CD3δ Establishes a Functional Link between the T Cell Receptor and CD8*

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The differentiation of CD4 CD8 double-positive (DP) thymocytes into CD8 single-positive (SP) T cells requires appropriate signals from the TCR and the coreceptor CD8 (1, 2). DP thymocytes and CD8 SP peripheral T cells express TCRβ that are associated with three signal-transducing units, namely CD3δ and CD3γε heterodimers and a disulfide-linked ζ chain homodimer (3–5). The CD3γε and ε subunits contain in their cytoplasmic tail a single immunoreceptor tyrosine-based activation motif, whereas the tail of the ζ chain harbors three immunoreceptor tyrosine-based activation motifs. For surface expression of TCRαβ, their association with CD3ε, γ, and ζ but not with CD3δ is required (6–9). Accordingly, knockout of CD3ε, γ, and ζ chain arrests T cell development at early stages (6–11). By contrast, in CD3δ knockout mice T cell development proceeds to the DP stage, but positive selection of CD8 (and CD4) SP T cells is severely compromised (9, 12). During TCRβ assembly the TCRβ chain first associates with CD3δε and the TCRα chain with CD3ε, and the resulting trimers then associate and the TCRβ disulfide bond is formed (13). Although CD3δ is physically associated with the pre-TCR complex, it is not required for pre-TCR signaling, which is essential for the transition of double-negative (DN) to DP thymocytes (10, 14, 15).

A conserved motif in the TCRα chain-connecting peptide domain, which connects the transmembrane and the Ig domains, referred to as αCPM, plays a crucial role in positive selection of CD8 and CD4 SP T cells (16–18). The αCPM consists of seven highly conserved amino acids (FETDXXNLN) and is present in TCRδ but not in TCRγ (16). In mice expressing TCR in which the αCPM is replaced by the corresponding sequence of the TCRδ chain, positive selection of SP T cells is greatly impaired, whereas negative selection is normal (16–18). These variant TCRs, referred to as αvβIII (16) TCR, exhibit impaired association with CD3δ, ζ-chain phosphorylation, defective activation of p56lck and extracellular signal-regulated kinase, impaired phosphorylation and recruitment of ZAP-70, p56lck (Lek), and LAT to lipid rafts (17–19). Very similar findings were obtained in CD3δ knockout mice (12, 15), arguing that the defects observed for the αCPM variant TCR are mainly accounted for by their impaired association with CD3δ.

On the other hand, the β chain of CD8 plays a key role for positive selection of CD8 SP T cells. Although CD8 can be readily expressed as the CD8α homodimer, the number of CD8 SP T cells in CD8δ knockout mice is greatly reduced (20, 21). In a milder form, positive selection is also compromised in mice overexpressing tailess CD8δ, and activation is impaired in CD8+ T cells expressing tailess CD8δ (22, 23). Given the similarity in impaired activation and positive selection of CD8 SP T cells in mice lacking CD8β or CD3δ, we examined here whether this is accounted for by the same mechanism, i.e. whether CD3δ couples TCR with CD8αβ. Several studies indicated that CD69 (and CD4) associates with TCR-CD3. For example, the proximity between CD8 (and CD4) and the TCR has been demonstrated on cells by using fluorescence resonance...
energy transfer (FRET) (24, 25). In other studies, CD8 was co-immunoprecipitated with anti-TCR-CD3 antibodies (26–29). According to one of these studies the tail of CD8β is involved in the association of CD8 with TCR-CD8 (29), and another suggested that CD8β can be selectively co-immunoprecipitated with CD8 (and CD4) (26).

To investigate whether and how CD8β establishes a functional link between the TCR-CD3 and CD8, we used thymocytes and T cells from transgenic mice expressing the T1 TCR. This TCR recognizes the Plasmodium berghei circumsporozoite (PcBS) peptide 252–260 (SYIPSAEK) containing photoactive 4-azidoobenzoic acid on Lys-259 (PcBS(ABA)) in the context of KIR (30). Photoactivation of the ABA group results in cross-linking of the T1 TCR with KIR-CD8 fluorescence associated with live cells was measured on a FACSCalibur at 580 nm upon excitation at 488 nm (E1), at 670 nm after excitation at 630 nm (E2), and at 670 nm after excitation at 488 nm (E3). The transfer of fluorescence was calculated as FRET units as follows: FRET unit = (E1both - E3both) x (E1mono - E3mono) x (E2mono/E2both) - (E3 mono - E3both) x (E1 mono/E1 both). The different fluorescence values (E) were corrected for unspecifically labeled cells (Emono), or cells labeled with PE (Epolychrome), Cy5 (Epolychrome), or Cy5 and PE (Eboth).

Soluble KIR-peptide Complexes—KIR-137/2ASA-YIPSAEKABAI complexes (about 2000 Ci/mmol) were prepared as described previously (31). Non-radioactive KIR-PCBS(ABA) complexes were obtained by refolding of KIR heavy chain and human β2 microglobulin, produced in Escherichia coli using the dilution method (35, 36). The refolded monomers were biotinylated, purified, and reacted with PE-labeled extravidin (Sigma) as described previously (35, 36).

Intracellular Calcium Mobilization—P815 mastocytes (1 × 10^5 cells/ml) were pulsed with graded amounts of IASA-YIPSAEK-ABAII for 2 min at 37 °C and UV-irradiated at ×350 nm for 90 s to cross-link the peptide to KIR1 TCR hybridomas or T1 thymocytes (1 × 10^5 cells/ml) were incubated with 5 μM Indo-1 (Sigma, Buchs, Switzerland) at 37 °C for 10 min, washed in DMEM, and incubated with P815 cells for 1 min at 37 °C at an E:T ratio of 1/3. Calcium dependent Indo-1 fluorescence was measured on a FACStarTM as described (37).

TCR Photoaffinity Labeling—T1 TCR hybridomas or T1 TCR thymocytes (7 × 10^5 cells/ml) were incubated in a 12-well plate with KIR-137/2ASA-YIPSAEKABAI (0.5–1.5 × 10^5 cpm/3 × 10^5 cells). After 1 h of incubation at 26 °C and UV irradiation at 312 ± 40 nm for 30 s with 90 watts, the cells were washed twice and lysed for >60 min on ice in 1 ml of PBS containing 50 μg/nl n-octylglucoside and a mixture of protease inhibitors (Roche Molecular Biochemicals, Rotkreuz, Switzerland). TCR were immunoprecipitated using Sepharose-conjugated anti-KIR IgG antibody, and the immunoprecipitates were resolved on SDS-PAGE (10% reducing), and radioactivity was quantified by phosphorimaging analysis using a Fuji BAS1000 (29–32).

Isolation of Lipid Rafts—T1 TCR hybridomas or T1 TCR thymocytes (5 × 10^5 cells/ml) photoaffinity-labeled with KIR-137/2ASA-YIPSAEK-ABAII were lysed in 1 ml of MN buffer (25 mM MES, pH 6.5, 150 mM NaCl) containing 0.5% Brij96 (Sigma) and 50 mM ethylmaleimide (Sigma). The refolded complexes were incubated in 3% sodium dodecyl sulfate (SDS) (100 μl) on ice for 30 min, transferred onto a nitrocellulose membrane, and Western blotted using antibodies specific for KIR (30).

Co-immunoprecipitation and Western Blotting—T1 TCR hybridomas or T1 thymocytes (1.5 × 10^5 cells) were lysed on ice for 2 h in Tris (20 mM, pH 8.0) containing 0.5% Triton X-100 and protease inhibitors (Roche Molecular Biochemicals). The lysates were spun at 10,000 × g for 10 min, and the supernatants were immunoprecipitated using monoclonal antibodies specific for CD8β (KT112), CD8α (53.6.72), or TCR (H57). The immunoprecipitates were washed twice with Tris buffer, pH 8.0, containing n-octylglucoside (50 mM) (Sigma) or as described with Tris buffer, pH 8.0, containing Triton X-100 (0.15%), supplemented with EDTA (5 mM), ethylenimine (5 mM) (Sigma), or NacN (0.5 μM). Immunoprecipitates were resolved on SDS-PAGE (15%, reducing), transferred onto a nitrocellulose membrane, and Western blotted using antibodies specific for anti-CD3, anti-CD8, or anti-CD8α antibodies. For detection the enhance chemiluminescence (ECL) (Amersham Biosciences) was used as described (29).
Modeling of CD3εb—An homologous model of the CD3εb complex was built based on the CD3εγ 3D structure and CD3ε sequence alignment (5), using the MODELLER program (39). The conformations of the connecting loops of the immunoglobulin fold were refined using an ab initio method based on simulated annealing (40).

RESULTS

CD8αβ and Wild Type TCR αCPM Are Required for Efficient Intracellular Calcium Mobilization.—To examine the role of CD8 and the TCR αCPM for T cell activation we first assessed intracellular calcium mobilization in T cell hybridomas expressing CD8αβ and wild type T1 TCR or T1 TCR in which the αCPM was replaced with the corresponding sequence of TCRδ (T1 TCR αIV/βIII). As shown in Fig. 1A, hybridomas expressing the wt TCR and CD8αβ exhibited strong calcium mobilization upon incubation with P815 cells pulsed with 10^-6 to 10^-5 M IASA-YIPSAEK(ABA)I. This calcium flux was stable over the assayed period of 15 min. By contrast, no significant calcium mobilization was observed in the presence of the CD8δ blocking antibody H35. Similarly, on hybridomas expressing the T1 TCR αIV/βIII, only scant calcium mobilization was observed, which was reduced to background levels in the presence of mAb H35 (Fig. 1B).

Using the same method we next examined thymocytes from T1 TCR transgenic mice. These mainly DP cells exhibited strong and stable intracellular calcium mobilization upon incubation with IASA-YIPSAEK(ABA)I-pulsed P815 cells (Fig. 1C). Maximal response was observed at 10^-7 M IASA-YIPSAEK(ABA)I. The stronger calcium responses observed on T1 thymocytes, as compared with CD8αβ T1 T cell hybridomas, is explained at least in part, by the higher surface expression of TCR and CD8 (see “Experimental Procedures”). In the presence of mAb H35, no marked calcium mobilization was observed, indicating that CD8 was required for this response (Fig. 1C). Taken together these results indicate that, for efficient calcium mobilization, CD8αβ and CD3δ TCR are required, which is in accordance with previous studies showing that CD8 and the αCPM are crucial for efficient TCR signaling and positive selection of CD8 SP T cells (16–22).

CD8 Increases MHC-peptide Binding on Cells Expressing Wild Type but Not αCPM Variant TCR—TCR photoaffinity labeling with soluble monomeric K^d^{125I}-IASA-YIPSAEK(ABA)I complexes allows direct assessment of TCR-ligand binding and its dependence on CD8 (30–32). Using this technique we compared TCR-ligand binding on T cell hybridomas expressing the wild type T1 TCR or the T1 TCR αIV/βIII. As shown in Fig. 2A, TCR photoaffinity labeling was reduced by over 6-fold in the presence of Fab fragments of anti-K^d mAb SF1–1.1.1, which block CD8 binding to K^d (32). A slightly larger inhibition was observed on T1 thymocytes and on cloned T1 CTL (Fig. 2B and Ref. 32). The same reductions were observed upon blocking of CD8 with anti-CD8β mAb H35 (data not shown).

Remarkably, on hybridomas expressing T1 TCR αIV/βIII, K^d-Pbscs(ABA) binding was over 4-fold lower than on cells expressing wild type T1 TCR and blocking of CD8 binding to K^d caused only a small reduction. The nonspecific labeling, as seen in the presence of anti-K^d mAb 20–8–4S, which blocks binding of K^d to TCR (32), was in the range of 3% on the hybridomas and below 1% on thymocytes. Upon blocking of CD8 binding to K^d, TCR photoaffinity labeling was slightly lower on hybridomas expressing T1 TCR α IV/β III, as compared with hybridomas expressing the wild type T1 TCR (Fig. 2A). Because the TCR expression is slightly lower on the former as compared with the latter cells (see “Experimental Procedures”), it seems that both TCR bind K^d-Pbscs(ABA) with very comparable efficiency. This is consistent with the fact that both TCR have the same variable and constant domains and argues that the poor TCR photoaffinity labeling observed on T1 TCR α IV/β III is accounted for by inefficient participation of CD8 in TCR ligand binding.
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binding. Because CD8-mediated increase in TCR-ligand binding relies on association of CD8 with TCR-CD3 (29), this argues that the T1 TCR αIVβIII associates poorly with CD8.

Wild Type but Not αCPM Variant TCR Associates with CD8 on Intact Cells—To validate this conclusion, we assessed the proximity of TCR-CD3 and CD8 by FRET. To this end we stained hybridomas expressing CD8 and wild type T1 TCR in the cold with Cy5-labeled anti-CD8α mAb KT112 and PE-labeled K<sup>a</sup>-PbCS(ABA) multimers and measured the FRET value from PE to Cy5. As shown in Fig. 3A, on CD8<sup>+</sup> hybridomas expressing the wild type T1 TCR, FRET was 2.2 units but only 0.4 unit on hybridomas expressing the T1 TCR αIVβIII. The nonspecific signal, as recorded on the corresponding CD8<sup>−</sup> hybridomas, was about 0.03 unit. When using PE-labeled anti-CD3ε mAb 17A2, slightly less efficient FRET was observed (1.5 units, Fig. 3B). Remarkably, this FRET value was enhanced very little when soluble K<sup>a</sup>-PbCS(ABA) monomers were present in the incubation at saturating concentration, indicating that the proximity of CD8 and TCR-CD3 on these hybridomas was not induced by MHC-peptide, i.e. it was constitutive.

To investigate which chain of CD8 was important for coupling of CD8 with TCR-CD3, we performed FRET experiments on hybridomas expressing CD8αβ or CD8αα. Upon staining of CD8αβ<sup>+</sup> hybridomas with PE-labeled K<sup>a</sup>-PbCS(ABA) multimers and Cy5-labeled anti-CD8β KT112, a 4.3-fold stronger FRET was observed than on CD8αα<sup>+</sup> hybridomas stained with Cy5-labeled anti-CD8α mAb 53.6.72 (Fig. 3C). The nonspecific signal in this experiment, as assessed on CD8<sup>−</sup> hybridomas, was 0.04 unit.

Strong FRET was observed on T1 thymocytes following staining with PE-labeled K<sup>a</sup>-PbCS(ABA) multimers and Cy5-labeled anti-CD8β mAb KT112 (37 units, Fig. 3D). About one-third lower FRET (25 units) was recorded when using Cy5-labeled anti-CD8α mAb 53.6.72. Because thymocytes express only CD8αβ, but CD8-transfected T cell hybridomas always express high levels of CD8αα (see “Experimental Procedures” and Refs. 29 and 33), the over 4-fold reduced FRET observed on CD8αα<sup>+</sup> T cell hybridomas indicates that CD8αβ couples with TCR-CD3 more extensively than does CD8αα (Fig. 3C). This is consistent with the finding that CD8αβ is co-immunoprecipitated with the TCR more efficiently as compared with CD8αα (29). In the presence of anti-CD8β mAb H35, this FRET was reduced to 6.2 units, indicating that mAb H35 impedes the association of TCR and CD8. Moreover, faint FRET (5 units) was observed when using Cy5-labeled anti-CD4 mAb GK1.5 as acceptor, which was about 2-fold above background, as recorded when using Cy5-labeled anti-Thy-1 antibody (Fig. 3D). This is consistent with the observation that CD4 also associates with TCR-CD3 (25, 26). This FRET was not reduced in the presence of mAb H35, indicating that this antibody does not impair the PE K<sup>a</sup>-PbCS(ABA) multimer staining. The differences in FRET values observed in the different experiments in Fig. 3 are accounted for in part by variations in TCR and CD8 expression of the different cells (see “Experimental Procedures”). Together these results indicate that CD8 and TCR-CD3 are in close proximity in T1 thymocytes and in T1 T cell hybridomas, given they express CD3<sup>−</sup> TCR and CD8αβ.

Wild Type but Not αCPM Mutant T1 TCR Dock to Raft-associated CD8—Because wild type T1 TCR, but not variant T1 TCR αIVβIII, associates with CD8αβ (Figs. 2 and 3) and CD8αβ is a raft constituent (33, 36), we examined the raft

Fig. 3. Wild type, but not αCPM variant TCR, associates with CD8. A, T cell hybridomas expressing wild type (wt) or αCPM variant T1 TCR (αIVβIII) and CD8αβ (+) or not (−) were stained with PE-labeled K<sup>a</sup>-PbCS(ABA) multimer and Cy5-labeled anti-CD8α mAb KT112. B, alternatively, staining was performed with PE-labeled anti-CD3ε mAb 17A2 and Cy5-labeled mAb KT112 in the absence (−) or presence (+) of soluble K<sup>a</sup>-PbCS(ABA) monomer (1 μg/ml). C, T cell hybridomas expressing CD8αβ, CD8αα, or no CD8 were stained with PE-labeled K<sup>a</sup>-PbCS(ABA) multimer and Cy5-labeled anti-CD8α KT112 or anti-CD8α mAb 53.6.72. D, thymocytes from T1 TCR transgenic mice were stained in the absence (−) or presence (+) of anti-CD8α mAb H35 (15 μg/ml) with PE-labeled K<sup>a</sup>-PbCS(ABA) multimers and Cy5-labeled mAb KT112, 53.6.72, anti-CD4 mAb GK1.5 or anti-Thy-1 mAb III/5. Cells were analyzed by FACS using excitation at 488 and 630 nm. FRET units were calculated from the fluorescence emissions at 580 and 670 nm (see “Experimental Procedures”). Mean values and S.D. were calculated from two to four experiments, each performed in triplicate.
ents. A significantly larger fraction of Kd-125 detergent-soluble and -insoluble components on sucrose gradients (fractions 2–6) was found exclusively in the detergent-soluble dense gradient components, whereas glycosylphosphatidylinositol-linked Thy-1 was detected only in the detergent-insoluble light fractions (fractions 6–10) (Fig. 4). Indeed CD45 which are known markers for the detergent-soluble and detergent-insoluble raft fractions, respectively (41, 42). Indeed CD45 was analyzed by SDS-PAGE (10%, reducing) and Western blotted with anti-CD45 antibodies specific for CD45 (A–D). Aliquots of the fractions from A were analyzed by SDS-PAGE (10%, reducing) and Western blotted with antibodies specific for CD45 (E) or Thy-1 (F). Detergent-insoluble components were mainly found in fractions 2–4, and detergent-soluble components were in fractions 6–10. Each experiment was repeated at least once.

Association of these two TCR. To this end we TCR photoaffinity-labeled CD8αβ⁺ T cell hybridomas expressing wild type or T1 TCR αIV/βIII, lysed the cells in Brij96, and fractionated the detergent-soluble and -insoluble components on sucrose gradients. A significantly larger fraction of Kd-125-IASA-YIPSAEK(ABA)I labeled TCR was found in the detergent-insoluble light fractions 2–4 on hybridomas expressing the wild type T1 TCR as compared with hybridomas expressing the T1 TCR αIV/βIII (Fig. 4, A and B). To verify that our fractionation procedure was correct, we assessed the distribution of CD45 and Thy-1, which are known markers for the detergent-soluble and detergent-insoluble raft fractions, respectively (41, 42). Indeed CD45 was found exclusively in the detergent-soluble dense gradient fractions (fractions 6–10), whereas glycosylphosphatidylinositol-linked Thy-1 was detected only in the detergent-insoluble light fractions (fractions 2–4) (Fig. 4, E and F).

In T1 thymocytes an appreciable fraction of photoaffinity-labeled TCR was found in the light fractions (Fig. 4C). This fraction was greatly diminished when TCR photoaffinity labeling was performed in the presence of the CD8β-blocking mAb H35 (Fig. 4D). These findings indicate that a fraction of TCR is raft-associated due to association of TCR-CD3 with raft-resident CD8. This is consistent with the findings that αCPM variant TCR (18) or TCR from CD3- knockout mice (12) exhibit impaired raft-association and argues that CD3δ couples TCR-CD3 with CD8αβ and hence with lipid rafts.

Cross-linking of TCR-CD3 Adducts Results in the Formation of Large TCR-CD8 Aggregates—We next investigated what consequences TCR-CD3 cross-linking has on TCR aggregation. As assessed by confocal microscopy, incubation of T cell hybridomas expressing CD8αβ and wild type T1 TCR with PE-labeled Kd-PbCS(ABA) multimers resulted in extensive patch formation and internalization (Fig. 5A). The aggregate formation, but not the internalization, also took place when the incubation was performed in the cold (Fig. 5B), suggesting that it does not require cell activation. Strong TCR-CD8 aggregate formation was also seen on T1 thymocytes upon incubation with Kd-PbCS(ABA) multimers and on CD8αβ⁺, T1 TCR⁺ hybridomas after incubation with anti-TCR, and anti-CD8β antibodies (Fig. 5, A and C). By contrast, on hybridomas expressing T1 TCR αIV/βIII, aggregate formation and internalization were greatly reduced. The same diffuse, mainly surface staining, was also observed when CD8αβ⁺, T1 TCR⁺ hybridomas, or T1 thymocytes were pretreated with methyl-β-cyclodextrin, which destabilizes lipid rafts (Fig. 5A). Taken collectively, these findings demonstrate.

**Fig. 5.** CD3δ and CD8αβ are required for cross-linking-induced formation of large TCR-CD8 aggregates. A, T cell hybridomas expressing CD8αβ and wild type (wt), T1 TCR (αIV/βIII), or thymocytes from T1 TCR transgenic mice, pretreated or not with methyl-β-cyclodextrin (MCD), were incubated at room temperature for 20 min with PE-labeled Kd-PbCS(ABA) multimers and examined using confocal microscopy. B, alternatively, the staining of the hybridomas was performed for 40 min at 4°C. C, the hybridomas were incubated for 20 min at room temperature with anti-CD8β mAb KT12, anti-TCR mAb H57, and FITC-labeled rabbit anti-rat IgG antibody and analyzed by confocal microscopy. Representative images are shown from over ten cells analyzed per condition and from at least two different experiments.
To obtain further information on the association of CD3δ with CD3ε and CD8, we washed the CD8 immunoprecipitates twice with different buffers and assessed the amount of co-precipitated CD3δ and CD3ε by Western blotting. The association of CD3δ with CD8 was substantially reduced (to 36%) upon washing with ethylmaleimide, which alkylates free cysteines (Fig. 6B). A smaller reduction (to 55%) was observed upon washing with EDTA-containing buffer, which chelates divalent cations. About 40% reduction was noted upon washing with n-octylglucoside, which disrupts association of transmembrane proteins (41). StrIKingly, washing with 0.5 M NaCl had no marked effect, suggesting that the association of CD3δ with CD8 is not ionic in nature. By contrast, washing with this buffer removed most of the CD3ε from the immunoprecipitates. The other buffers affected the co-precipitation of CD3ε in the same way as the co-precipitation of CD3δ.

In CD8αβ⁺ T1 TCR⁺ T cell hybridomas, similar as with T1 thymocytes (Fig. 6A), anti-CD8β antibody efficiently co-immunoprecipitated CD3δ but little CD3ε (Fig. 6C). In contrast to the T1 thymocytes, there was less CD8 co-immunoprecipitated with the TCR, especially when compared with the amount of CD8 in the lysate. However, although thymocytes express only CD8αβ, the hybridomas express CD8αβ heterodimers and CD8αα homodimers (29, 33), which accounts for the large amount of CD8α detected in the lysate. Taken together these results indicate that association of CD3δ with CD8 is remarkably strong and resists washing with n-octylglucoside and high salt, which combined effectively disrupt the association of CD3δ with CD3ε. On the other hand, the association of CD8 with CD3δ (and CD3ε) is sensitive to alkylation or chelating of divalent cations, suggesting that it involves free cysteines and chelate complexes.

**DISCUSSION**

A key finding of the present study is that CD3δ mediates a functional link between TCR-CD3 and CD8 and that this is crucial for efficient TCR triggering and activation of CD8⁺ T cells. CD3δ knockout mice or mice expressing an αCPM variant TCR, which lacks the δ chain in their CD3 complex, exhibit strongly impaired positive selection and TCR-mediated activation of CD8 SP T cells (Fig. 1 and Refs. 12, 15, 18). The same findings were obtained on CD8α knockout mice (20, 21) and in a milder form, on mice expressing tailless CD8β (22, 23, 29). The present study shows that these signaling defects are explained by the same molecular mechanism, namely that CD3δ couples the TCR with the coreceptor CD8. Several observations support this conclusion. Using TCR photoaffinity labeling with soluble monomeric K⁺-PbCS(AβA) complexes, we find that on cells expressing the αCPM variant T1 TCR, CD8 fails to markedly increase TCR-ligand binding (Fig. 2). It is known that CD8 increases the avidity of TCR-ligand interactions by binding to TCR-associated MHC complexes and that this coordinate binding requires association of TCR and CD8 (29, 32, 43). For example, CD8αα or CD8ββ lacking the tail of CD8β (CD8αβ), poorly associate with TCR-CD3 and therefore inefficiently increase TCR-ligand binding (29). Because T1 TCR αIV/βIII lacks the δ chain of their CD3 complex (17), our TCR photoaffinity labeling experiments argue that CD3δ mediates association of TCR-CD3 with CD8. Consistent with this are our FRET data showing that in T cell hybridomas wild type T1 TCR is in close proximity to CD8 whereas T1 TCR αIV/βIII is not (Fig. 3).

Moreover, because CD8αβ is palmitoylated and partitions in lipid rafts, a fraction of T1 TCR-CD3 is raft-associated, due to its association with CD8 (Fig. 4 and Ref. 29). Several observations indicate that raft association of TCR-CD3 is mediated by CD3δ and CD8αβ. First, TCR lacking CD3δ, due either to disruption of the CD3δ gene (12) or to αCPM replacement,
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exhibit no or little raft association (Fig. 4 and Ref. 18). Second, no significant TCR raft association was observed on T cell hybridomas lacking CD8β or on thymocytes upon blocking of CD8 (Fig. 4 and Ref. 29). Third, CD8δ was selectively co-immunoprecipitated with CD8 (and CD4) (Fig. 6 and Ref. 26). Because CD8δ is known to associate with CD3ε (4, 5), this implies that the association of CD8δ with the coreceptor is stronger than with CD3ε, i.e., is remarkably avid.

How does CD8δ associate with the coreceptor? Because CD8αδ poorly associates with TCR-CD3 (Fig. 3 and Ref. 29), CD8β is important for this interaction. It has been shown that the tail of CD8β is involved in coupling CD8 with TCR-CD3 (29), but it is unclear what other portions of CD8 are involved in this interaction and in what way. Moreover, CD8δ can also be selectively immunoprecipitated with CD4 (26), which is surprising, given the striking structural differences between CD4 and CD8. Because CD4 and CD8 have in common that they associate with Lck and LAT (12, 18, 19). This has been attributed to their poor association with the tail of CD8, which greatly increases the binding of CD8 to raft-resident CD8 or, more precisely, with CD8/Lck, because CD8 associates with Lck in rafts (29, 33). Although they are small in resting cells rafts, they dramatically increase in size upon TCR triggering, which greatly increases the separation of kinases and phosphatases and hence the efficiency of TCR signaling (41, 42). Our confocal studies show that co-cross-linking of TCR and CD8 by soluble MHC-peptide multimers or anti-TCR-CD3 and CD8 antibodies results in the formation of large aggregates of TCR and CD8 (Fig. 5). This was also observed under conditions where cell activation is prevented, e.g. in the cold or in the presence of Src kinase inhibitors (Fig. 5 and Ref. 45). 2 This aggregate formation was also inhibited by methyl-β-cyclodextrin or similar agents, which disrupt lipid rafts, and was not observed on cells expressing the CD3γ-α-TCR (Fig. 5). Taken together these findings argue that cross-linking of raft-associated TCR-CD3 addsucts with CD8/Lck results in strong TCR aggregation and the formation of large rafts and that this is essential for efficient TCR signal induction. Consistent with this is the observation that disruption of rafts greatly diminishes multimer staining of CD8+ CTL, because TCR and CD8 aggregate formation increases the binding of soluble MHC-peptide complexes (45). Furthermore, in cells lacking CD8αβ or upon blocking of CD8, raft association of TCR is diminished and TCR cross-linking-mediated TCR aggregation is strongly impaired, just as it is in cells expressing CD3 ε TCR-CD3 or upon disruption of rafts (Fig. 5 and Refs. 12, 18, 29, 36, and 45).

In conclusion, the present study shows that CD8δ serves to

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Fig. 7. Concepts of CD3δ association with CD8. A, transmembrane and adjacent extracellular and cytoplasmic sequences of CD8α, CD8β, CD8ε, CD2α, CD2γ, CD3ε, and CD4. The sequences and definition of the transmembrane regions were taken from Swiss-Prot (available at www.expasy.ch/sprot). The spanning regions are shown in gray boxes, and the numbers indicate their N- and C-terminal residues. Cysteines are shown in boldface, basic residues in black boxes, and acidic ones are in ovals. B, the electrostatic potential of the outer surface (opposite to CD3ε) of the CD3γ structure (left) and the CD3δ model (right) shown from top to bottom. Acidic domains are shown in red, and basic ones are in blue. The images were produced using the software GRASP (49).
establish a functional link between the TCR and the coreceptor CD8 and that this is essential for efficient TCR signaling, which in turn is needed for activation and positive selection of CD8 SP T cells. A similar conclusion was reached in a related study using a different approach (50). Even though strikingly different in structure, CD4 seems also to associate with CD3, thus forming a similar link with the TCR (25, 26). Indeed, mice expressing CD3ΔΔ TCR αβVIII also exhibit impaired positive selection of CD4 SP T cells (16, 17). In accord with this is the finding that Lck plays a crucial role in T cell development and that for positive selection of CD4 and CD8 SP T cells, Lck must be associated with the coreceptors CD4 and CD8, respectively (1, 2, 46). Finally, it has been shown that the negative selection of CD8 (and CD4) SP T cells is normal in mice expressing CD3ΔΔ TCR (16–18). It is interesting to note that TCR-mediated apoptosis of CD8+ cells is CD8-independent, i.e. in contrast to cell activation and positive selection it is not impaired by the lack of CD8 co-engagement (47, 48).

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