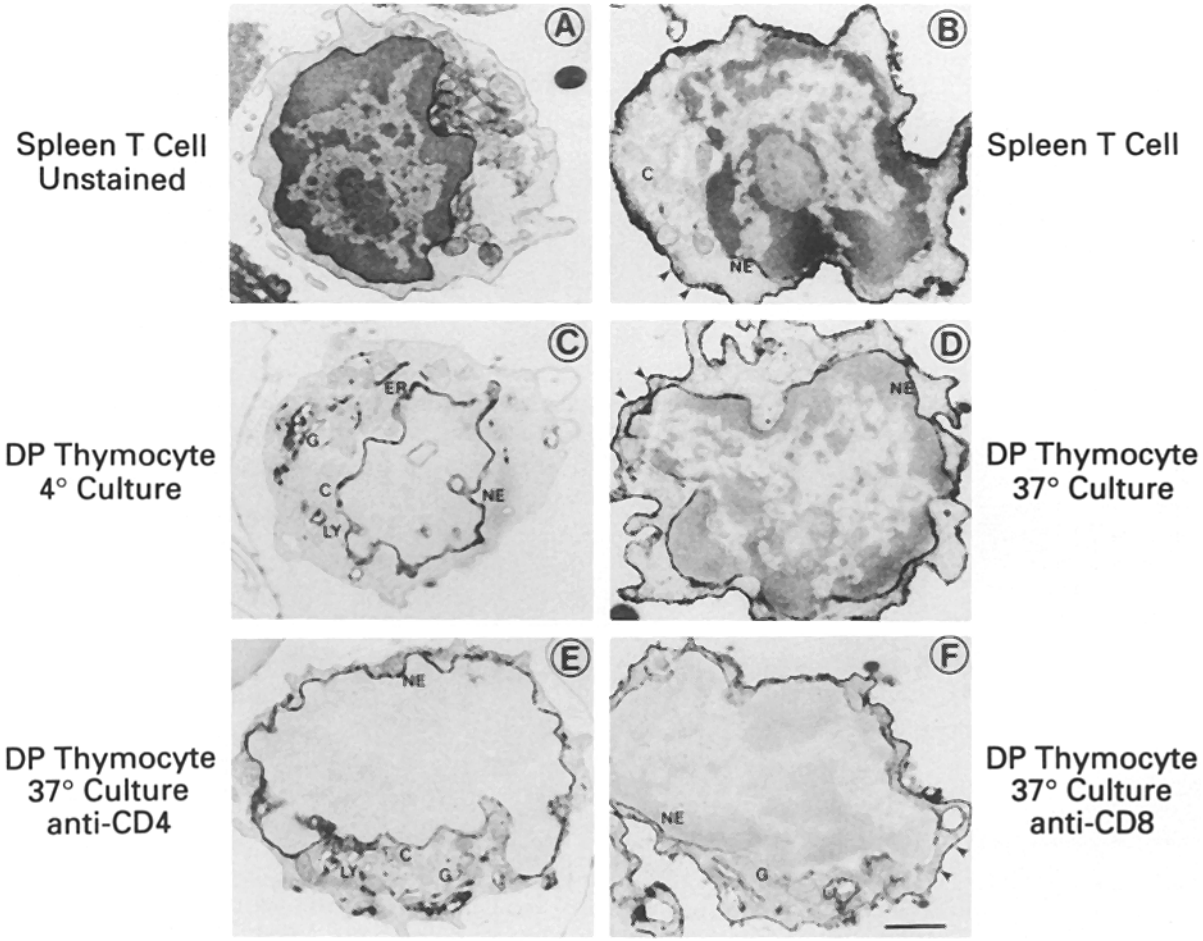

Figure 1. Intracellular distribution of TCR components in immature CD4$^+$CD8$^+$ thymocytes is regulated by CD4. CD3ε was localized within mature spleen T cells and immature CD4$^+$CD8$^+$ (*DP*) thymocytes by immunoelectron microscopy by staining with anti-CD3ε (500A.2 mAb), goat anti–hamster IgG, and horseradish peroxidase–conjugated avidin. CD4$^+$CD8$^+$ thymocytes were cultured for 14 h in single-cell suspension in the absence or presence of IgM anti-CD4 or IgM anti-CD8 mAb as indicated. (*Arrowheads*) Cell surface staining. (*C*) Centriole; (*G*) Golgi apparatus; (*LY*) lysosome; (*ER*) endoplasmic reticulum; (*NE*) nuclear envelope. Bar, 1 μm.

is equivalent to the ER (Fig. 1 *C*). In <10% of $CD4^+$ $CD8^+$ thymocytes, CD3ε chains were also detected in Golgi (*G*) membranes and lysosomes (*L*), but always with significantly less intensity than the staining of the ER (Fig. 1 *C*). Induction of $CD4^+CD8^+$ thymocytes in short-term 37°C cultures resulted in a significant fraction of CD3ε chains shifting from the ER to the plasma membrane (Fig. 1 *D*), although some ER staining persisted, unlike mature spleen T cells. The molecular redistribution of CD3ε from the ER to the plasma membrane during induction was effectively prevented by mAb cross-linking of surface CD4 molecules (Fig. 1 *E*) but was unaffected by mAb cross-linking of surface CD8 molecules (Fig. 1 *F*). Immunoperoxidase staining, though not strictly quantitative, nevertheless clearly revealed differences between intracellular and plasma membrane expression of CD3ε in these different groups of $CD4^+CD8^+$ thymocytes. Indeed, these results identify the ER as the organelle retaining most CD3ε chains in uninduced $CD4^+$ $CD8^+$ thymocytes and demonstrate that the subcellular distribution of TCR components in $CD4^+CD8^+$ thymocytes is regulated by CD4-mediated signals.

The cytoplasmic tail of CD4 is associated with the *src* family tyrosine kinase lck, and CD4 cross-linking activates intracellular lck molecules to tyrosine phosphorylate a number of substrates (10, 12). To determine if CD4 regulation of TCR expression in immature $CD4^+CD8^+$ thymocytes is mediated by tyrosine kinases such as lck, we examined the effect of tyrosine kinase inhibitors on the ability of CD4 to regulate TCR expression. We measured the abundance of TCR-α/β heterodimers in $CD4^+CD8^+$ thymocytes by performing immunoblots on detergent-solubilized cell lysates with an anti-TCR-α mAb. The abundance of TCR-α/β significantly increased on induction of $CD4^+CD8^+$ thymocytes (Fig. 2 *A*, lanes *1* and *2*), and this increase was specifically inhibited by signals generated by multivalent anti-CD4 mAb but not anti-CD8 mAb (Fig. 2 *A*, lanes *3* and *4*). Interestingly, the ability of anti-CD4 mAb to regulate TCR levels in $CD4^+CD8^+$ thymocytes was largely eliminated by both HA and its structural homolog GDA ([29]; Fig. 2 *A*, lanes *5–8*), which function by targeting selected tyrosine kinases for degradation (30). Indeed, these drugs did reduce cellular lck levels in these $CD4^+CD8^+$ thymocytes (Fig. 2 *B*). Analysis of TCR surface expression by flow cytometry yielded analogous results (data not shown).

Because CD4 regulation of TCR expression in immature $CD4^+CD8^+$ thymocytes most likely involved CD4-associated lck molecules, simply dissociating lck from CD4 should be sufficient to interfere with CD4 regulation of TCR expression, even without changing total cellular amounts of lck. Consequently, we used the metal chelator OP, because

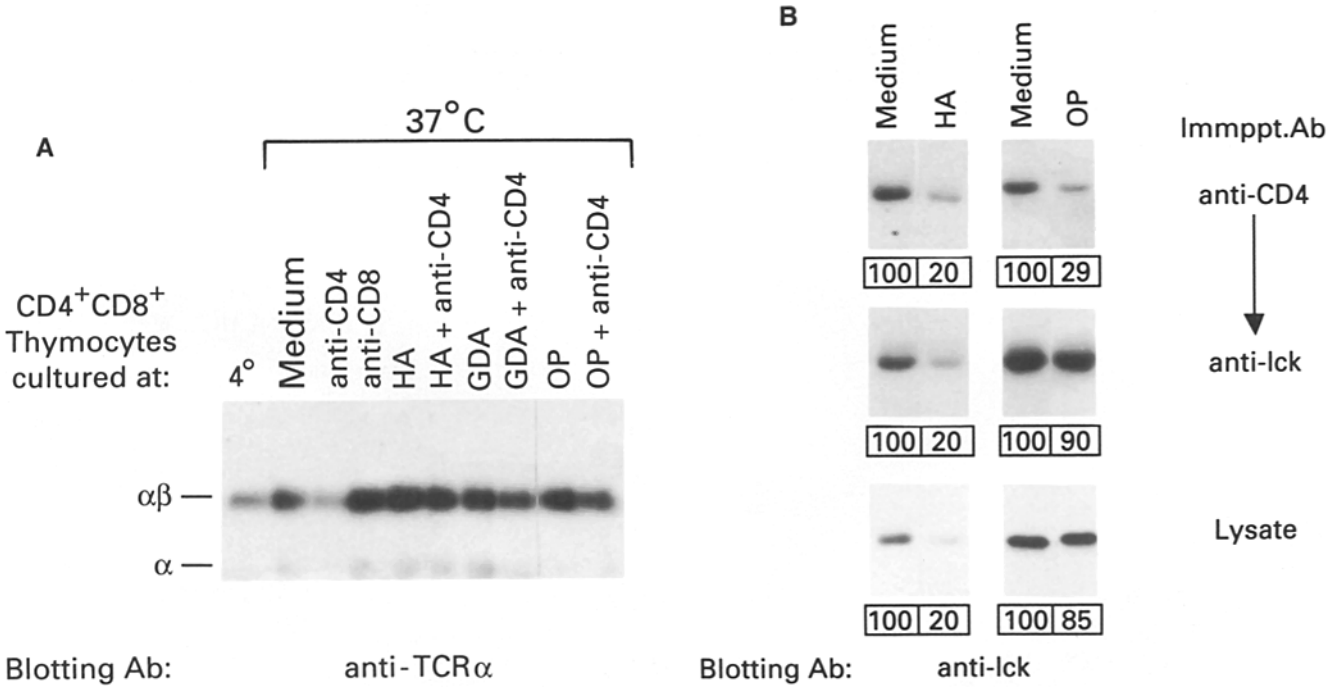

Figure 2. TCR regulation by CD4 requires CD4-associated tyrosine kinase activity. $CD4^+CD8^+$ thymocytes were cultured for 14 h in suspension at either 4°C or 37°C in either the absence or presence of Ab or drug supplements. The added Ab were IgM anti-CD4 (RL172.4) and IgM anti-CD8 (3.155); the added drugs were HA (500 nM), GDA (50 nM), or OP (5 μM). All drugs were solubilized in DMSO and, at the doses indicated, represented 1:2,000-fold dilutions of stocks. DMSO was added as a vehicle control at a final concentration of 0.05% to all cultures not containing a drug supplement. (*A*) TCR-α levels were determined in cell lysates that were electrophoresed nonreduced, blotted with anti-TCR-α (H28-710) mAb, and visualized by ^{125}I-protein A followed by autoradiography. (*B*) lck abundance was determined in cell lysates either directly (*bottom*) or after sequential immunoprecipitations with anti-CD4 (GK1.5) (*top*) and rabbit anti-lck Ab (*middle*). Samples were electrophoresed under reducing conditions, blotted with rabbit anti-lck Ab, and visualized by ^{125}I-protein A followed by autoradiography. Relative band intensities were determined by densitometry. ($\alpha\beta$) Disulfide-linked TCR-α/β heterodimers; (α) TCR-α monomers.

OP has been shown to dissociate lck from CD4 in cell-free extracts as well as having other effects (31). OP treatment of viable cells (with no effect on cell viability) significantly decreased the amount of CD4-associated lck in $CD4^+CD8^+$ thymocytes without affecting total cellular levels of lck (Fig. 2 *B*). Interestingly, OP treatment abrogated the ability of CD4 to regulate TCR expression in $CD4^+CD8^+$ thymocytes (Fig. 2 *A*, lanes *9* and *10*), and did so in a dose-dependent manner that paralleled disruption of lck–CD4 associations (data not shown). Taken together, these data indicate that CD4 regulation of TCR expression in immature $CD4^+CD8^+$ thymocytes is dependent on a CD4-associated tyrosine kinase, presumably lck, because lck is the only tyrosine kinase known to associate with CD4.

Preferential Association of lck with CD4 in $CD4^+CD8^+$ Thymocytes. Because lck can associate with the cytoplasmic tails of both CD4 and CD8, the ability of CD4 but not CD8 to regulate TCR expression in $CD4^+CD8^+$ thymocytes required explanation. To understand this functional disparity between CD4 and CD8, we performed two kinds of experiments: we examined the fraction of CD4 and CD8 molecules on $CD4^+CD8^+$ thymocytes that were associated with lck, and, reciprocally, we examined the fraction of lck molecules in $CD4^+CD8^+$ thymocytes that were associated with CD4 and CD8.

Because lck activation is thought to require lck aggregation, the ability of CD4 or CD8 cross-linking to activate lck is critically dependent on the percentage of surface CD4 or CD8 molecules that are associated with lck. Consequently, we measured the fraction of surface CD4 and CD8 molecules on freshly isolated $CD4^+CD8^+$ thymocytes that were immunoprecipitable by anti-lck Ab (Fig. 3 *A*). Interestingly, in different experiments, 25–50% of surface-iodinated CD4 molecules were associated with lck, whereas only 2% of surface-iodinated CD8-α molecules on $CD4^+CD8^+$ thymocytes were associated with lck. CD8β chains, which cannot associate with lck, were present in anti-lck immunoprecipitates because of their disulfide linkage to CD8α, which can associate with lck (32). Thus, the occupancy of surface CD4 molecules by lck is greater than the occupancy of CD8 by lck in $CD4^+CD8^+$ thymocytes. These results predict that CD4 cross-linking would far more effectively aggregate and activate associated lck molecules in $CD4^+CD8^+$ thymocytes than would CD8 cross-linking.

Reciprocal experiments comparing the relative amounts of lck associated with CD4 and CD8 molecules revealed only marginal preferential association of lck with CD4 in uninduced $CD4^+CD8^+$ thymocytes (Fig. 3 *B*, lanes *1* and *3*). Abundance of lck protein in anti-CD4 precipitates was only 2.5 times that in anti-CD8 precipitates. The relative distribution of lck between CD4 and CD8 that we observed in Fig. 3 *B* was less skewed toward CD4 than we expected given the large difference in the fraction of surface CD4 and CD8 molecules associated with lck that we observed in Fig. 3 *A*.

One possible explanation was that surface expression of CD8 on uninduced $CD4^+CD8^+$ thymocytes was significantly greater than that of CD4. Consequently, we measured the surface densities of CD4 and CD8 by indirect immunofluorescence and quantitative flow cytometry under conditions in which all reagents were saturating and at equilibrium. Uninduced $CD4^+CD8^+$ thymocytes were stained with saturating doses of two sets of isotype-matched rat anti-mouse Ab reactive with either CD4 or CD8 and visualized by a single fluoresceinated goat anti-rat developing reagent (Table 1). We found that uninduced $CD4^+CD8^+$ thymocytes expressed approximately four times as much sur-

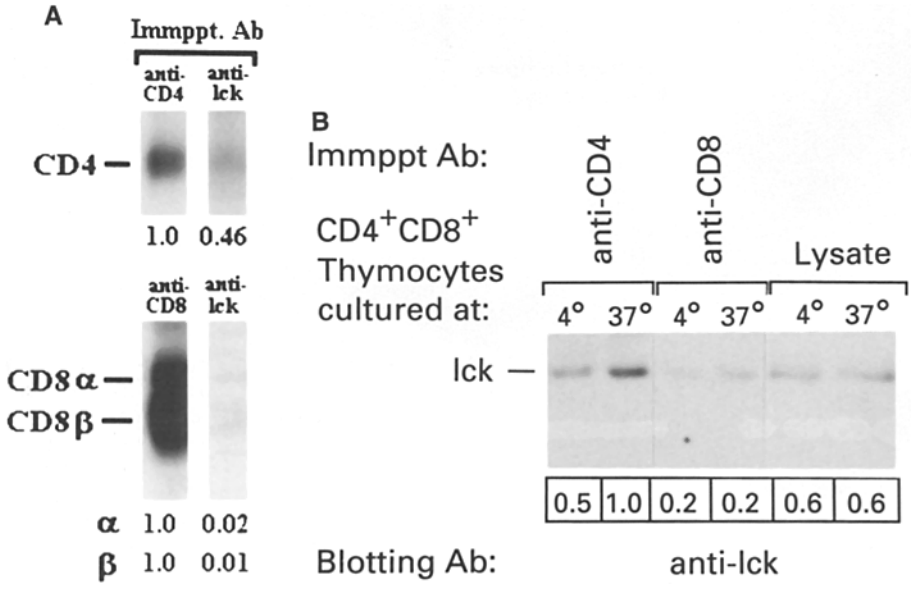

Figure 3. Association of lck with CD4 and CD8 molecules in immature $CD4^+CD8^+$ thymocytes. (*A*) Fractional association of surface CD4 and CD8 molecules with intracellular lck in $CD4^+CD8^+$ thymocytes. Freshly isolated $CD4^+CD8^+$ thymocytes were surface labeled with ^{125}I after treatment with Bolton Hunter reagent. Triton X-100 extracts were immunoprecipitated with anti-CD4 (GK1.5), anti-CD8 (53-6-72), or anti-lck COOH-terminal serum (UBI), electrophoresed under reducing conditions, and visualized and quantified by phosphorimaging. (*B*) Association of lck with CD4 and CD8 in $CD4^+CD8^+$ thymocytes. $CD4^+CD8^+$ thymocytes were cultured in single-cell suspension for 14 h at either 4°C or 37°C, after which the pool sizes of CD4-associated, CD8-associated, and total lck were determined. Triton X-100 extracts were immunoprecipitated with anti-CD4 (GK1.5), anti-CD8 (53-6-72), or anti-lck and evaluated by blotting with anti-lck as in Fig. 2 *B*. Lysate samples (3×10^6 cell equivalents) were blotted directly and contain only one quarter the material of that in immunoprecipitated samples.

Table 1. *Relative Surface Densities of CD4 and CD8 on CD4⁺CD8⁺ Thymocytes*

Antibody	Specificity	Isotype	FU × 10^{-3}
2.43	Anti-CD8	IgG2b	6,137
GK1.5	Anti-CD4	IgG2b	1,800
53-6	Anti-CD8	IgG2a	5,831
MT4	Anti-CD4	IgG2a	1,656

Cells were stained with saturating doses of the above antibodies (23, 24), which were then visualized with a fluoresceinated goat anti-rat second antibody. Fluorescence intensity was translated to linear fluorescence units (FU) such that FU = cell frequency × median intensity. Median intensity was derived by conversion of median logarithmic channel numbers to linear units via a calibration curve empirically derived for each logarithmic amplifier used. Negative control antibody fluorescence was subtracted.

face CD8 as CD4, providing one explanation for why lck distribution was less skewed toward CD4 than expected.

We also considered the possibility that recent in vivo CD4 signals in CD4⁺CD8⁺ thymocytes had reduced the amount of lck associated with CD4 in these cells. Indeed, placement of CD4⁺CD8⁺ thymocytes in 37°C induction cultures in order to permit them to recover from in vivo signaling events did result in an additional twofold increase in CD4-associated lck, whereas the abundance of CD8-associated lck was unaffected by the induction cultures (Fig. 3 *B*, lanes *2* and *4*). It should be noted that total levels of lck in cell lysates did not change in the induction cultures (Fig. 3 *B*, lanes *5* and *6*).

These results demonstrate that lck associates preferentially with CD4 in CD4⁺CD8⁺ thymocytes, but this preferential association with CD4 is actually obscured by two factors: (*a*) surface expression of CD8 is approximately fourfold greater than that of CD4, and (*b*) the amount of lck associated with CD4 in uninduced CD4⁺CD8⁺ thymocytes is half that observed in induced CD4⁺CD8⁺ thymocytes, possibly as a result of previous in vivo cell interactions and signaling events.

Cross-Linking of CD4 on CD4⁺CD8⁺ Thymocytes Activates Associated lck Molecules, Which Then Dissociate. To examine the possibility that CD4 signals generated in vivo might have been responsible for the unexpectedly low amount of CD4-associated lck detected in CD4⁺CD8⁺ thymocytes, we examined the effect of acute and prolonged CD4 cross-linking on the activity and abundance of CD4-associated lck. As measured by autophosphorylation of lck and transphosphorylation of enolase, the kinase activity of CD4-associated lck in induced CD4⁺CD8⁺ thymocytes increased significantly within 2 min of anti-CD4 cross-linking and, in a separate experiment, remained elevated even after 120 min of anti-CD4 cross-linking (Fig. 4). However, by 180 min of anti-CD4 cross-linking, the kinase activity of CD4-associated lck declined (Fig. 4, *top*). As measured in protein immunoblots, anti-CD4 cross-linking for ≥60 min reduced the amount of CD4-associated lck protein, with the amount decreasing progressively the longer the cross-linking persisted (Fig. 4, *bottom*). Despite the decrease in amount of CD4-associated lck, normalization of CD4-associated lck kinase activity to CD4-associated lck protein levels at each time point after cross-

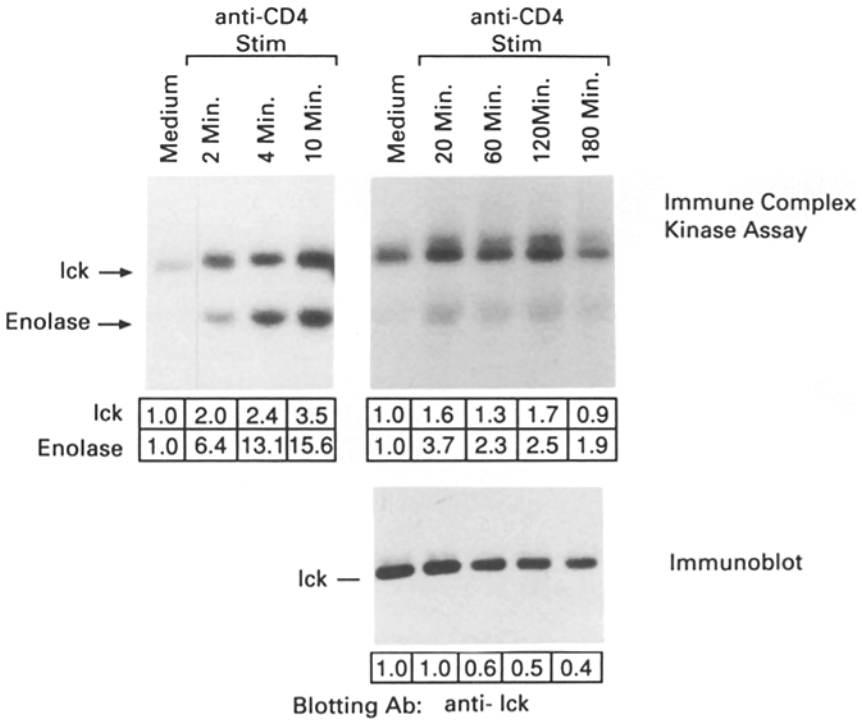

Figure 4. Kinetics of lck activation and dissociation after CD4 engagement. CD4⁺CD8⁺ thymocytes were cultured in single-cell suspension for 14 h at 37°C after which they were stimulated for varying times with IgM anti-CD4 (RL172.4). Triton X-100 extracts were immunoprecipitated with anti-CD4 (GK1.5) and evaluated for enzyme activity by immune complex assay and for lck abundance by blotting as in Fig. 3. Relative band intensities were determined by densitometry. Enolase was used as an exogenous substrate added to the immune kinase assay.

linking revealed that the specific activity of CD4-associated lck molecules was increased by anti-CD4 cross-linking and remained high for the duration of CD4 cross-linking. Thus, mAb engagement of surface CD4 molecules activates CD4-associated lck molecules, which then dissociate from CD4. Indeed, dissociation of activated lck from CD4 may be an integral part of the signal transduction process.

Differential Ability of Anti-CD4 and Anti-CD8 Cross-Linking to Induce Tyrosine Phosphorylation of Associated lck Molecules in CD4⁺CD8⁺ Thymocytes. Although anti-CD4 cross-linking affected lck activity as measured in vitro by the immune kinase assay, it was important to verify that anti-CD4 cross-linking affected lck activity in vivo in $CD4^+CD8^+$ thymocytes. To do so, we examined the ability of anti-CD4 cross-linking to induce in vivo tyrosine phosphorylation of CD4-associated lck molecules, as lck activation is correlated with its phosphorylation of tyr394 (33, 34). In agreement with the in vitro kinase assay, anti-CD4 cross-linking acutely induced tyrosine phosphorylation of CD4-associated lck molecules in $CD4^+CD8^+$ thymocytes, and phosphorylation decreased after 120 min of cross-linking (Fig. 5 *A*). Susceptibility of the phospho-lck band to digestion with alkaline phosphatase confirmed that antiphosphotyrosine reactivity was indeed due to phosphorylation (Fig. 5 *B*). Phosphorylation of CD4-associated lck molecules by anti-CD4 cross-linking was specific in that it was not induced by anti-CD3 cross-linking (Fig. 5 *A*). Importantly, unlike anti-CD4 cross-linking, anti-CD8 cross-linking was unable to induce tyrosine phosphorylation of associated lck molecules (Fig. 5 *A*). Thus, anti-CD8 cross-linking, unlike anti-CD4 cross-linking, is unable to efficiently activate associated lck molecules, consistent with low fractional occupancy of CD8 by lck in $CD4^+CD8^+$ thymocytes.

$CD4^+CD8^+$ Thymocytes from MHC Class II^- Mice Lacking the Natural Ligand for CD4 Have Induced Levels of TCR and CD4-associated lck. Prolonged anti-CD4 cross-linking of $CD4^+CD8^+$ thymocytes in vitro replicated the lck phenotype of uninduced $CD4^+CD8^+$ thymocytes in vivo in that it resulted in reduced amounts, activity, and tyrosine phosphorylation of CD4-associated lck. Consequently, we wished to determine if the phenotype of uninduced $CD4^+CD8^+$ thymocytes in vivo was the result of chronic CD4 engagement in the thymus, presumably from interaction with thymic epithelial cells, which express MHC class II, the natural ligand for CD4. The generation of MHC class II^- mice by targeted disruption of $I\text{-}A^b_\beta$ genes made it possible to examine $CD4^+CD8^+$ thymocytes from mice whose surface CD4 molecules could not have been engaged by intrathymic MHC class II (16).

Surface TCR expression is known to be increased on $CD4^+CD8^+$ thymocytes from MHC class II^- mice (35), consistent with the absence in vivo of inhibitory CD4 signals.

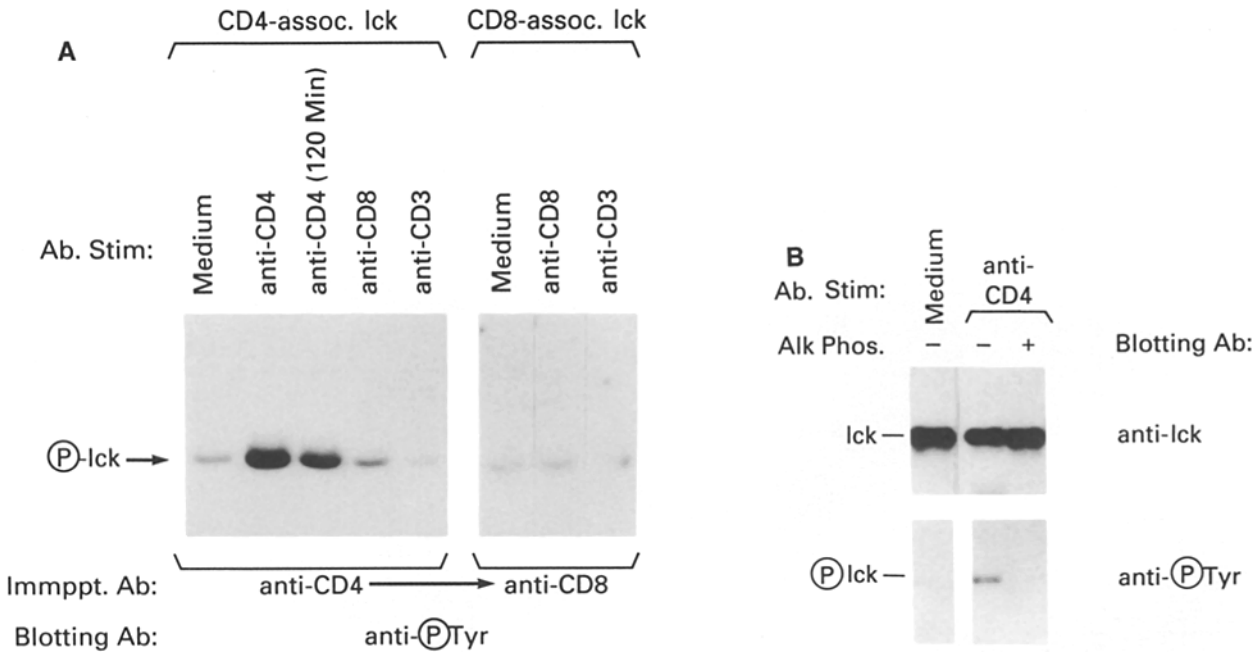

Figure 5. Relative abilities of CD4 and CD8 on $CD4^+CD8^+$ thymocytes to induce autophosphorylation of intracellular lck. Cultured $CD4^+CD8^+$ thymocytes were stimulated for 20 min or as indicated with IgM anti-CD4 (RL172.4), IgM anti-CD8 (3.155), or anti-CD3 (145-2C11). (*A*) Triton X-100 extracts were sequentially immunoprecipitated with anti-CD4 (GK1.5) and anti-CD8 (53-6-72), electrophoresed under reducing conditions, and then blotted with antiphosphotyrosine mAb (4G10). (*B*) Anti-CD4 immunoprecipitates of Triton X-100 extracts were either mock digested (–) or digested (+) with alkaline phosphatase (*Alk Phos.*), electrophoresed under reducing conditions, and blotted with either anti-lck or antiphosphotyrosine (4G10). The stimulating Ab, which did not induce phosphorylation of lck, were otherwise active in that anti-CD3 stimulation induced the phosphorylation of TCR-ζ in $CD4^+CD8^+$ thymocytes, and anti-CD8 stimulation activated associated lck molecules in $CD8^+$ splenic T cells. (*Ⓟlck*) phospho-lck; (*anti-PTyr*), antiphosphotyrosine antibody.

Because such signals are thought to contribute to the retention and degradation of newly synthesized TCR components, total cellular as well as surface TCR levels should be increased in $CD4^+CD8^+$ thymocytes from MHC class II^- mice. We found that uninduced $CD4^+CD8^+$ thymocytes from MHC class II^- (−/−) mice did contain signfiicantly higher levels of TCR components, as indicated by increased amounts of TCR-α/β dimers in cell lysates, and did contain more assembled TCR complexes, as determined by increased amounts of CD3-associated TCR-ζ chains (Fig. 6 *A*). In fact, TCR levels in uninduced $CD4^+CD8^+$ thymocytes from MHC class II^- mice were comparable to those from fully induced $CD4^+CD8^+$ thymocytes from normal mice (+/+) (Fig. 6 *A*) and did not exhibit any significant additional increase on placement in 37°C induction cultures. Thus, in the absence of intrathymic MHC class II molecules to induce CD4 signaling, TCR expression in $CD4^+CD8^+$ thymocytes is increased in vivo and does not change significantly in short-term 37°C induction cultures.

Finally, we used MHC class II^- thymocytes to confirm the effect of intrathymic CD4 signaling on intracellular association of lck with CD4. The amount of CD4-associated lck in uninduced $CD4^+CD8^+$ thymocytes from MHC class II^- mice (−/−) was significantly higher than that from normal mice (+/+) (Fig. 6 *B*, lanes *1* and *4*), and was even higher than the induced levels of CD4-associated lck in normal thymocytes after 37°C suspension cultures (Fig. 6 *B*, lanes *2* and *4*). Indeed, placement of MHC class II^- $CD4^+CD8^+$ thymocytes into 37°C suspension cultures did not increase the amount of CD4-associated lck (Fig. 6 *B*, lanes *4* and *5*), consistent with MHC class II^- $CD4^+CD8^+$ thymocytes being already fully induced. Importantly, surface CD4 molecules on MHC class II^- thymocytes were functionally competent, as indicated by the fact that anti-CD4 cross-linking induced lck dissociation, precisely as in normal thymocytes (Fig. 6 *B*, lanes *3* and *6*). Finally, surface CD4 levels were also increased on uninduced $CD4^+CD8^+$ thymocytes from MHC class II^- mice and did not increase further in induction culture (Table 2), as would be expected when CD4 is not previously engaged in vivo.

These results confirm four important points: (*a*) in vivo regulation of TCR levels by CD4 in $CD4^+CD8^+$ thymocytes is dependent on tyrosine kinase activity and involves, at least in part, intrathymic interactions with MHC class II, the natural ligand for CD4; (*b*) MHC class II–induced CD4 signals in $CD4^+CD8^+$ thymocytes promote the dissociation

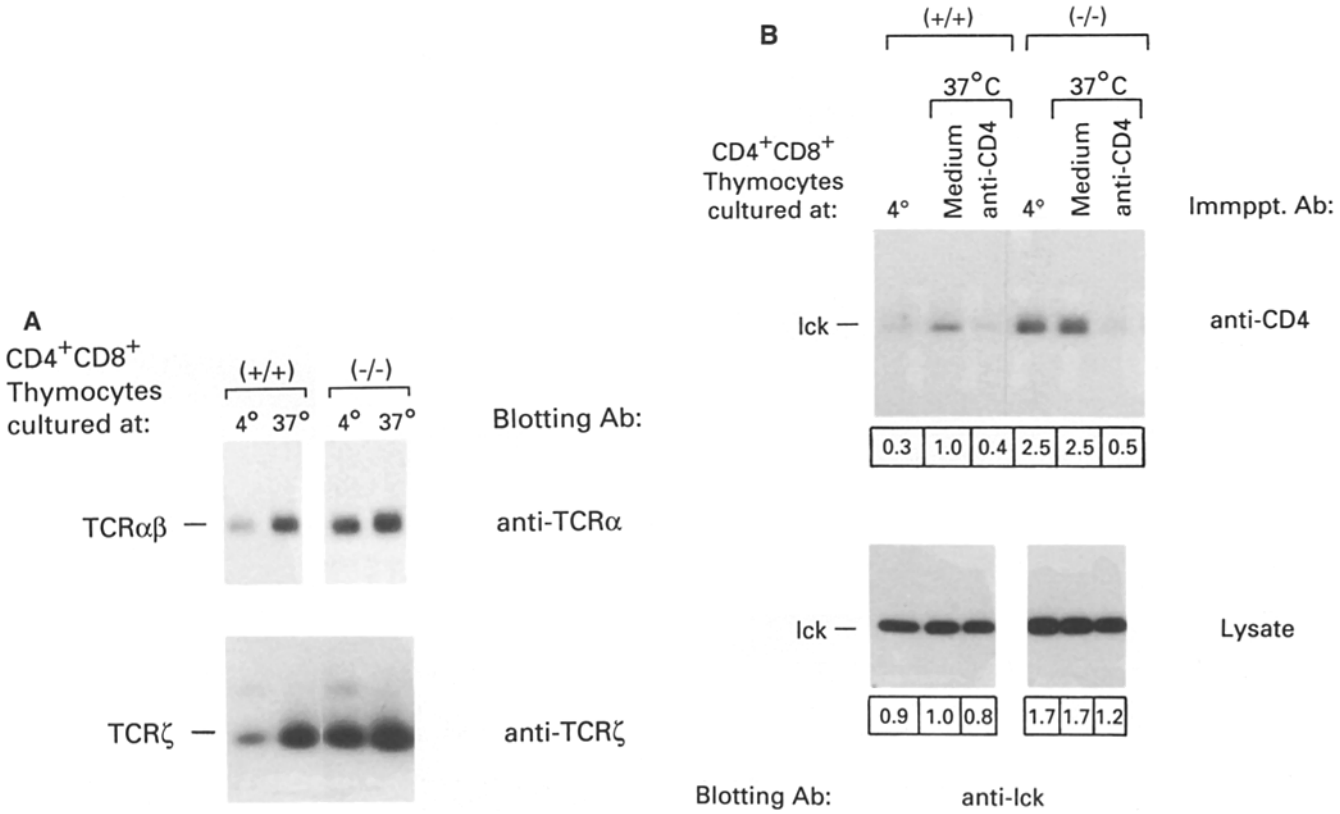

Figure 6. Abundance of TCR and lck in immature $CD4^+CD8^+$ thymocytes from normal and MHC class II^- mice. $CD4^+CD8^+$ thymocytes from normal (+/+) and MHC class II^- (−/−) mice were cultured in single-cell suspension for 14 h at either 4°C or 37°C. (*A*) TCR-α levels were determined in cell lysates that were electrophoresed, nonreduced, and blotted with anti-TCR-α (H28-710) mAb. TCR-ζ levels were determined in anti-CD3 immunoprecipitates, electrophoresed under reducing conditions, and blotted with anti-TCR-ζ antiserum. Both plots were visualized by ^{125}I-protein A followed by autoradiography. (*B*) Triton X-100 extracts were either immunoprecipitated with anti-CD4 (GK1.5, *top*) or resolved directly (*bottom*) on SDS–PAGE and blotted with anti-lck, which was visualized by ^{125}I-protein A. Relative band intensities were determined by densitometry.

Table 2. *Surface Expression of CD4 on CD4⁺CD8⁺ Thymocytes from Normal (+/+) and MHC Class II⁻ (−/−) Mice*

Cultured $CD4^+CD8^+$ thymocytes	Antibody added to culture	Surface expression of CD4	
		(+/+)	(−/−)
		FU × 10^{-3}	
4°C		276	526
37°C		349	474
37°C	Anti-CD4	183	222

Cells were stained with FITC-conjugated anti-CD4 mAb (RM4.5) which binds to a CD4 epitope distinct from that of RL172.4, the anti-CD4 mAb added to culture. Fluorescence intensity was translated to FU as in Table 1.

of lck from CD4; (*c*) MHC class II engagement of surface CD4 molecules on $CD4^+CD8^+$ thymocytes reduces surface CD4 expression; and (*d*) the inductive effect of 37°C short-term suspension cultures on $CD4^+CD8^+$ thymocytes permits their recovery from intrathymic CD4 signaling events, because such cultures had no discernible effect on cells from MHC class II^- mice in which in vivo CD4 engagement had not occurred.

Discussion

This study has investigated the role of CD4-associated lck in regulating TCR expression in immature $CD4^+CD8^+$ thymocytes, and has examined the activation of CD4-associated lck molecules by CD4 engagement. CD4, but not CD8, cross-linking regulated the intracellular distribution and abundance of TCR molecules in immature $CD4^+CD8^+$ thymocytes, and this regulation required a CD4-associated tyrosine kinase. The only tyrosine kinase known to associate directly with CD4 is lck, which can associate with either CD4 or CD8, both of which are present on $CD4^+CD8^+$ thymocytes (13). However, lck occupancy of surface CD4 molecules was significantly greater than lck occupancy of surface CD8 molecules on $CD4^+CD8^+$ thymocytes, thus explaining the differential abilities of CD4 and CD8 to regulate TCR expression through lck activation. Importantly, the preferential association of lck with CD4 on $CD4^+CD8^+$ thymocytes in vivo was obscured by chronic intrathymic CD4 engagement, which resulted in the activation of CD4-associated lck molecules that then dissociated from CD4. Indeed, comparison of CD4-associated lck levels in $CD4^+CD8^+$ thymocytes from normal mice and MHC class II^- mice indicated that surface CD4 molecules on normal $CD4^+CD8^+$ thymocytes were chronically engaged by intrathymic MHC class II, resulting in lck activation/dissociation and low TCR expression. Thus, this study demonstrates that TCR expression in immature $CD4^+CD8^+$ thymocytes is regulated by lck, and provides one mechanism by which lck activation can influence thymocyte development.

The identification of lck as the signal-transducing molecule regulating TCR expression in $CD4^+CD8^+$ thymocytes is an important step in understanding how TCR expression and T cell development are regulated during thymic development, because it indicates that tyrosine phosphorylation of a critical intracellular substrate in immature thymocytes affects either the assembly or intracellular transport of nascent TCR chains. Because TCR ζ chains are tyrosine phosphorylated upon lck activation (12) and also influence TCR expression (36, 37), it was a particularly attractive possibility that lck might regulate TCR expression by regulating the phosphorylation status of TCR ζ chains. However, we have recently excluded this possibility, because tyrosine-phosphorylated TCR ζ chains are only associated with cell surface TCR complexes in $CD4^+CD8^+$ thymocytes and so cannot directly regulate assembly or transport of nascent TCR complexes (38). Consequently, the identity of the tyrosine-phosphorylated substrates that regulate TCR assembly or transport in $CD4^+CD8^+$ thymocytes remains to be elucidated. Whereas tyrosine kinases are typically thought of as signal-transducing molecules, tyrosine kinase activity has been shown to regulate intracellular transport and degradation of the epidermal growth factor receptor (39).

Although CD4 is expressed at significantly lower levels than CD8 on $CD4^+CD8^+$ thymocytes, a relatively large fraction of surface CD4 molecules are associated with lck, allowing CD4 to efficiently activate lck. Thus, the signaling competence of CD4 on $CD4^+CD8^+$ thymocytes results from the high fractional occupancy of CD4 by lck, which is maintained by removing from the cell surface those CD4 molecules lacking lck. The association of lck with the cytoplasmic tail of CD4 prevents the entry of CD4:lck into coated pits, thus preventing endocytosis (40). Conversely, "empty" CD4 molecules lacking associated lck do enter coated pits and are rapidly cleared form the cell surface (41). In this study, we have shown that chronic engagement of CD4 activates associated lck molecules, which subsequently dissociate from CD4, generating "empty" CD4 molecules; empty CD4 molecules are then internalized, ensuring that lck occupancy of surface CD4 remains high and that surface CD4 remains competent to transduce intracellular signals. Indeed, release of $CD4^+CD8^+$ thymocytes from intrathymic CD4 engagement (either by placement in induction cultures or by development in an MHC class II^- thymus) results in increased lck association with CD4 and increased CD4 surface levels.

Whereas CD8 is abundant on the surface of $CD4^+CD8^+$ thymocytes, most surface CD8 molecules are not associated with lck. Low lck occupancy of CD8 in $CD4^+CD8^+$ thymocytes may result from several factors: (*a*) because CD4 and CD8 are coexpressed within the same cell and lck preferentially binds to CD4, CD4 may compete directly with CD8 for available lck molecules (42); (*b*) CD8α is the CD8 component that binds lck but, in $CD4^+CD8^+$ thymocytes, CD8α exists in equal amounts of two alternative mRNA splice forms, one of which contains a truncated cytoplasmic tail lacking the lck binding site (43); and (*c*) "empty" CD8

molecules lacking lck do not appear to be selectively cleared from the cell surface. Thus, low occupancy of CD8 by lck provides an explanation for the inability of CD8 on $CD4^+CD8^+$ thymocytes to activate associated lck molecules (34), which, in turn, explains the failure of CD8 to regulate TCR expression in $CD4^+CD8^+$ thymocytes.

Differentiation of immature $CD4^+CD8^+$ thymocytes into mature $CD4^+$ or $CD8^+$ T cells involves a selection step based on the specificity of the TCR molecules that individual $CD4^+CD8^+$ thymocytes express. This process is thought to be dependent on signals transduced by TCR molecules as well as CD4 and CD8 coreceptor molecules, although the molecular identity of these signals is currently obscure (for a review see reference 44). Our study is consistent with the possibility that thymic selection involves CD4-mediated coreceptor signals transduced by lck as well as TCR signals, but raises serious questions as to the nature of CD8-mediated coreceptor signals that might be involved in thymic selection. It is possible that lck might be the transducing molecule for CD8 in thymic selection if those surface CD8 molecules that are bound to intracellular lck preferentially associate with the TCR complex (45), creating a high local concentration of CD8-associated lck capable of transducing coreceptor signals on coengagement of TCR. Alternatively, CD8-mediated coreceptor signals might be transduced by a previously undescribed signaling molecule other than lck.

Taking this study together with previous studies in experimentally constructed transgenic animals, it is clear that lck regulates TCR expression in developing thymocytes at different levels. lck regulates TCR-β gene rearrangement and expression, as observed in mice expressing a transgene encoding constitutively activated lck molecules (46), and it regulates the assembly or transport of TCR proteins in normal thymocytes, as observed in this study. The critical role performed by lck in intrathymic development is emphasized by the profound developmental arrest observed in lck^- mice whose *lck* gene locus was disrupted by homologous recombination (47). It might be thought that thymic development would also be arrested by the absence of CD4, because without surface CD4, developing thymocytes could be severely impaired in their ability to activate intracellular lck. In fact, thymic development is not arrested in $CD4^-$ mice whose CD4 gene locus was disrupted by homologous recombination (48), presumably because other surface molecules are able to substitute for CD4 and bind intracellular lck, a function for which there are many contenders (31).

In conclusion, this study demonstrates a novel function for an intracellular tyrosine kinase in regulating TCR distribution and expression in immature $CD4^+CD8^+$ thymocytes. The tyrosine kinase involved in this activity is lck, which is chronically activated in immature $CD4^+CD8^+$ thymocytes by MHC class II–CD4 interactions in the thymus.

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