CD8β Chain Influences CD8α Chain-associated Lck Kinase Activity

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Summary

The CD8 molecule plays an important role in the differentiation of $CD8^+$ T cells in the thymus and in their normal function in the periphery. CD8 exists on the cell surface in two forms, the αα homodimer and the αβ heterodimer. Recent studies indicate an important role for the CD8β chain in thymic development of $CD8^+$ T cells and suggest that signaling via CD8αβ may be distinct from CD8αα. To better understand these differences, we introduced the CD8β gene into a T cell hybridoma which only expressed the CD8αα homodimer. In the parent hybridoma, cross-linking of the CD8α chain led to minimal enhancement of CD8-associated Lck tyrosine kinase activity. In the $CD8\beta^+$ transfectants, several observations suggested that CD8β modifies CD8α-associated Lck tyrosine kinase activity: (*a*) in in vitro kinase assays, antibody-mediated crosslinking of CD8 alone, or CD8 cross-linking with the TCR, resulted in 10-fold greater activation of Lck kinase activity, compared to cells expressing CD8αα alone; (*b*) in vivo, markedly enhanced tyrosine phosphorylation of several intracellular proteins was observed upon CD8 cross-linking with the TCR in CD8αβ-expressing cells, compared to cells expressing CD8αα alone; and (*c*) Lck association with CD8α was stabilized by the coexpression of CD8β. Thus, the differential Lck kinase activation and tyrosine phosphorylation seen with CD8αα vs. CD8αβ may reflect the unique signaling capabilities of the CD8β molecule. These differences in signaling may, in part, account for the diminished ability to generate CD8 single positive thymocytes in mice bearing a homozygous disruption of the CD8β gene.

The CD8 molecule is a cell surface glycoprotein that is expressed predominantly on class I MHC-restricted T cells. It is composed of a 38-kD α chain and a 30-kD β chain (1). CD8 is expressed either as a disulfide-linked αα homodimer, the predominant form expressed on natural killer cells and intestinal γδ T cells, or as an αβ heterodimer, the form found on most thymocytes and peripheral T cells (1–3). Much of the work establishing the importance of CD8 as a coreceptor has found that the expression of CDα, alone, is sufficient for its interaction with the α3 nonpolymorphic region of the MHC class I molecule and for the enhancement of antigen responses mediated via the TCR (4, 5). Furthermore, the CD8α molecule, like CD4, associates intracellularly with the src-family protein tyrosine kinase Lck (6).

The CD8β molecule is expressed on the cell surface only in association with the CD8α chain (7, 8) and does not associate with Lck (9). There is growing evidence to suggest that the CD8β chain may play a distinct role in antigen responsiveness. First, the CD8β chain may influence the avidity and/or specificity of the interaction of CD8 with the MHC (10). Second, hybridomas expressing the CD8αβ heterodimer, in comparison to those expressing the CD8α chain alone, were observed to produce greater amounts of IL-2 in response to stimulator cells (11). More recently, several groups have have identified an important role for the CD8β chain in thymic development. Nakayama et al. (12) described a significant decrease in the number of peripheral $CD8^+$ T cells in chimeric mice bearing a homozygous disruption of the CD8β gene. In these mice, there was interference with T cell development subsequent to the $CD4^+$ $CD8^+$ stage. Crooks and Littman (13) showed that in mice completely defective for CD8β expression, the number of $CD8^+$ T cells was also significantly reduced. Positive and negative selection of thymocytes expressing a specific transgenic TCR were impaired in the absence of CD8β gene expression. However, the disturbance in maturation of $CD8^+$ T cells observed in the absence of CD8β expression may be limited to thymus-derived T cells. Although the number of $CD8^+$ T cells was reduced in the thymus in CD8β-deficient mice generated by Fung-Leung et al. (14), the numbers of $CD8^+$ intestinal intraepithelial lymphocytes, which are proposed to be generated extrathymically, were normal.

It was speculated earlier that the extracellular domain of CD8β mediates most of its function. This was based on the observations that: (*a*) cells expressing chimeric molecules consisting of the extracellular domain of CD8β and the transmembrane and intracellular domains of CD8α were equally capable of enhanced IL-2 production (11); and (*b*) the 19 amino

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acid cytoplasmic tail of CD8β is not known to associate with Lck or any other intracellular molecule. However, a cytoplasmic tail–deleted form of the CD8β gene, when expressed as a transgene in mice, was found to act as a dominant negative mutation which interfered with the normal development of CD8$^+$ T cells (15). Therefore, the cytoplasmic tail also appears to be important in signaling via the CD8$\alpha\beta$ heterodimer.

These studies suggested that CD8$\alpha\beta$ generates signals that are distinct from those of CD8$\alpha\alpha$. To better understand the signaling capabilities of the CD8β chain, we transfected the human CD8β gene into hybridomas that expressed only the CD8$\alpha\alpha$ homodimer and examined signaling via the $\alpha\alpha$ and $\alpha\beta$ forms of CD8. Our data suggest that the CD8β chain stabilizes the interaction of Lck with CD8α and enhances CD8/Lck-dependent tyrosine kinase activity. This effect may reflect part of the signaling capabilities of the CD8β molecule.

Materials and Methods

Cell Lines and Transfections. BYDP, a murine T cell hybridoma expressing human CD4 and CD8α molecules (16, 17), was transfected with the human CD8β.1 gene (gift of D. Littman, New York University School of Medicine, NY), which was subcloned into the EcoRI site of the expression vector pMH-Neo (18). Cell lines that efficiently expressed the CD8$\alpha\beta$ heterodimer (BY4/8$\alpha\beta$) were generated in several independent transfections. Cells transfected with the pMH-Neo vector alone served as controls. For all transfections, 5×10^6 cells were electroporated with 10–20 μg of DNA linearized with XmnI. Selection with 2 mg/ml G418 solution was started 48 h after electroporation and transfectants were selected for ~2 wk. The data presented are from representative CD8$\alpha\beta$-expressing clones 6.1 and 10.1.

Assay for IL-2 Production. Stimulation of T cells and the assessment of IL-2 production were performed as described previously (19). Briefly, 96-well plates were coated with rabbit anti–mouse Ig (RAMG)[1]. Subsequently, varying concentrations of F23.1 (anti-TCR) Ab and either anti-CD4 (Leu3a, 500 ng/ml), anti-CD8α (Leu2a, 500 ng/ml), or anti-CD8$\alpha\beta$ Ab (2ST8-5H7, 1:250 dilution of ascites, gift of Dr. E. Reinherz, Dana-Farber Cancer Institute) were added and incubated for 1 h at room temperature. The plates were washed and incubated with hybridoma cells (5×10^4 cells/well) for 24 h at 37°C. The level of IL-2 in the supernatants was measured using the IL-2–dependent cell line, CTLL-20.

Stimulation, Immunoprecipitations, and In Vitro Kinase Assays. Cells (2×10^7 cells/ml) were incubated for 10 min on ice with anti-TCR Ab (500 ng/ml) and/or anti-CD4 (500 ng/ml), anti-CD8α (500 ng/ml), or anti-CD8$\alpha\beta$ Ab (1:250 dilution). After addition of RAMG (10 μg/ml final concentration) and an additional incubation on ice for 10 min, the cells were stimulated at 37°C for 3 min, washed, and lysed, as described previously (19).

Immunoprecipitations and in vitro kinase assays were performed as described previously (19). Lysates were incubated with 50 μl of a 50% solution of protein A-Sepharose beads without further addition of antibodies for 2–18 h at 4°C. The beads were then washed, resuspended in 50 μl of kinase reaction buffer (10 mM $MnCl_2$, 5 mM Hepes, 5 mM *p*-nitrophenylphosphate, 10 μCi γ-[^{32}P] ATP, 0.1 mM $Na_3V_4O_7$, and 10 μg/ml each of aprotinin and leupeptin), and incubated at 30°C for 3 min. The proteins were resolved by 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA), and developed by autoradiography.

Antiphosphotyrosine and Anti-Lck Immunoblotting. Immunoprecipitations with antiphosphotyrosine Ab 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) were performed using lysates from 1×10^7-stimulated cells/sample. Lysates were incubated with 2 μg of 4G10 Ab and 50 μl of protein A-Sepharose beads (preincubated with RAMG) for 2–18 h at 4°C. The beads were washed, and the bound proteins were eluted with 10 mM *p*-nitrophenylphosphate, as described previously (19). The proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, immunoblotted with antiphosphotyrosine Ab (RC20H; Transduction Laboratories, Lexington, KY), and developed by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Immunoprecipitations with anti-Shc Ab (Transduction Laboratories) were performed as described previously (20).

For anti-Lck immunoblotting, 1×10^7 stimulated cells/sample were lysed in either 1% Brij 96, or NP40 detergent, immunoprecipitated with Leu3a, Leu2a or mouse IgG1 (1.25 μg), resolved by 8% SDS-PAGE, and immunoblotted with anti-Lck Ab directed to the COOH terminus (Upstate Biotechnology, Inc.).

Results and Discussion

Generation of T Cell Hybridoma Cells Expressing Functional CD8$\alpha\beta$ Heterodimers. Previous work from our laboratory (19) has provided evidence for differential signaling mechanisms for the CD4 and CD8 coreceptors. We observed that antibody-mediated cross-linking of CD4 initiated greater tyrosine kinase activity than CD8 cross-linking. However, these comparisons were made between CD4 and the CD8$\alpha\alpha$ homodimer in the absence of expression of the CD8β gene. To delineate the signaling cascades that may be initiated through the heterodimeric form of CD8, we generated T cell hybridoma cell lines (BY4/8$\alpha\beta$) that coexpress human CD4, CD8α, and CD8β. The human CD8β gene was transfected into parent BYDP cells (19), which express both human CD4 and CD8$\alpha\alpha$. The surface expression of CD4, CD8α, and CD8β was examined by flow cytometry using antibodies to CD4 (Leu3a), CD8α (Leu2a), and CD8$\alpha\beta$ (2ST8-5H7) (Fig. 1 *A*). While Leu2a recognizes both the $\alpha\alpha$ and $\alpha\beta$ forms of CD8, 2ST8-5H7 specifically recognizes the CD8$\alpha\beta$ heterodimer (21). Nearly equivalent expression of CD4, CD8α, and CD8$\alpha\beta$ molecules was observed with antibody staining of several independent CD8β transfectants. The CD8β chain was not expressed on the parent cells or the clones transfected with the pMH-Neo vector alone (BY4/8$\alpha\alpha$). Biochemically, the expression of CD8$\alpha\beta$ heterodimers on the surface was confirmed by biotinylation of cell surface proteins, followed by immunoprecipitation with Leu2a or 2ST8-5H7, and immunoblotting with streptavidin (data not shown). Furthermore, the murine CD8β molecule was not expressed either in the parent cells, or the transfectants (data not shown).

IL-2 production in response to TCR cross-linking with CD4, CD8α, or CD8$\alpha\beta$ was assessed for control BY4/8$\alpha\alpha$ cells and BY4/8$\alpha\beta$ cells. When cross-linked with the TCR by antibody-mediated cross-linking, CD4, CD8α, and CD8$\alpha\beta$ were all capable of enhancing IL-2 production several-fold

[1] *Abbreviation used in this paper:* RAMG, rabbit anti–mouse Ig.

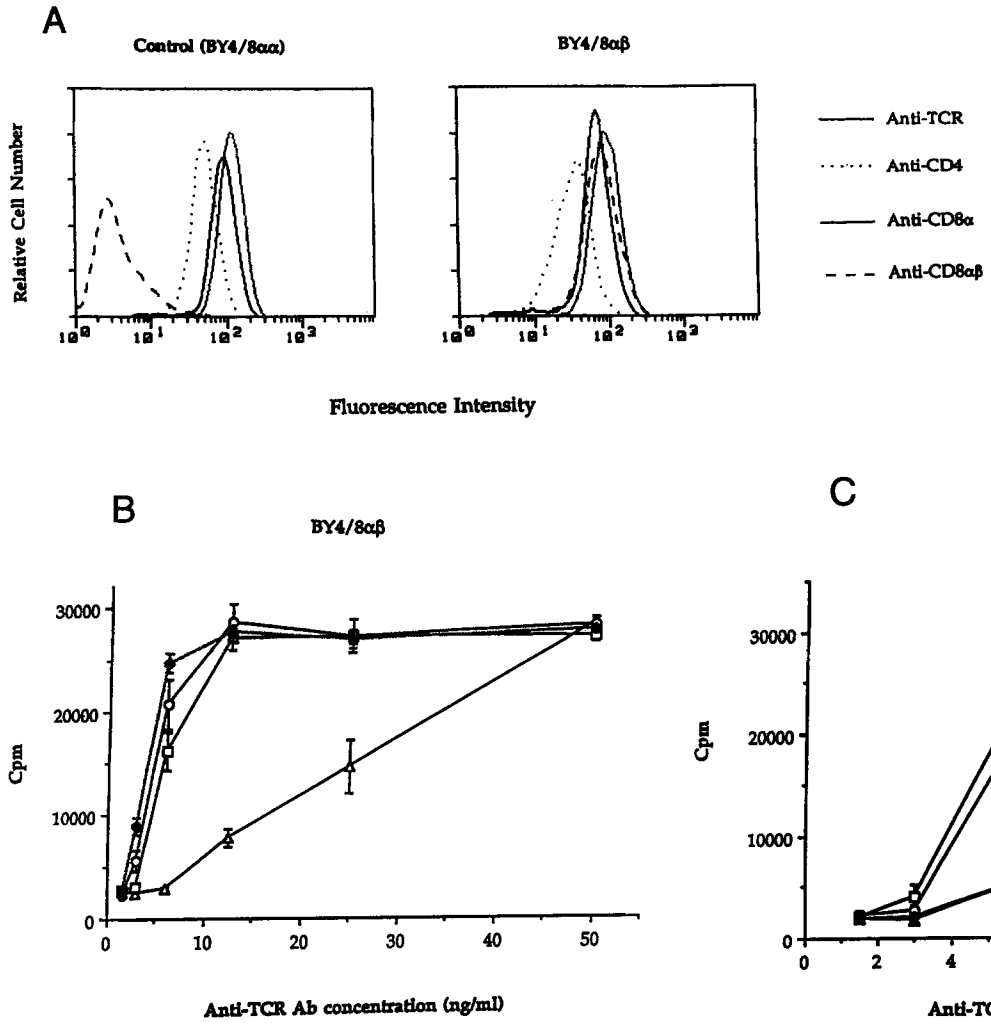

Figure 1. Expression and characterization of functional CD8$\alpha\beta$ in a T cell hybridoma. (*A*) BY4/8$\alpha\beta$ cells or control BY4/8$\alpha\alpha$ cells were stained with anti-TCR (F23.1), anti-CD4 (Leu3a), anti-CD8α (Leu2a), or anti-CD8$\alpha\beta$ Ab (2ST8-5H7). FITC-labeled goat anti-mouse Ig was used as a secondary Ab, and the cells were analyzed by flow cytometry. (*B*) BY4/8$\alpha\beta$ cells or (*C*) control BY4/8$\alpha\alpha$ cells (5×10^4 cells/well) were stimulated for 24 h in 96-well plates with varying concentrations of anti-TCR Ab, alone, or in combination with anti-CD4, anti-CD8α, or anti-CD8$\alpha\beta$ Ab. IL-2 in the supernatants was measured using the IL-2–dependent cell line, CTLL20. [^{3}H]Thymidine incorporation by CTLL20 cells is plotted. (*B* and *C*) Δ, TCR; $\square$, TCR + CD4; O, TCR + CD8α; $\bullet$, TCR + CD8$\alpha\beta$.

over TCR cross-linking alone (Fig. 1, *B* and *C*). Transfectants expressing CD8$\alpha\beta$ (Fig. 1 *B*) enhanced IL-2 production to an equivalent or slightly greater level when compared to control cells, which express only the CD8$\alpha\alpha$ homodimer (Fig. 1 *C*). Similar results were obtained with multiple clones from several independent transfections (data not shown). These data suggest that the BY4/8$\alpha\beta$ transfectants express CD8$\alpha\beta$ heterodimers on the cell surface that are functionally competent. The differences between our findings and previously reported data suggesting that CD8β markedly enhances IL-2 production (11) may be due, in part, to the differences in the experimental systems that were used. In the published report, antigen presentation by stimulator cells was used to stimulate IL-2 production by murine hybridoma cells expressing murine CD8β.

CD8-associated Lck Kinase Activity Is Enhanced in Cells Expressing CD8$\alpha\beta$. We previously observed that CD8$\alpha\alpha$ is capable of functioning as a coreceptor for IL-2 production without significant activation of Lck kinase activity as assessed by in vitro kinase assays (19). We also examined Lck kinase activity in BY4/8$\alpha\beta$ cells, using in vitro kinase assays (Fig. 2 *A*). BY4/8$\alpha\beta$ cells were stimulated at 37°C by antibody-mediated cross-linking of the TCR alone, CD4 or CD8 alone, or TCR with CD4 or CD8. Cells were then lysed in mild detergent conditions with Brij 96. Autophosphorylation of Lck, induced by CD8 cross-linking or TCR/CD8 cross-linking, attained levels that were comparable to that seen with TCR/CD4 cross-linking. In contrast, minimal activation of Lck kinase activity was seen in control BY4/8$\alpha\alpha$ cells with CD8 or TCR/CD8 cross-linking, using

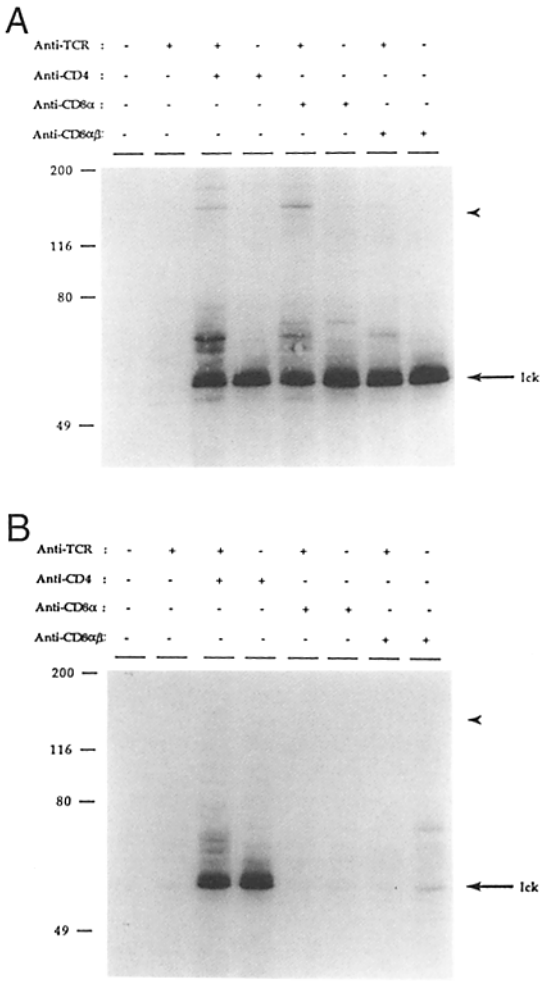

Figure 2. Activation of CD8-associated Lck kinase activity in cells expressing CD8$\alpha\beta$. (*A*) BY4/8$\alpha\beta$ cells, or (*B*) control BY4/8$\alpha\alpha$ cells were stimulated for 3 min at 37°C with anti-TCR Ab and/or anti-CD4, anti-CD8α or anti-CD8$\alpha\beta$ Ab cross-linking. The cells were lysed in 1% Brij 96 detergent, immunoprecipitated with protein A-Sepharose beads, and in vitro kinase assays were performed for 3 min at 30°C. Proteins were resolved using 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and developed by autoradiography. The 56-kD band was identified as Lck by reimmunoprecipitation with anti-Lck antibody (data not shown). β scoping indicated that incorporation of radioactivity in the 56-kD Lck band in BY4/8$\alpha\beta$ cells (*A*) was equivalent in lane *3* and lane *5* (TCR/CD4 vs. TCR/CD8), while, in BY4/8$\alpha\alpha$ cells (*B*), a 10-fold lower incorporation was measured in this band with TCR/CD8 cross-linking (lane *5*), compared with TCR/CD4 cross-linking (lane *3*).

the same anti-CD8α antibody (Fig. 2 *B*). In these cells, the level of Lck kinase activity achieved with cross-linking of CD8 alone, or with the TCR, was at least 10-fold less than that achieved with TCR/CD4 cross-linking. Although it has been proposed that CD4 may bind more efficiently to Lck than CD8, this does not account for the diminished Lck tyrosine kinase activity associated with CD8$\alpha\alpha$, since we have made a similar observation previously in CD4$^-$CD8$\alpha\alpha^+$ cells (19). The greater Lck kinase activity seen with TCR/CD8 cross-linking in cells expressing the CD8β molecule most likely resulted from the recognition and engagement of the CD8$\alpha\beta$ heterodimer by the anti-CD8α antibody, Leu2a. This difference in CD8-associated Lck tyrosine kinase activity between BY4/8$\alpha\beta$ and BY4/8$\alpha\alpha$ cells was observed over a range of stimulation times (data not shown). It is, therefore, unlikely that stimulation of tyrosine kinase activity follows a different time course in cells expressing CD8$\alpha\beta$, compared to those expressing CD8$\alpha\alpha$. In addition to greater levels of Lck autophosphorylation, several other phosphorylated proteins were seen with TCR/CD8 cross-linking in BY4/8$\alpha\beta$ cells which were not observed in BY4/8$\alpha\alpha$ cells in in vitro kinase assays. These data suggest that the expression of the β chain and the formation of CD8$\alpha\beta$ heterodimers on the cell surface appears to enhance Lck tyrosine kinase activity associated with the CD8α chain.

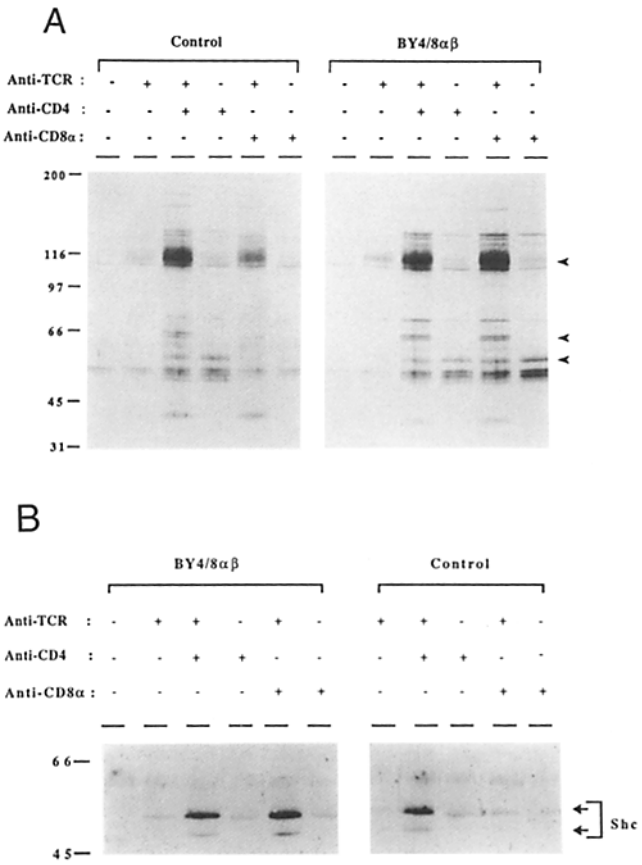

Figure 3. Comparison of tyrosine phosphorylation of intracellular proteins after TCR/CD8 cross-linking of BY4/8$\alpha\beta$ or BY4/8$\alpha\alpha$ cells. (*A*) BY4/8$\alpha\beta$ cells or control BY4/8$\alpha\alpha$ cells were stimulated at 37°C for 3 min with anti-TCR Ab cross-linking, alone, or in combination with anti-CD4, anti-CD8α or anti-CD8$\alpha\beta$ Ab. Cells were lysed in 1% Brij 96 detergent and immunoprecipitated with antiphosphotyrosine Ab (4G10) and protein A-Sepharose beads (preincubated with RAMG). The bound proteins were eluted using 10 mM *p*-nitrophenylphosphate, resolved by 6–12% SDS-PAGE, immunoblotted with antiphosphotyrosine Ab (RC20H), and developed by enhanced chemiluminescence. Several proteins which undergo differential phosphorylation, including a diffuse band seen around 116 kD, are indicated by arrowheads on the right. (*B*) BY4/8$\alpha\beta$ cells or control BY4/8$\alpha\alpha$ cells were stimulated as described above. The lysates were immunoprecipitated with anti-Shc Ab and immunoblotted with antiphosphotyrosine Ab.

The greater activity of Lck seen in in vitro kinase assays also correlated with enhanced tyrosine phosphorylation of several intracellular proteins in vivo (Fig. 3 *A*). We compared the pattern of tyrosine phosphorylation after stimulating BY4/8$\alpha\beta$ cells or BY4/8$\alpha\alpha$ cells by cross-linking the TCR alone, or with CD4, CD8α, or CD8$\alpha\beta$. After activation, the lysates were immunoprecipitated with antiphosphotyrosine Ab (4G10) and immunoblotted with antiphosphotyrosine Ab. Phosphorylation of several proteins was observed in BY4/8$\alpha\beta$ cells upon TCR/CD8 cross-linking, which was not seen in control BY4/8$\alpha\alpha$ cells. Many of these proteins appear to be similar in molecular weight to those seen upon TCR/CD4 cross-linking in BY4/8$\alpha\beta$ or BY4/8$\alpha\alpha$ cells.

The identity of all of these newly phosphorylated proteins and whether they are substrates specific to Lck have yet to be determined. However, one of the proteins which is a likely substrate for Lck and whose phosphorylation is enhanced upon TCR/CD4 cross-linking is Shc (20, 22). To determine if the phosphorylation of Shc is enhanced by TCR/CD8 cross-linking in transfectants expressing CD8β, Shc was immunoprecipitated from lysates after activation and assessed by immunoblotting with antiphosphotyrosine Ab (Fig. 3 *B*). In the CD8β-expressing BY4/8$\alpha\beta$ cells, stimulation via CD8 resulted in enhanced tyrosine phosphorylation of Shc. This enhancement correlates with the enhanced activity of Lck associated with the CD8α chain in the presence of the β chain.

CD8β May Stabilize Lck Association with CD8α. Since the cytoplasmic region of the CD8β chain does not associate with Lck, the greater activity of Lck seen after TCR/CD8$\alpha\beta$ cross-linking cannot be explained by simple stoichiometry. We, therefore, considered the possibility that the β chain in the CD8$\alpha\beta$ heterodimer may play a role in stabilizing the association of Lck with the α chain, thereby allowing for greater activation of its kinase activity after CD8 cross-linking.

All of the experiments, thus far, have been performed after lysis with Brij 96, a mild detergent that preserves many of the molecular complexes with the TCR, CD4, and CD8 (23). Complexes that remain preserved under lysis conditions with a harsher detergent, such as NP40, were examined (Fig. 4). Lysates from BY4/8$\alpha\beta$ and control BY4/8$\alpha\alpha$ cells were immunoprecipitated with antibodies to CD4, CD8α, or CD8$\alpha\beta$ and immunoblotted for associated Lck. Under NP40 lysis conditions, there was minimal Lck associated with CD8 in cells lacking CD8β, while considerably greater amounts of Lck remained associated with CD8 in BY4/8$\alpha\beta$ cells. The possibility that there may be a difference in Lck expression between BY4/8$\alpha\beta$ and BY4/8$\alpha\alpha$ cells was ruled out when whole cell lysates were immunoblotted with anti-Lck antibody and found to contain comparable amounts of Lck (data not shown). The CD8β molecule may, therefore, enable the α chain to adopt a conformation that allows a more stable association with Lck. The greater tyrosine kinase activity observed after cross-linking may result from this more stable association.

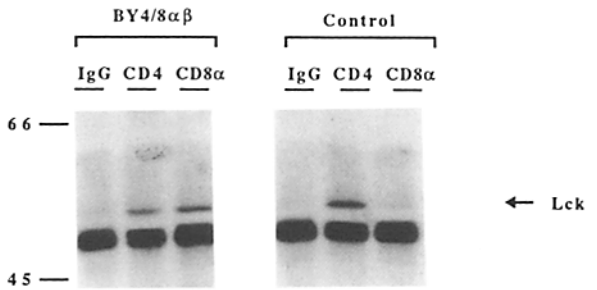

Figure 4. Stabilization of Lck association with CD8 in CD8β^+ cells. BY4/8$\alpha\beta$ or control BY4/8$\alpha\alpha$ cells were lysed with 1% NP40 and immunoprecipitated with anti-CD4 Ab (Leu3a), anti-CD8α Ab (Leu2a), or mouse IgG_1. The proteins were resolved by 8% SDS-PAGE and immunoblotted with anti-Lck Ab (anti–C-terminus). The Ig chain is seen in all lanes.

Only a fraction of CD4 or CD8 molecules are found on the cell surface in association with Lck (24). While the presence of Lck is necessary for T cell maturation in the thymus (25), the relative importance of Lck association with the coreceptors CD4 and CD8 remains unclear. Mice deficient in endogenous CD4 or CD8, but expressing mutant forms of either CD4 or CD8 as a transgene (which no longer associate with Lck) were able to support normal positive and negative selection (26, 27). However, these mutant molecules had to be overexpressed to observe this effect. While the need for Lck association with the coreceptors can be overcome by overexpression of the mutant forms of CD4 or CD8, the association with Lck may be important at physiological levels of the coreceptors. In fact, overexpression of a full length CD4 as a transgene in mice interfered with CD8-dependent selection, presumably through the sequestration of Lck away from CD8 (28).

Thus, the precise role of Lck and its tyrosine kinase activity in CD8-dependent thymocyte selection remains to be resolved. Our data suggest that the CD8β chain stabilizes the Lck association with CD8α and enhances the Lck tyrosine kinase activity. The crucial role of the CD8β chain in thymocyte development, reported recently by several groups, may be linked to its ability to modulate CD8α-associated Lck kinase activity. However, the relative requirement for CD8-associated Lck kinase activity may differ for immature vs. mature T cells. While the CD8$\alpha\beta$ form is expressed on both immature and mature T cells, surprisingly, the deficiency of β chain significantly affects the development of T cells in the thymus, but does not affect the cytotoxic ability of mature T cells that reach the periphery (13, 14). Although more subtle alterations in the functions of these cells are possible, this is consistent with earlier reports by others, as well as with our own (Fig. 1 *C*) (5), that CD8$\alpha\alpha$ homodimers alone can enhance IL-2 production (4).

We have previously shown that, in T cells, tyrosine phosphorylated Shc interacts with Grb2 and the guanine nucleotide exchange factor, mSOS (20). mSOS has previously been shown to convert Ras to its active form by exchanging GDP for GTP (29). Antibody-mediated cross-linking of the TCR with CD8$\alpha\beta$ leads to enhanced tyrosine kinase activity and Shc phosphorylation, which, in turn, may lead to enhanced Ras activation. Enhanced Ras activation, provided by engage-

ment of the CD8$\alpha\beta$ coreceptor, may be required for thymic differentiation of $CD4^+CD8^+$ thymocytes to $CD4^-CD8^+$ thymocytes, but may not be essential for enhanced IL-2 production. Future investigations will aim to elucidate the precise role of Lck tyrosine kinase activity in various CD8-dependent functions.

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