CD8β Endows CD8 with Efficient Coreceptor Function by Coupling T Cell Receptor/CD3 to Raft-associated CD8/p56Lck Complexes

Alexandre Arcaro,1 Claude Grégoire,2 Talitha R. Bakker,3 Lucia Baldi,4 Martin Jordan,4 Laurence Goffin,1 Nicole Boucheron,1 Florian Wurm,4 P. Anton van der Merwe,3 Bernard Malissen,2 and Immanuel F. Luescher1

1Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland
2Centre d’Immunologie de Marseille-Luminy, Institut National de la Sante et de la Recherche Médicale, Centre National de la Recherche Scientifique, University of Marseille, Marseille 13288 Cedex 9, France
3Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom
4Swiss Federal Institute of Technology, Lausanne 1015, Switzerland

Abstract
The extraordinary sensitivity of CD8+ T cells to recognize antigen impinges to a large extent on the coreceptor CD8. While several studies have shown that the CD8β chain endows CD8 with efficient coreceptor function, the molecular basis for this is enigmatic. Here we report that cell-associated CD8αββ, but not CD8αβα or soluble CD8αββ, substantially increases the avidity of T cell receptor (TCR)-ligand binding. To elucidate how the cytoplasmic and transmembrane portions of CD8β endow CD8 with efficient coreceptor function, we examined T1.4 T cell hybridomas transfected with various CD8β constructs. T1.4 hybridomas recognize a photoreactive Plasmodium berghei circumsporozoite (PbCS) peptide derivative (PbCS (4-azidobezoic acid [ABA])) in the context of H-2Kd, and permit assessment of TCR-ligand binding by TCR photoaffinity labeling. We find that the cytoplasmic portion of CD8β, mainly due to its palmitoylation, mediates partitioning of CD8 in lipid rafts, where it efficiently associates with p56Lck. In addition, the cytoplasmic portion of CD8β mediates constitutive association of CD8 with TCR/CD3. The resulting TCR-CD8 adducts exhibit high affinity for major histocompatibility complex (MHC)-peptide. Importantly, because CD8αβ partitions in rafts, its interaction with TCR/CD3 promotes raft association of TCR/CD3. Engagement of these TCR/CD3-CD8/lck adducts by multimeric MHC-peptide induces activation of p56Lck in rafts, which in turn phosphorylates CD3 and initiates T cell activation.

Key words: T lymphocytes • cytotoxicity • phosphorylation • protein-tyrosine kinase • plasmon resonance

Introduction
The coreceptor CD8 plays a key role in the activation of mature CD8+ T cells. In the absence of CD8, or upon blocking of CD8, activation of CD8+ T cells requires considerably higher and longer TCR engagement (1–4). While CD8 on peripheral T cell consists of a disulfide-linked α and β chains, intestinal T cells, γδ T cells, and NK cells express homodimeric CD8αα (1, 2). Heterodimeric CD8αβ is a much more potent coreceptor than homodimeric CD8αα, but there is considerable controversy on what domains of CD8β and by what mechanism CD8β endows CD8 with efficient coreceptor function (1–8).

Using Kd-restricted CD8+ T cells, which recognize a photoreactive derivative of the Plasmodium berghei circumsporozoite (PbCS)* peptide 252–260 (SYIPSAEKI)
cells, the CD8\(\text{a}\) binding CD8\(\text{a}\) with LAT (5, 23).

Important, because cross-linking-mediated lck activation in
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To the transmembrane and/or cytoplasmic portions of
(ABA), indicating that the CD8-mediated increase in T1
binding of soluble CD8 and TCR to MHC-peptide are
similar studies on soluble CD8 have shown that CD8\(\text{a}\) and CD8\(\text{b}\) bind class I molecules with similar affinities (13, 14). By contrast, cell-associated CD8\(\text{a}\) seems to bind MHC class I molecules more avidly than CD8\(\text{a}\) (3–5). According to one study, soluble CD8\(\text{a}\) enhances the binding of soluble TCR to MHC-peptide (13). However, another study indicated that soluble CD8\(\text{a}\) has no effect on TCR-ligand binding, i.e., that binding of soluble CD8 and TCR to MHC-peptide are two independent events (15). Here we report that the extracellular portion of CD8\(\text{a}\) in soluble form does not strengthen the interaction of the T1 TCR with K\(\text{a}\)-PbCS (ABA), indicating that the CD8-mediated increase in T1 TCR-ligand binding observed on cells (4, 10) is attributed to the transmembrane and/or cytoplasmic portions of CD8\(\text{a}\).

The tail of CD8\(\text{a}\) is 30 residues long and contains two vacuolar cysteines, which interact with the Src kinase p56\(\text{ck}\) (lck) by means of a zinc chelate complex (1, 2). The tail of CD8\(\text{b}\) contains 19 residues and seems to play an important role for the positive selection and activation of CD8\(\text{a}\) T cells (5, 6–8, 16). We have shown previously that the CD8\(\text{b}\) tail is palmitoylated at a membrane-proximal cysteine and mediates CD8 partitioning in lipid rafts (6). Rafts, also called detergent-insoluble membranes (DIMs), are ordered microdomains, enriched in sphingolipids and cholesterol, which include molecules containing short saturated fatty acids, such as GPI-linked proteins, or on the inner membrane leaflet palmitoylated molecules, like CD8, lck, p59\text{fyn} (fyn), and the linker for activation of T cells (LAT) (6, 17–22). Other molecules are excluded from rafts, namely phosphatases (e.g., CD45), which makes rafts privileged sites for the induction of TCR signaling. Raft localization of CD8 increases its association with lck, which is important, because cross-linking-mediated lck activation in rafts is a crucial initial event in T cell activation (6, 20). The CD8\(\text{b}\) tail has also been implicated in association of CD8 with LAT (5, 23).

In this study we prepared T1 T cell hybridsomas expressing CD8\(\text{a}\) and various CD8\(\text{b}\) variants. We show that on cells, the CD8\(\text{b}\) cytoplasmic portion mediates association of CD8 with TCR/CD3. The resulting TCR/CD3 aducts with CD8/lck are raft associated, exhibit increased avidity for MHC-peptide, and upon cross-linking, induce phosphorylation of lck and CD3 and mobilization of intracellular calcium.

### Materials and Methods

**Antibodies and Cell Culture.** The following antibodies were obtained from American Type Culture Collection: anti-CD8\(\text{a}\) mAb 53.6.72, anti-CD8\(\text{b}\) mAb H35–17, anti-CD8\(\text{b}\) mAb 53.5.81, anti-TCR\(\text{a}\) mAb H57, anti-TCR\(\text{c}\) mAb H28, and anti-CD3\(\text{c}\) mAb HAM146. Anti-phospho-tyrosine (pY) mAb 4G10 and anti-lck mAb 3A5 were from Upstate Biologicals. Anti-LAT, anti-lck, and anti-CD3\(\text{e}\) polyclonal antibodies were from Santa Cruz Biotechnology, Inc. Transfected Chinese hamster ovary (CHO) cells were maintained in Glasgow minimum essential medium (Life Technologies) supplemented with 25 \(\mu\)M methionine sulfoximine and 5% dialyzed FCS, as described (14). T cell hybridsomas were cultured in DMEM (Life Technologies) supplemented with 5% FCS (Life Technologies), penicillin/ streptomycin/neomycin (PSN; Life Technologies), and 50 \(\mu\)M \(\beta\)-mercaptoethanol.

**Soluble CD8\(\text{a}\), T1 TCR, and Biotinylated K\(d\)-PbCS(ABA).** Soluble T1 TCR and CD8 were prepared essentially as described (14). In brief, the cDNA encoding the extracellular portions of the T1 TCR \(\alpha\) and \(\beta\) chains were fused to the sequences encoding the basic and acidic units of a leucine zipper, respectively. The constructs were cloned into the pEE14GS vector using the EcoR1 site and stably transfected into CHO-K1 cells with yields of \(\sim\)8 mg/l. The T1 TCR was purified on H28-Sepharose, using acid elution (pH 3.1). The extracellular portions of murine CD8\(\text{a}\) (residues 1–156) and CD8\(\text{b}\) (residues 1–146) were fused to basic and acidic leucine zipper, cloned into pEE14GS, and stably expressed in CHO-K1 cells, with yields of \(\sim\)10 mg/l. CD8 was purified on H35–17 Sepharose, using acid elution (pH 3.1). Correct folding of the purified CD8, was confirmed by ELISA using five different anti-CD8 monoclonal antibodies (anti-CD8\(\text{a}\) mAb 53.6.72, 19/178 and anti-CD8\(\text{b}\) mAb 53.5.8, KT112, H35–17). Monomeric covalent K\(d\)-PbCS (ABA), indicating that the CD8-mediated increase in T1 TCR-ligand binding observed on cells (4, 10) is attributed to the transmembrane and/or cytoplasmic portions of CD8\(\text{a}\).

The tail of CD8\(\text{a}\) is 30 residues long and contains two vacuolar cysteines, which interact with the Src kinase p56\(\text{ck}\) (lck) by means of a zinc chelate complex (1, 2). The tail of CD8\(\text{b}\) contains 19 residues and seems to play an important role for the positive selection and activation of CD8\(\text{a}\) T cells (5, 6–8, 16). We have shown previously that the CD8\(\text{b}\) tail is palmitoylated at a membrane-proximal cysteine and mediates CD8 partitioning in lipid rafts (6). Rafts, also called detergent-insoluble membranes (DIMs), are ordered microdomains, enriched in sphingolipids and cholesterol, which include molecules containing short saturated fatty acids, such as GPI-linked proteins, or on the inner membrane leaflet palmitoylated molecules, like CD8, lck, p59\text{fyn} (fyn), and the linker for activation of T cells (LAT) (6, 17–22). Other molecules are excluded from rafts, namely phosphatases (e.g., CD45), which makes rafts privileged sites for the induction of TCR signaling. Raft localization of CD8 increases its association with lck, which is important, because cross-linking-mediated lck activation in rafts is a crucial initial event in T cell activation (6, 20). The CD8\(\text{b}\) tail has also been implicated in association of CD8 with LAT (5, 23).

### Calcium Measurements and TCR Photoaffinity Labeling.

P815 cells (10\(^6\)/ml) were pulsed or not with \(1 \mu\M\) IASA-YISSAEK(ABA)I or IASA-YISSAEK(ABA)I (P255S) and then UV irradiated at \(>350\) nm as described (6, 25). T cell hybridsomas (10\(^6\)/ml) were incubated with \(5 \mu\M\) Indo-1/AM (Sigma-Aldrich).
at 37°C for 45 min, washed in DMEM, and incubated with P815 cells at an E/T ratio of 1/3 for 2 min. Calcium-dependent Indo-1 fluorescence was measured on a FACStar™ as described (25). TCR photoaffinity labeling of T1.4 hybridomas was performed as described (9–11, 20). In brief, hybridomas (10^7/ml) were incubated with K^d-L^125IASA-YIPSAEK(ABA)I for 2 h at 0–4°C. After UV-cross-linking at 312 nM the cells were washed and lysed in RIPA buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5 mM sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μM pepstatin A, 5 mM benzamidine, 2 μg/ml aprotinin) for 30 min on ice. The detergent soluble fractions were immunoprecipitated with H57-Sepharose and the samples analyzed by SDS-PAGE (10%), reducing) and PhosphorImaging using a Fuji BAS1000 PhosphorImager.

Isolation of DIM. T cell hybridomas (5 × 10^7) were lysed for 30 min on ice in MNE buffer (25 mM MES [pH 6.5], 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 (Sigma-Aldrich). Alternatively, 0.5% Brij58 (Fluka) without EDTA was used (21). The lysates were homogenized with a Dounce homogenizer (10 strokes) and fractionated on sucrose density gradients as described (6). The gradients were fractionated from the top in 10 fractions. In some experiments the fractions 2–4 and 6–9 were pooled and referred to as DIM and detergent soluble membrane (M) fractions, respectively. Surface biotinylation was performed as described (6). For immunoprecipitation the fractions were incubated with Sepharose-conjugated anti-CD8α mAb 53.6.72, anti-CD8β mAb H35–17, or anti-TCR mAb H57–1 for 2 h at 37°C. The immunoprecipitates were resolved on SDS-PAGE (10%, reducing) and Western blotted with streptavidin–horseradish peroxidase (HRP; GIBCO BRL) or anti-CD8α antiserum. This material was immunoprecipitated with anti-TCR Cβ mAb H57 or anti-TCR Cα mAb H28, confirming the heterodimeric nature of the sT1 TCR. To ascertain the biological activity of the soluble CD8αβ, it was tested as inhibitor in a cytolytic assay using PbCS(ABA) pulsed P815 cells as targets. Increasing concentrations of sCD8αβ inhibited the lysis of P815 cells by cloned PbCS(ABA)-specific S14 CTL, with a half maximal inhibition at 0.66 mg/ml (10 μM), and maximal inhibition of 80% at 2 mg/ml (data not shown), indicating that sCD8αβ competes with cell-associated CD8αβ.

Results

Soluble CD8αβ and T1 TCR Bind Independently to K^d-PbCS(ABA). To assess whether the extracellular portion of CD8αβ affects TCR-ligand binding, we prepared soluble CD8αβ and T1 TCR. Purified soluble sT1 TCR was tested for ligand binding by TCR photoaffinity labeling with soluble K^d-L^125IASA-YIPSAEK(ABA)I (9–11). After UV irradiation it exhibited on reducing SDS-PAGE gels one labeled specie of 80–90 kD corresponding to the complex of sK^d-PbCS (ABA) and one chain of the T1 TCR. This material was immunoprecipitated with anti-TCRCβ mAb H57 or anti-TCR Cα mAb H28, confirming the heterodimeric nature of the sT1 TCR. To ascertain the biological activity of the soluble CD8αβ, it was tested as inhibitor in a cytolytic assay using PbCS(ABA) pulsed P815 cells as targets. Increasing concentrations of sCD8αβ inhibited the lysis of P815 cells by cloned PbCS(ABA)-specific S14 CTL, with a half maximal inhibition at 0.66 mg/ml (10 μM), and maximal inhibition of 80% at 2 mg/ml (data not shown), indicating that sCD8αβ competes with cell-associated CD8αβ.

SPR studies were performed to assess the interactions of sT1 TCR and sCD8αβ with immobilized K^d-PbCS(ABA). As shown in Fig. 1 A, sT1 TCR bound to K^d-PbCS(ABA) in a dose-dependent manner with an equilibrium constant K_D of 4 ± 0.4 μM. No binding was detectable when HLA-A2-flu matrix peptide complexes or CD4 were used instead of K^d-PbCS(ABA) or sT1 TCR (data not shown). sCD8αβ bound also in a dose-dependent manner to immobilized K^d-PbCS(ABA) (Fig. 1 B), but with low affinity (K_D 99 ± 10 μM), which is in agreement with published values (13, 14). To determine whether CD8αβ affects TCR-ligand binding, sT1 TCR was tested for binding to immobilized K^d-PbCS(ABA) in the presence or absence of 80 μM of sCD8αβ. As shown in Fig. 1 C, sCD8 did not increase the binding of sT1 TCR to K^d-PbCS(ABA). Consistent with this we found that sCD8αβ has no effect on TCR photoaffinity labeling on CD8^- T1.4 cell hybridomas (data not shown). These findings demonstrate that sCD8αβ does not affect the interaction of T1 TCR with K^d-PbCS(ABA). This is in accordance with a study showing that CD8α has no effect on TCR-ligand binding (15), but not with another study claiming that soluble CD8αβ does (13).
The CD8β Transmembrane and Cytoplasmic Portions Are Required for Efficient Intracellular Calcium Mobilization in CD8-transfected T1.4 Hybridomas. To evaluate the role of CD8β transmembrane and cytoplasmic portions for CD8 coreceptor function, we transfected CD8α+ T1.4 hybridomas (6, 10) with CD8β or the CD8β variants shown in Fig. 2. While the T1 CTL clone expresses only CD8αβ, the hybridomas express substantially more CD8α than CD8β, i.e., express homodimeric CD8αα and heterodimeric CD8αβ. The different hybridomas were tested for intracellular calcium mobilization upon incubation with P815 cells sensitized with IASA-YIPSAEK(ABA)I. CD8αβ cells exhibited higher calcium mobilization than the other cells (Fig. 3 A). More striking differences among the different cells emerged, when the peptide variant IASA-YISSAEK(ABA)I (P255S) was used, which is a weak agonist for T1 CTL (25). In this case, the CD8αβ cells displayed nearly the same calcium mobilization as observed for the wild-type peptide, whereas no change was observed in CD8αα or CD8αβα cells. Hybridomas expressing CD8αββ or CD8ααβ responded less efficiently than those expressing CD8αββα or CD8ααβα.

We also measured intracellular calcium mobilization of the indicated hybridomas after incubation with Kd-SYIPSAEK(ABA)I tetramer. As shown in Fig. 3 B, only cells expressing CD8αβ bind to Kd-PbCS(ABA) without affecting its interaction with T1 TCR. For affinity measurements sT1 TCR (A) and sCD8αβ (B) were injected for 30 s at the indicated concentrations over surfaces expressing Kd-SYIPSAEK(ABA)I (4,500 RU) or sCD4 (4,200 RU). Binding was calculated as the difference in the observed equilibrium response between the Kd-SYIPSAEK(ABA)I and the control sCD4 flow cells. The solid lines represent nonlinear fits of the Langmuir binding isotherm to the data, which yielded the indicated Kd values. The maximal binding was 2,600 RU for sT1 TCR and 1,200 RU for sCD8αβ. The insets show Scatchard transformations of the same data. (C) The indicated concentrations of sT1 TCR were injected for 30 s over Kd-SYIPSAEK(ABA)I (2,000 RU) or CD4 (2,300 RU) coated sensor chips in the absence (■) or presence (○) of added sCD8αβ (80 μM). The calculated difference in the responses observed with sT1 alone versus sT1 plus sCD8αβ are also shown (▲). The binding level observed with sCD8αβ alone at 80 μM is shown as dashed line, indicating that the effect of adding sCD8αβ on sT1 binding was never more than additive.

The CD8β Mediates Association of CD8 with TCR/CD3

The CD8β Transmembrane and Cytoplasmic Portions Are Required for Efficient Intracellular Calcium Mobilization in CD8-transfected T1.4 Hybridomas. To evaluate the role of CD8β transmembrane and cytoplasmic portions for CD8 coreceptor function, we transfected CD8α+ T1.4 hybridomas (6, 10) with CD8β or the CD8β variants shown in Fig. 2. While the T1 CTL clone expresses only CD8αβ,
Triton X-100 soluble (M) and insoluble (DIM) fractions and their CD8 content assessed. As shown in Fig. 4 A, CD8α/β and C8ααβ did not. Thus, mutation of Cys179 of CD8αβ impaired CD8 association with rafts as dramatically as deletion of the CD8αβ tail. Interestingly CD8αβ was significantly more raft-associated as compared with CD8ααβ and CD8ααβ/β, indicating that the transmembrane portion of CD8αβ is also involved in the recruitment of CD8 to rafts. Essentially the same results were obtained, whether or not EDTA was present in the lysis buffer, i.e., whether or not CD8 could associate with lck (data not shown). These results show that raft-association of CD8 is essentially mediated by the CD8αβ tail and this mainly due to palmitoylation of its Cys 179.

We next examined the association of CD8 with lck by coimmunoprecipitation. For simplicity we used here total Brij96 lysates, rather than M and DIM fractions, as this detergent has been used previously to assess CD8 association with lck (6–8). As the CD8 transfectants under study express CD8α/β transmembrane and cytoplasmic portions and their CD8 content assessed. As shown in Fig. 4 A, CD8αβ and C8ααβ, efficiently partition in DIM, whereas CD8αβ/β and CD8αβ/β/β did not. Thus, mutation of Cys179 of CD8β impaired CD8 association with rafts as dramatically as deletion of the CD8β tail. Interestingly CD8αβ was significantly more raft-associated as compared with CD8αβ and CD8αβ/β, indicating that the transmembrane portion of CD8β is also involved in the recruitment of CD8 to rafts. Essentially the same results were obtained, whether or not EDTA was present in the lysis buffer, i.e., whether or not CD8 could associate with lck (data not shown). These results show that raft-association of CD8 is essentially mediated by the CD8β tail and this mainly due to palmitoylation of its Cys 179.

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As it has been reported that CD8 associates with LAT, in a manner similar as it associates with lck (5, 23), we Western blotted the same CD8 immunoprecipitates with anti-LAT antibody. Even though LAT was well detectable in total lysates, no LAT was found in the CD8 immunoprecipitates (data not shown).

**CD8β Tail Is Required for CD8-mediated Increase in TCR-ligand Binding.** To assess the ability of the various CD8 constructs to strengthen TCR-ligand binding, we TCR photoaffinity labeled the different cells with soluble, monomeric Kd-125"ISA"-YIPSAEK(ABA)I. As shown in Fig. 5, T1 TCR photoaffinity labeling was highest on CD8αβ+ cells. In the presence of anti-CD8β mAb H35–17, which blocks binding of CD8 to TCR-associated Kd-PbCS(ABA) (10), TCR photoaffinity labeling was reduced by 12-fold, to the same low levels observed on CD8αβ− cells. CD8αα was quite unable to increase TCR-ligand binding, which is consistent with previous findings in a related system (4). Importantly, deletion of the cytoplasmic domain of CD8β (CD8αββ) or substitution with the one of CD8α (CD8αβα) caused a significant (67%) reduction in TCR photoaffinity labeling, demonstrating that the CD8β tail is required for efficient CD8-mediated increase of TCR-ligand binding. By contrast, mutation of CD8β Cys 179 (CD8αβ') caused only a modest decrease in TCR photoaffinity labeling. This variant poorly associates with lck (6; Fig. 4), it appears that CD8αββ supports TCR-ligand binding somewhat better than CD8αβ alone. It is noteworthy that CD8αββ and CD8αβα exhibit partially increased TCR photoaffinity labeling as compared with CD8αα, indicating that the extracellular and/or transmembrane regions of CD8β also play a role in enhancing TCR interactions with MHC-peptide.

The CD8β Tail Mediates Constitutive Association of CD8 with TCR/CD3. Our results so far indicate that CD8αββ increases the avidity of TCR-ligand binding on cells, but not in solution and that the tail of CD8β is needed for this effect (Figs. 1 and 5). We next examined whether this is so, because on cells CD8 associates with the TCR. To this end, we lysed hybridomas expressing no CD8, CD8αα,
CD8αβ, CD8αβ⁺, or CD8αβ⁻ in 0.3% NP-40 and immunoprecipitated TCR. As shown in Fig. 6 A, the amount of CD8α coimmunoprecipitated with TCR was substantially higher on CD8αβ⁺ or CD8αβ⁻⁺ cells, than on CD8αα⁺ or CD8αβ⁺ cells. These differences were not accounted for by variations of TCR or CD8 expression, as all cells expressed comparable levels of CD3ε, CD8α, and CD8β (Fig. 6, B and C).

To find out whether Kd-PbCS(ABA) complexes strengthen association of CD8 with TCR, CD8αβ/H9252/H9251 cells were photo–cross-linked with soluble Kd-SYIPSAEK(ABA)I complexes and analyzed as described in the previous paragraph. No significant increase in CD8 association with TCR/CD3 was observed (Fig. 6 D), indicating that the interaction of CD8αβ with TCR/CD3 is constitutive and not induced by MHC-peptide. Moreover, the coimmunoprecipitation was not impaired by the SH2 peptide pYEEI (data not shown), which has been shown to disrupt activation-induced coupling of CD8 to TCR/CD3 via lck and ZAP-70 (27). Constitutive association of CD8 (and CD4) with TCR/CD3 has been reported previously and low concentrations of NP-40 or Triton X-100 have been recommended to preserve these interactions (28–30). Essentially the same findings where obtained when Brij78 was used, which unlike NP-40 and Triton X-100 solubilizes lipid rafts, indicating that raft integrity was not required for this interaction (data not shown).

**Figure 6.** The CD8β tail mediates CD8 association with TCR/CD3. (A–C) T1.4 hybridomas with the indicated CD8 expression were lysed in 0.3% NP-40, and lysates immunoprecipitated with anti-TCR mAb H57 (A and B), anti-CD8α mAb 53.6.72 (C, left), or with anti-CD8β mAb H35–17 (C, right). The samples were analyzed by SDS-PAGE and Western blotting with anti-CD8α antiserum (A and C) or anti-CD3ε antiserum (B). (D) T1.4 T cell hybridomas expressing CD8αβ or CD8αβ’ were incubated with monomeric Kd-SYIPSAEK(ABA)I complexes (1.16 μM) for 2 h at 0–4°C. After UV irradiation the cells were lysed in 0.3% NP-40. Lysates were immunoprecipitated with anti-TCR mAb H57 or anti-CD8α mAb 35.6.72, as indicated and the immunoprecipitates analyzed by SDS-PAGE and Western blotting with anti-CD8α antiserum.

**Figure 7.** CD8αβ mediates raft association of TCR/CD3. T1.4 hybridomas (5 × 10⁷) expressing no CD8 (A), CD8αβ (B), CD8αβ’ (C), or CD8αβ” (D) were photoaffinity labeled at 26°C with Kd-125“IASA”-YIPSAEK(ABA)I (0.5–1.5 × 10⁸ cpm/7 ml). After UV irradiation the washed cells were lysed in 0.5% Brij58 and the lysates fractionated on sucrose density gradients. Brij58 was used rather than Triton X-100, because it better preserves weak molecular interaction of membrane proteins (21). This difference becomes especially apparent after long periods of incubation, such as fractionation on sucrose density gradients. As shown in Fig. 7, the DIM-containing light fractions exhibited substantial amounts of photoaffinity labeled TCR on CD8αβ⁺ cells, but not on cells expressing CD8αβ’, CD8αβ”, or no CD8. Cells expressing CD8αβα
also lacked significant amounts of photoaffinity labeled TCR in the light fractions (data not shown). The gradient fractions displayed the expected distributions of thy-1, which is a marker of rafts and CD45, which is excluded from rafts (data not shown, and references 17–21). These findings indicate that the association of TCR/CD3 with CD8 prom...
trostatic interactions with the CD8 tails (Fig. 9). Remarkably, this LAT sequence becomes highly negatively charged upon activation-dependent phosphorylation of its tyrosines and serine/threonines (37–39) and thus is expected to undergo strong electrostatic interactions with CD8. Differences in LAT phosphorylation may explain why we were unable to detect association of LAT with CD8, as has been observed in other systems (5, 23). The membrane proximal cytoplasmic sequences of CD3γ, CD3δ, and CD3ζ, similarly become acidic upon phosphorylation of their serines/threonines.

Association of CD8 (and CD4) with TCR/CD3 has been previously observed in different systems (28–30). Of particular interest is a study showing that anti-CD8 immunoprecipitates contain a high proportion of CD3δ(28), suggesting that CD8 associates with TCR via CD3δ (28). This may explain why mice lacking CD3δ (32), or expressing a variant TCR which fails to associate with CD3δ (33), exhibit severely impaired activation and positive selection of CD8+ T cells. It is important to note that the association of CD8 with TCR/CD3 described here (Fig. 6) and in previous studies (28–30) is constitutive, i.e., is not, or little, induced by MHC-peptide.

Moreover, the tail of CD8β mediates raft localization of CD8 (6, 20; Fig. 4). The finding that point mutation of CD8β Cys 179 (CD8αβ') abolishes this (Fig. 4 A), demonstrates that this is attributed essentially to palmitoylation of the CD8β tail. As lck is myristoylated and dipalmitoylated, it also efficiently partitions in rafts (17–19, 35, 36). Thus, the observation that efficient association of CD8 with lck takes place in rafts (6; Fig. 4), is most likely explained by increased concentrations of both molecules in microdomains, which constitute only a small fraction of the cell membrane (17). By contrast, association of CD8 with TCR/CD3 and hence TCR-ligand binding were greatly impaired upon deletion of the CD8β tail, but hardly by mutation of CD8β Cys 179 (Figs. 5 and 6). This demonstrates that the tail of CD8β exerts two different functions: (a) it mediates raft localization of CD8 and hence efficient association of CD8 with lck, and (b) it mediates association of CD8 with TCR/CD3. Because CD8β exerts both functions at the same time, it promotes raft-association of TCR/CD3 (Fig. 7).

While TCR/CD3 per se do not partition significantly in rafts (19–22), this study indicates that they become raft-associated by interacting with raft-resident CD8–lck complexes. Small fractions of TCR/CD3 have been shown to be raft-associated in resting cells in a fractionation study (21) and by confocal microscopy (40). Although CD3 components seem to bind weakly to raft-associated Src kinases in resting cells, which may be important for costimulatory-independent T cell activation (41), we show here that CD8 substantially strengthens raft-association of TCR/CD3 (Fig. 7). As association of TCR/CD3 with CD8/lck also enhances the avidity of TCR-ligand binding (20; Fig. 5), it appears that raft-associated TCR have an increased avidity for MHC-peptide, i.e., are those that are engaged first by MHC-peptide, particularly when these are scarce or of low affinity. Thus, for the induction of TCR signaling this constitutive TCR/CD3 association with CD8/lck is important, as it provides a significant increase in TCR-ligand binding and at the same time raft association of engaged TCR/CD3 (Figs. 5–8).

Because phosphatases are excluded from rafts, they are privileged sites for the induction of TCR signaling (19–22). Indeed we find that tetrameric K+–PbCS(ABA) complexes efficiently induce tyrosine phosphorylation of lck and CD3ζ in rafts on CD8αβ+ hybridoma, but much less so on hybridomas expressing no CD8, CD8αα, CD8αβ', or CD8αβ'' (Fig. 8). As lck is activated by cross-linking, even when Y505 is phosphorylated (6–8, 20, 42), our data propose that TCR signaling is induced by engagement of raft-associated TCR/CD3 adducts with CD8/lck by multimeric MHC-peptide complexes. While it is clear that once TCR signaling is induced, SH2-mediated interactions provide additional coupling of the TCR/CD3 with CD8, LAT, ZAP-70, and other molecules (1, 27, 34), the present study shows that CD8β significantly facilitates TCR signal.
induction, by increasing the avidity of TCR-ligand binding and by docking CD8/CD3 to rafts.

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