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 CD8α-associated Lck tyrosine kinase activity. In the CD8β+ 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 CD8αβ was stabilized by the coexpression of CD8β. Thus, the differential Lck kinase activation and tyrosine phosphorylation seen with CD8αβ vs. CD8αα 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.
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 CD8αβ heterodimer.

These studies suggested that CD8αβ generates signals that are distinct from those of CD8αα. To better understand the signaling capabilities of the CD8β chain, we transfected the human CD8β gene into hybridomas that expressed only the CD8αα homodimer and examined signaling via the αα and αβ forms of CD8. Our data suggest that the CD8β chain stabilizes the interaction of Lck with CD8αα 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 CD8αα 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 CD8αβ heterodimer (BY4/8αβ) were generated in several independent transfections. Cell lines 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 transfectedants were selected for ~2 wk. The data presented are from representative CD8αβ-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-CD8αβ 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 × 10^6 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 × 10^6 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-CD8αβ 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 MnCl₂, 5 mM HEPES, 5 mM p-nitrophenylphosphate, 10 μCi γ-[32P] ATP, 0.1 mM NaVO₄, 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 × 10^6-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-nitrophenolphosphate, 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 CD8αβ Heterodimers.** Previous work from our laboratory (19) has provided evidence for differential signaling mechanisms for 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 CD8αα 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 (BY4/8αβ) that coexpress human CD4, CD8α, and CD8β. The human CD8β gene was transfected into parent BYDP cells (19), which express both human CD4 and CD8αα. The surface expression of CD4, CD8α, and CD8β was examined by flow cytometry using antibodies to CD4 (Leu3a), CD8α (Leu2a), and CD8αβ (2ST8-5H7) (Fig. 1 A). While Leu2a recognizes both the αα and αβ forms of CD8, 2ST8-5H7 specifically recognizes the CD8αβ heterodimer (21). Nearly equivalent expression of CD4, CD8α, and CD8αβ molecules was observed with antibody staining of several independent CD8β transfecteds. The CD8β chain was not expressed on the parent cells or the clones transfected with the pMH-Neo vector alone (BY4/8αα). Biochemically, the expression of CD8αβ heterodimers on the surface was confirmed by bioninylation of cell surface proteins, followed by immunoprecipitation with Leu2a or 2ST8-5H7, and immunoblotting with streptavidin (data not shown). Furthermore, the human CD8β molecule was not expressed either in the parent cells, or the transfecteds (data not shown).

IL-2 production in response to TCR cross-linking with CD4, CD8α, or CD8αβ was assessed for control BY4/8αα cells and BY4/8αβ cells. When cross-linked with the TCR by antibody-mediated cross-linking, CD4, CD8α, and CD8αβ were all capable of enhancing IL-2 production several-fold.
Figure 1. Expression and characterization of functional CD8αβ in a T cell hybridoma. (A) BY4/8εβ cells or control BY4/8εα cells were stained with anti-TCR (F23.1), anti-CD4 (Leu3a), anti-CD8α (Leu2a), or anti-CD8αβ Ab (25T-8-SH7). FITC-labeled goat anti-mouse Ig was used as a secondary Ab, and the cells were analyzed by flow cytometry. (B) BY4/8εβ cells or (C) control BY4/8εα cells (5 × 10⁴ 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-CD8αβ Ab. IL-2 in the supernatants was measured using the IL-2-dependent cell line, CTLL20. [³H]Thymidine incorporation by CTLL20 cells is plotted. (B and C) Δ, TCR; □, TCR + CD4; O, TCR + CD8α; ●, TCR + CD8αβ.

CD8-associated Lck Kinase Activity Is Enhanced in Cells Expressing CD8αβ. We previously observed that CD8αα 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 BY4/8εβ cells, using in vitro kinase assays (Fig. 2 A). BY4/8εβ 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 BY4/8εα cells with CD8 or TCR/CD8 cross-linking, using...
difference in CD8-associated Lck tyrosine kinase activity between BY4/8αβ and BY4/8αα 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 CD8αβ, compared to those expressing CD8αα. In addition to greater levels of Lck autophosphorylation, several other phosphorylated proteins were seen with TCR/CD8 cross-linking in BY4/8αβ cells which were not observed in BY4/8αα cells in in vitro kinase assays. These data suggest that the expression of the β chain and the formation of CD8αβ heterodimers on the cell surface appears to enhance Lck tyrosine kinase activity associated with the CD8α chain.

Figure 2. Activation of CD8-associated Lck kinase activity in cells expressing CD8αβ. (A) BY4/8αβ cells, or (B) control BY4/8αα cells were stimulated for 3 min at 37°C with anti-TCR Ab and/or anti-CD4, anti-CD8α or anti-CD8αβ 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). Scoring indicated that incorporation of radioactivity in the 56-kD Lck band in BY4/8αβ cells (A) was equivalent in lane 3 and lane 5 (TCR/CD4 vs. TCR/CD8), while, in BY4/8αα 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).

Figure 3. Comparison of tyrosine phosphorylation of intracellular proteins after TCR/CD8 cross-linking of BY4/8αβ or BY4/8αα cells. (A) BY4/8αβ cells or control BY4/8αα 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-CD8αβ Ab. Cells were lysed in 1% Brij 96 detergent and immunoprecipitated with anti-phosphotyrosine 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 anti-phosphotyrosine 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) BY4/8αβ cells or control BY4/8αα cells were stimulated as described above. The lysates were immunoprecipitated with anti-Shc Ab and immunoblotted with anti-phosphotyrosine 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. 3A). We compared the pattern of tyrosine phosphorylation after stimulating BY4/8αβ cells or BY4/8αα cells by cross-linking the TCR alone, or with CD4, CD8α, or CD8αβ. After activation, the lysates were immunoprecipitated with antiphosphotyrosine Ab (4G10) and immunoblotted with antiphosphotyrosine Ab. Phosphorylation of several proteins was observed in BY4/8αβ cells upon TCR/CD8 cross-linking, which was not seen in control BY4/8αα cells. Many of these proteins appear to be similar in molecular weight to those seen upon TCR/CD4 cross-linking in BY4/8αβ or BY4/8αα 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 CD8αβ, Shc was immunoprecipitated from lysates after activation and assessed by immunoblotting with antiphosphotyrosine Ab (Fig. 3B). In the CD8αβ-expressing BY4/8αβ 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.

**CD8α May Stabilize Lck Association with CD8αβ.** Since the cytoplasmic region of the CD8αβ chain does not associate with Lck, the greater activity of Lck seen after TCR/CD8αβ cross-linking cannot be explained by simple stoichiometry. We, therefore, considered the possibility that the β chain in the CD8αβ 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 BY4/8αβ and control BY4/8αα cells were immunoprecipitated with antibodies to CD4, CD8α, or CD8αβ and immunoblotted for associated Lck. Under NP40 lysis conditions, there was minimal Lck associated with CD8 in cells lacking CD8αβ, while considerably greater amounts of Lck remained associated with CD8 in BY4/8αβ cells. The possibility that there may be a difference in Lck expression between BY4/8αβ and BY4/8αα 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 CD8αβ 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.

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 CD8α-dependent selection, presumably through the sequestration of Lck away from CD8α (28).

Thus, the precise role of Lck and its tyrosine kinase activity in CD8α-dependent thymocyte selection remains to be resolved. Our data suggest that the CD8αβ chain stabilizes the Lck association with CD8α and enhances the Lck tyrosine kinase activity. The crucial role of the CD8αβ chain in thymocyte development, reported recently by several groups, may be linked to its ability to modulate CD8αβ-associated Lck kinase activity. However, the relative requirement for CD8α-associated Lck kinase activity may differ for immature vs. mature T cells. While the CD8αβ form is expressed on both immature and mature T cells, surprisingly, the deficiency of the 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. 1C) (5), that CD8α 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 CD8αβ leads to enhanced tyrosine kinase activity and Shc phosphorylation, which, in turn, may lead to enhanced Ras activation. Enhanced Ras activation, provided by engage-
mment of the CD8αβ coreceptor, may be required for thymic
differentiation of CD4+CD8+ thymocytes to CD4−CD8+
thymocytes, but may not be essential for enhanced IL-2 produc-
tion. Future investigations will aim to elucidate the precise
role of Lck tyrosine kinase activity in various CD8-dependent
functions.

We thank Dr. Dan R. Littman for providing the human CD8β1 gene and Dr. Ellis Reinherz for providing the 2ST8-SH7 Ab. We thank Dr. Georg Hollander for initial work on this project.

This work was supported by National Institutes of Health grant AI-17258 (to S. J. Burakoff) and a grant from F. Hoffman-LaRoche. K. S. Ravichandran is supported by a fellowship from The Medical Foundation of the Charles King Trust. H. Y. Irie was supported by a National Research Service Award T32 GM07753.

Address correspondence to Dr. Steven J. Burakoff, DANA 1840, Division of Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

Received for publication 25 August 1994 and in revised form 14 November 1994.

References
novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell.* 70:93–104.


