CD8+ cytotoxic T lymphocyte (CTL) can recognize and kill target cells that express only a few cognate major histocompatibility complex class I-peptide (pMHC) complexes. To better understand the molecular basis of this sensitive recognition process, we studied dimeric pMHC complexes containing linkers of different lengths. Although dimers containing short (10–30 Å) linkers efficiently bound to and triggered intracellular calcium mobilization and phosphorylation in cloned CTL, dimers containing long linkers (≥80 Å) did not. Based on this and on fluorescence resonance energy transfer experiments, we describe a dimeric binding mode in which two T cell receptors engage in an anti-parallel fashion two pMHC complexes facing each other with their constant domains. This binding mode allows integration of diverse low affinity interactions, which increases the overall binding and, hence, the sensitivity of antigen recognition. In proof of this, we demonstrated that pMHC dimers containing one agonist and one null ligand efficiently activate CTL, corroborating the importance of endogenous pMHC complexes in antigen recognition.

A hallmark of CD8+ CTLs is their extraordinary sensitivity in recognizing and killing target cells that express only very few cognate pMHC complexes (1, 2). Although soluble TCR and CD8 have been shown to bind pMHC complexes typically with low affinities, fast dissociation kinetics, and in an independent manner (3, 4), the molecular basis of this highly sensitive recognition process is not clear.

For several hormone, cytokine, and chemokine receptors, it has been shown that receptor dimerization is a means to strengthen receptor ligand binding and to promote receptor signaling (5, 6). Dimerization and aggregation, pMHC driven or not, have also been proposed for TCR (7–10); however, the evidences provided never gained general acceptance, leaving this issue open (11–13). In addition, none of these studies provided a plausible structural explanation for TCR dimerization. In view of the wealth of structural information on pMHC, TCR, CD8, pMHC-TCR, and pMHC-CD8 complexes, there should be a structural explanation for TCR dimerization, if it indeed exists. The crystal structure of TCR has uncovered three features that are unique to TCR, i.e. are not found in other immunoglobulin (Ig) super-family members. 1) In the Vα domain, the C′ strand forms hydrogen bonds with the D strand and not with the C′′ strand (14). 2) Cα has only 12–18% sequence homology with other Ig-constant domains, and 12–15 residues are missing, resulting in a less ordered structure and a flatter outer surface as compared with Cβ (15). 3) Cβ has a prominent, surface-exposed FG loop (15). Because of these structural features, TCRα chains are more likely to dimerize than TCRβ chains (14).

Because TCR are naturally membrane-integrated and associated with CD3 units, studies on TCR dimerization/aggregation should be performed on cells (11). The CD3εγδ chains each contain an extracellular Ig domain and a cytoplasmic tail harboring one immunotoyrosine-based activation motive (12, 13). The ζ chain forms a disulfide-linked homodimer, has only a nine-amino-acid-long extracellular portion, and its cytoplasmic tail contains three immunotoyrosine-based activation motives. CD3ζ and ζζ are associated with the TCRα chain, whereas CD3εγ associates with the TCRβ chain (12, 13). Mutations in the highly conserved TCRα chain connecting the peptide motif (CPMα) and the transmembrane domain have profound effects on TCR association with CD3ζ and the ζ chain and severely impair positive selection of thymocytes and TCR signaling (16–18). It seems therefore that the TCRα chain plays a more important role in TCR signaling than the β chain. There are indications that TCR engagement elicits rearrangement of CD3 units and structural changes in their cytoplasmic portions and that this is important for TCR signaling (3, 19, 20).

Soluble pMHC complexes have been used to elucidate the TCR and co-receptor-mediated activation events. These studies concur that activation of CD8+ (and CD4+) T cells by soluble pMHC requires that these are at least dimeric and co-engage the co-receptor (21, 22). The co-receptor CD8 is associated with the cytoplasmic tyrosine kinase p56lck (Lck), which is activated by cross-linking-mediated autophosphorylation (23). In addition, CD8 strengthens the interaction of pMHC with TCR by binding to TCR-associated pMHC (24). Because Lck mediates the initial phosphorylation of immunotoyrosine-based activation...
To address the question of whether CTL activation relies on structurally defined pMHC-driven TCR aggregation, we studied dimeric pMHC complexes containing linkers of different lengths. Such complexes were prepared by alkylation of a free cysteine at the C terminus of the MHC heavy chain (position 275) (26) with bis-maleimides containing linkers of different lengths. To know the actual length of the longer linkers, we incorporated polyprolylproline, which in aqueous media, assume a proline II helix in which one residue spans 3.1 Å (27, 28). Because when in excess of 5–9 residues, polyproline II helices form stable rigid structures containing only trans amide bonds, polyprolylprolines have been used as “ruler” molecules to assess distances, e.g., within large DNA molecules or between the combining sites of immunoglobulin binding sites (27–29). As cells, we used cloned CD8+ S14 CTLs, which recognize the PbCS(ABA) peptide 252–260 (SYIPSAEKI) containing photoreactive 4-azido-benzoic acid on Lys-259 (PbCS(ABA)) in the context of H-2Kd (Kd) (30). These CTLs are amenable to pMHC-TCR photo cross-linking, which when using fluorescent-labeled pMHC, allows detailed binding studies with monomeric or dimeric pMHC complexes as well as fluorescence resonance energy transfer (FRET) experiments, a powerful means to gauge the proximity of suitably fluorescent-labeled molecules (31, 32).

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies—**Cloned S14 CTLs were cultured and used as described previously (22, 24, 26, 30). Polyclonal rabbit anti-actin (AA20–33) and monoclonal anti-diphosphorylated Erk1 and Erk2 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-ZAP-70 mAb was from BD Biosciences; anti-Vav-1 and anti-phosphotyrosine (4G10) mAbs were from Upstate Biotechnology (Waltham, MA); and anti-Fyn phosphospecific (Tyr-417) antibody from BIOSOURCE (Camarillo, CA). The anti-phosphotyrosine (pTyr) mAbs (P-Tyr-01 and P-Tyr-02) were a gift from Dr. V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). Anti-pY-Sepharose was prepared by coupling P-Tyr-01 and P-Tyr-02 mAbs to CNBr-activated-Sepharose 4B (Amersham Biosciences) according to the manufacturer’s recommendations. For blocking of CD8 binding to Kd, Fab fragments of anti-Kd mAbs SP1–1.1.1 were used (24).

**Synthesis of Maleimide-containing Linkers—**All peptides were prepared by solid phase peptide synthesis using Fmoc for transient N-termination and diisoproylethylamine (H9251). For the synthesis of the linker type Mal-(NH-(CH2)2-O-(CH2)2-NH-Mal, bis(2-aminoethyl)-ether trityl resin was used, and for the others, proline-2-chlorotrityl resin (Merck, Darmstadt, Germany) was used. Sequential couplings were performed with 2.5 molar excess of diphenylpropionic anhydride and diisoproylethylamine. Peptides were cleaved from the resin with 5% trifluoroacetic acid in dichloromethane containing 5% triethylamine (H9251). Peptides were then reacted at their free amino groups with a 10-fold molar excess of N,N-diisopropylcarbodiimide (H11003) and diisoproylethylamine (H2-2-glucopyranoside (H9262) and 1% 1-hydroxybenzotriazole (H11002), for 3 h at room temperature under argon. The alkylated Kd/PbCS(ABA) complexes were purified by gel filtration on a Superdex S200 column (Amersham Biosciences). Dimeric Kd/PbCS(ABA) complexes were obtained by reacting the monomeric complexes with a 3-fold molar excess of reduced Kd/PbCS(ABA)-Cys and purified by gel filtration on a Superdex S200 column (26).

To manufacture soluble dimeric Kd complexes containing one agonist peptide (SYIYASEK(ABA)I) and one null peptide (SIYPASEK or SYFPEITHI), monomeric Kd complexes containing the null peptide were first alkylated with DapS bismaleimide linker and the resulting monomers purified by gel filtration on a Superdex S200 column. The alkylated Kd/PbCS(ABA)I complexes were then reacted with Kd/PbCS(ABA)-Cys, and the mixed dimeric Kd complexes were purified likewise. This procedure precludes contamination of the mixed dimers with disulfide-linked homodimers containing two PbCS(ABA) peptides.

**Ca2+ Mobilization and Binding Studies—**Calcium mobilization experiments were performed using the calcium-sensitive dye Indo-1 and flow cytometry as described previously (26, 33). For binding studies, S14-CTL (1 × 106 cells/ml) in Hank’s buffered salt solution supplemented with 0.5% fetal calf serum, 10 mM HEPES, 2 μg/ml human β2-microglobulin, 10 μM PbCS peptide (FACS buffer) were incubated in the absence or presence of the inhibitors with the indicated concentrations of fluorescent Kd-PbCS(ABA) complexes for 5 min at 37 °C. In all experiments, the cells were pre-incubated for 15 min at 37 °C with 100 μM Brefeldin A to inhibit the transfer of peptide from soluble to cell-associated pMHC (36, 37). After UV irradiation (430 nm for 30 s at 310 ± 40 nm), the cells were washed twice in cold FACS buffer and analyzed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences). Data were analyzed using the CellQuest software (BD Biosciences). The nonspecific binding was assessed using the corresponding irrelevant Kd-Cw3 170–179 peptide complexes.

**Western Blotting and Immunoprecipitation—**S14 CTL (1 × 106 cells/ml) were incubated in Opti-MEM I (H9252) containing human β2-microglobulin and 10 μM PbCS peptide in the presence or absence of Kd-PbCS(ABA) monomers or dimers at 37 °C. After washing, the cells were lysed on ice for 40 min in lysis buffer (20 mM Tris/HCl, pH 8.2, containing 100 mM NaCl, 10 mM EDTA, 1% octyl β-D-glucopyranoside (Sigma), 50 mM sodium fluoride, 1 mM orthovandate, and a protease inhibitor mixture (Roche Applied Science). After lysis, cytosolic fractions were resolved on SDS-PAGE and immunoblotted with anti-pY mAb 4G10 as described previously (22, 33). For identification of individual phosphorylated proteins, these were immunoprecipitated with a mixture (1:1) of anti-pY mAbs (P-Tyr-01 and P-Tyr-02), and the immunoprecipitates were immunoblotted using the indicated antibodies (Lck, ZAP-70, PLCγ1, and Vav-1) or, after Western blotting of post-nuclear detergent-soluble fractions, using phosphospecific antibodies (Fyn, Erk1, and Erk2). For LAT, anti-LAT immunoprecipitates were immunoblotted using anti-pY antibody, 4G10.

**FRET Measurements—**S14 CTL (5 × 106/ml) were labeled with Kd-PbCS(ABA) DapS dimer (500 nM) containing the peptides (Dap(Cys)-YIASAEK(ABA)I) and (Dap(Cys)-SYIYASEK(ABA)I), as described above, mixed with paraformaldehyde (3%, 8 min at room temperature). FRET measurements on single cells and for comparison on DapS dimers in solution were performed on a modified epiluminescence wide-field microscope (Axiovert 100 TV, Zeiss). Spectra were recorded by integrating the fluorescence of the whole cell during 1 s on a charge-coupled device camera (LNCCD-576 Euv, Spectroscopy Instruments GmbH) coupled to a spectrometer (CP-140, Spectroscopy Instruments GmbH) with a monochromator (Perkin–Elmer). Dap(Cys) spectra were recorded after excitation at 514.5 nm using an Ar+ laser (Inova Sabre, Coherent) and at 637 nm using a diode laser (Radius 635, Coherent), respectively. FRET efficiencies (EFRET) were calculated as the ratio of the total acceptor emission and the emission due to direct acceptor excitation as described previously (31, 32). The correction for direct excitation of the acceptor was determined using DapS dimer containing only Dap(Cys)-YIASAEK(ABA)I peptides.
Molecular Modeling—The docked conformation of the 2C TCR-Kβ-dEV8 peptide dimer was obtained by an \textit{ab initio} Monte Carlo search using a soft-core van der Waals potential with a distance-dependent dielectric constant. All calculations were performed using the CHARMM program (38). The complex was refined using molecular dynamics simulations at 300 K.

RESULTS

Toward defining the molecular basis of TCR-mediated antigen recognition by CTL, we prepared well defined soluble dimeric pMHC complexes containing linkers of 10–148-Å lengths, connecting the C termini of their constant domains (Fig. 1). The short linkers are flexible, and the indicated spacer lengths refer to their fully extended conformation, i.e., the maximal distance. To define the length of the longer linkers, we incorporated polyproline spacers, which, in aqueous media in excess of 5–9 residues, assume a rigid proline II helix, in which one residue spans 3.1 Å (27–29). For the polyproline-containing dimers, full freedom of mobility of the pMHC entities was assured by short flexible spacers added at either end of the proline helix, as well as the flexible thioether linkage with the protein (Fig. 1B). The integrity of the complexes was verified by protein electrophoresis and gel filtration (Fig. 2).

The complexes were tested on cloned S14 CTL for their ability to elicit intracellular Ca$^{2+}$ mobilization, which is one of the earliest activation events in CTL. Incubation of cloned S14 CTL with graded concentrations of dimers showed that those containing 10–30-Å-long linkers efficiently induced Ca$^{2+}$ flux (Fig. 3A). This is in accordance with the finding that short (but not long) dimeric pMHC class II complexes efficiently activate CD4$^+$ T cells (39, 40). The ability of the dimers to elicit calcium mobilization rapidly decreased as the length of their linker increased. The P10 dimer induced an ~3-fold lower maximal Ca$^{2+}$ flux than the DapS dimer. The P20 dimer elicited a Ca$^{2+}$ mobilization that was slightly above background, and the P30 and the P40 dimers were essentially inactive.

Titration of the DapS dimer showed that half-maximal calcium mobilization required ~1 nM and maximal response ~10 nM pMHC (Fig. 3B). The same titrations performed with the other dimers indicated that the maximal calcium response was highest for the DapS dimer. However, the shorter dihydroxybutane (10.2 Å) and Dap (12.3 Å) as well as the longer Dap(SG)
The DapS dimer induced strong tyrosine phosphorylation; i.e. activation of the TCR proximal signaling molecules analyzed by the specific mAb, PLCγ1, and Vav-1 after immunoprecipitation (IP) on anti-pY mAb; pY (4G10) after immunoprecipitation with anti-LAT Ab; or Fyn, Erk1, and Erk2 using phosphospecific antibodies.

These findings argue that cross-linking of TCR and CD8 by pMHC complexes can be joined by short linkers. The finding that the short dimeric pMHC complexes bind well to and activate CTL poses the question of how two pMHC complexes can be joined by C-terminal linkers as short as 10 Å long.

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Fig. 5. DapS dimer binds better than P30 dimer and monomer. A, S14 CTLs were incubated at 37 °C for 5 min with the indicated concentrations of Cy5-labeled K\(^2\)-PbCS(ABA) monomer (diamonds), DapS dimer (squares), P30 dimer (triangles), or K\(^2\)-Cw3 peptide 170–179 DapS dimer (open squares) or P30 dimer (open triangles). After UV irradiation, the cells were washed and cell-associated fluorescence measured by flow cytometry. MFI, mean fluorescence intensity. B, the incubation of S14 CTLs with the indicated pMHC complexes at 100 nM in the absence (dark gray bars) or presence of EDTA (10 mM) and sodium azide (0.02%) (light gray bars), piceatannol (100 μM) (open bars), or Fab fragments of anti-K\(^4\) mAb SF1–1.1.1 (black bars). Mean values and S.D. were calculated from three to seven experiments.

Fig. 6. pMHC-TCR dimer formation demonstrated by FRET. A, S14 CTLs were labeled with (Dap(Cy3)-YIASAEK-(ABA)I) and (Dap(Cy5)-YIASAEK(ABA)I) containing DapS dimer (500 nM), and FRET was assessed on single cells. The gray line represents the emission spectrum recorded upon donor excitation at 514.5 nm after autofluorescence subtraction, and the black line represents the corresponding fit consisting of contributions from the donor (red line) and acceptor emission (blue line). High FRET efficiencies (E\(_{\text{FRET}}\); insets) are obtained when a large part of the total acceptor emission (green line) is because of FRET (blue line). The remaining acceptor emission is a result of direct excitation of the acceptor. Mean values and standard deviation were calculated from ten cells analyzed. B, the same analysis was performed on DapS dimer in solution. The schematics on the right side show the DapS as bound to TCR (top) or in solution (bottom), where the two pMHC move freely.

tides were recognized by S14 CTL identical to the wild type PbCS(ABA) peptide (data not shown), arguing that the fluorochromes do not interfere with the binding of the S14 TCR to the PbCS complex. Because the fluorescence of Cy3 can excite Cy5, these fluorochromes allow assessment of their proximity by FRET. In solution, the high flexibility of the DapS linker resulted in a broad distribution of distances and orientations between the two N termini, reflected in a low FRET efficiency (E\(_{\text{FRET}}\); of 5%) (Fig. 6B). However, upon photo cross-linking to cell-associated S14 TCR, the FRET efficiency increased to ~20% (Fig. 6A). Because FRET efficiencies decrease with the sixth power of the distance between the donor (Cy3) and the acceptor (Cy5), this marked increase in FRET demonstrated that, upon binding of DapS dimer to cell-associated TCR, the N termini of its two peptides come in close proximity. This validates our dimer model. As expected, no FRET was observed on S14 CTLs that were TCR photoaffinity-labeled with Cy3/Cy3 and Cy5/Cy5 DapS dimers (data not shown).

Any valid TCR dimerization/aggregation model must be consistent with the fact that CTL can recognize target cells expressing only very few cognate complexes amid a vast number of non-cognate ones. To validate our dimer model, we therefore tested dimeric DapS complexes that contain one PbCS(ABA) peptide and one null peptide, i.e., a peptide that binds to K\(^2\) but is unable to trigger S14 CTL (Fig. 7A). As null peptides, we chose the PbCS 252–260 peptide (SYFPEITHI), both of which are not recognized by S14 CTL and are unable to elicit calcium mobilization (Refs. 30, 43, 44, and data not shown). The DapS dimer containing one PbCS(ABA) and one PbCS peptide triggered Ca\(^{2+}\) mobilization in S14 CTL nearly as efficiently as DapS dimers containing two PbCS(ABA) peptides (Fig. 7B). The DapS dimer containing one PbCS(ABA) and one JAK1 peptide induced weaker (but still very significant) Ca\(^{2+}\) mobilization. By contrast, the DapS dimers containing two PbCS or two JAK1 peptides or monomeric K\(^4\)-PbCS(ABA) complexes elicited no Ca\(^{2+}\) flux, even at high concentrations (1 μM) (Fig. 7B and data not shown).

The binding of the different dimers to S14 CTL was in good agreement with their ability to trigger Ca\(^{2+}\) mobilization (Fig. 7C). As compared with the binding of the K\(^4\)-PbCS(ABA)/K\(^4\)-PbCS(ABA) dimer, the binding of the K\(^4\)-PbCS(ABA)/K\(^4\)-PbCS dimer was reduced by ~10%, the binding of the K\(^4\)-PbCS(ABA)/K\(^4\)-JAK1 dimer by 25%, and the K\(^4\)-PbCS(ABA) monomer by 70% (Fig. 7C). Strikingly, however, as assessed in a competition assay, K\(^4\)-PbCS monomer very weakly competed with the K\(^4\)-PbCS(ABA)/K\(^4\)-PbCS(ABA) monomer binding to S14 CTL, and for the K\(^4\)-JAK1 monomer, competition was barely detectable (Fig. 7D). Because the preparation of the mixed DapS dimer precludes any contamination of other dimers, these results clearly demonstrated that null ligands, such as the dominant JAK1 kinase-derived self-peptide, substantially augment the sensitivity of CTL antigen recognition. This validates the proposed dimeric antigen recognition mode, which stipulates that the overall
binding avidity is increased by integrating diverse weak interactions and provides a molecular explanation on how endogenous null ligands crucially contribute to the extraordinary sensitivity of CTL.

**DISCUSSION**

Although it has been recognized previously (22) that dimeric pMHC complexes are the minimal trigger for CD8+ T cells, the present study showed for the first time that the ability of soluble dimeric pMHC complexes to bind to and activate CTL is determined by the length of the linker they contain (Figs. 3 and 4). It has been reported that TCR on resting T cells occur as dimers or super-dimers (8, 9). However, it has been argued that this model is inconsistent with the stoichiometry of CD3 association with TCR (12, 13). The finding that monomeric pMHC complexes were unable to activate CTL (Figs. 3 and 4) and the lack of FRET, i.e., of close proximity between pMHC monomer and engaged TCR (unpublished results), indicate that tight TCR dimer formation occurs only upon TCR cross-linking. This, however, does not exclude that TCR/CD3 on resting T cells occurs in some pre-aggregated state; in fact such TCR pre-aggregation may facilitate pMHC binding.

The proposed dimeric pMHC/TCR binding mode described in this study is a logical consequence of diverse, converging evidences. First, our observation that pMHC dimers containing C-terminal linkers as short as 10 Å efficiently bind to and activate CTL (Figs. 3–5) imposes a great constraint on how two pMHC complexes can be joined, such that they can interact with cell-associated TCR. Considering the three-dimensional structure of the Kd-dev8 peptide complex, we found that this is possible only in configurations, in which the constant domains of the pMHC complexes face each other (Fig. 8A and Ref. 15). This is so, because the outer surface of the α3 domain together with the tip of the β2 microglobulin forms a nearly flat surface, which contains the α3 C terminus, via which the pMHC dimers are formed.

Second, as a result of the binding orientation of TCR to pMHC, the two TCRα chains face each other in the dimeric pMHC/TCR complex, resulting in stabilizing interactions between the Ca and Vα domains, including the fourth variable loop (Fig. 8B). This dimeric binding mode also emerged from docking simulations using the coordinates of the Kd-dev8-peptide-2C TCR complex (15) and an energy minimum-seeking Monte Carlo procedure. In the resulting stable binary complex, the C termini of the pMHC heavy chains were ~30 Å apart (Fig. 8A). This corresponds well with the maximal distance of the DapS dimer (17.2 Å for the linker plus 13 Å for the two thiioether linkages). The hallmark of the TCR dimer formation is the intimate proximity and contact formation of the two TCRα chains (Fig. 8B). This is made possible by two structural features unique to TCRα chains: (i) in the Vα domain, there is a strand shift that removes surface protrusions and allows tight Vα–Vα interactions (14) and (ii) in the Ca, 12–15 residues are missing as compared with Ig constant domains, which results in a less ordered, flatter surface and favors Ca–Ca interactions (15). We propose that these features have evolved to allow such TCR dimer formation. This TCR dimer formation is likely to be important for TCR signal initiation. The TCRα chain associates with the TCRβ chain, one ζ chain homodimer, and one CD3ε6 heterodimer (12, 13). The close proximity of the two TCRα chains induced by the TCR dimer formation thus may cause rearrangement and/or conformational changes of CD3 units in the TCR/CD3 complex. This view is supported by the findings that (i) mutations in the conserved TCRα chain connecting peptide and transmembrane sequences have deleterious effects for TCR function (16–18), that (ii) upon TCR triggering, ζ-chain dissociates from TCR/CD3 (19), and that (iii) structural changes occur in CD3ε upon TCR ligation (8).

Third, the FRET efficiency of 20%, as measured on cell-bound Dap(Cy3)-YLISAAEK(Aβ)-Dap(Cy5)-YLISAAEK(Aβ)I DapS dimers, corresponds to a distance of ~65 Å between the donor (Cy3) and the acceptor (Cy5) (Fig. 6A). This, however, is a gross overestimate. The TCR photo-affinity labeling efficiency on S14 CTL for Kd-PbCS(Aβ) monomer is ~50% (34, 35). This means that, upon photo-cross-linking, about one-third of the DapS dimer is cross-linked with two TCRs and two-thirds with only one TCR. In the mono-crosslinked DapS dimers, the free pMHC units have quite the same high freedom of mobility as on DapS dimers in solution, i.e., they contribute little to FRET. Thus, considering a double cross-linking efficiency of 33%, the average distance of Cy3 and Cy5 is estimated to be ~40 Å. Moreover, photo-cross-linking causes some photobleaching of Cy3 and namely Cy5, which further reduces the apparent FRET efficiency. Taken together, the FRET data indicate that Cy3 and Cy5 on the cell-bound DapS dimer are 30–40 Å apart. This strongly supports our dimer model, which predicts a distance of 30–35 Å (Fig. 8).

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**FIG. 7.** The TCR-pMHC dimer formation endows CTL with high sensitivity. A, schematic showing the pMHC complexes under study. B, Indo-1-labeled S14 CTLs were incubated at 37 °C with the indicated concentrations of the DapS dimeric complexes containing the peptides SYIASAEK(Aβ)I and SYIASAEK(Aβ)I (●), SYIASAEK(Aβ)I and SYIPSAEKI, (●), SYIASAEK(Aβ)I and SYIPFEITHI, (●), or monomeric Kd-SYIASAEK(Aβ)I complexes (●), and the maximal Ca2+ mobilization measured was as described for Fig. 2B. C, the binding of the Cy5-labeled DapS complexes (● circles), (●) squares), (●) circles), (●) diamonds), Kd-SYIASAEKI (circles), Kd-SYIPFEITHI (squares), or HLA-A2-melanA (open triangles). After UV irradiation and washing, cell-associated Cy5 fluorescence was measured by flow cytometry. Mean values and S.D. were calculated from three to five experiments.

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2 M. Cebecauer, P. Guillaume, S. Mark, O. Michielin, N. Boucheron, M. Bezard, B. H. Meyer, J.-M. Segura, H. Vogel, and I. F. Luescher, unpublished results.
X-ray crystallography provided invaluable information on the structure of pMHC complexes, TCR, CD8αα, as well as of pMHC-TCR and pMHC-CD8α complexes. In a few cases, pMHC molecules, TCR Va chains, or pMHC-TCR complexes crystallized in dimeric form, raising the question as to what extent such information provides insights on how these molecules interact on cell surfaces. It, however, turns out to be difficult to extract from such data a unifying structural consensus, and often the intermolecular associations seen in crystals are not compatible with the fact that pMHC complexes expressed on one cell have to interact with TCR and CD8 that are expressed on another cell. It is noteworthy that in a crystal of

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**Fig. 8. Structural model of pMHC-TCR dimer formation.** Two Kb-dEV8 peptide-2C TCR complexes were docked using an energy minimum-seeking Monte Carlo procedure. The side and top views are shown for the pMHC dimer (A), the pMHC-TCR dimer (B), and the pMHC-TCR-CD8 dimer (C). The peptide is shown in green, the heavy chain in gray-yellow, β2-microglobulin in light blue, TCRα chain in dark blue, TCRβ chain in gray, and CD8αα in red.
D\textsuperscript{b} with the LCMV peptide gp33, a similar “back-to-back” pMHC dimer was observed, as shown in Fig. 8, exhibiting 12 Å of distance between the α3 C termini (45). However, in other crystals other orientations were found. For example HLA-B8 crystallized as a dimer in which the two pMHC face each in an opposite orientation, compared with our model (i.e. one pMHC complex is turned by 180°) (Fig. 8) (46). The xenoreactive TCR AHIII 12.2 crystallized with its ligand, the HLA-A2 peptide p1049 complex, in a parallel configuration in which the α1 domain of one pMHC complex interacts with the α2 domain of the other (47). The distances of the α3 C termini in these two complexes are ~44–46 Å, which is inconsistent with our finding that the DB dimer containing a 10.2-Å-long linker (23.2 Å including the two thioether linkages) efficiently activates CTL (Figs. 3 and 4).

Using FRET and single molecule tracking, numerous studies concur that cell-associated pMHC class I complexes on antigen-presenting cells form dimers and higher aggregates (48–50). One of these studies, based on FRET experiments using fluorescent-labeled Fab fragments of anti-MHC heavy and light chain monoclonal antibodies and lipid probes as well as computer-assisted docking experiments, came up with a dimer model that is similar to ours (49). This study also proposed that pMHC-TCR dimers form dimers of dimers. We have single molecule tracking data on CTL demonstrating that TCR photo cross-linked DapS dimer (but not monomer) indeed form dimers of dimers.\textsuperscript{2} A better understanding of the molecular nature of such dimer-dimer association should provide new insights not only on pMHC-pMHC interactions relevant to CTL triggering but also on the role of CD8 in pMHC binding and CTL activation. The observation that blocking of CD8 co-engagement inhibited the binding of the short DapS dimer more effectively than the binding of the long P30 dimer or the monomer (Fig. 5), suggests that the striking differences in binding of these three pMHC complexes are related to CD8. To better understand how CD8 may participate in pMHC-TCR dimer and higher aggregate formation, we added CD8α to the TCR-pMHC dimer by superimposing the K\textsuperscript{β} coordinates of the K\textsuperscript{β}-dEV8-peptide-2C TCR (15) and the K\textsuperscript{α}-VSV peptide-CD8α (42) complexes (Fig. 8C). Although CD8 expressed not CD8α but CD8β, for which there are no structural data, this model suggests that CD8 is not directly involved in pMHC-TCR dimer formation but probably is involved in pMHC-TCR dimer-dimer association.

One of the enigmas of CD8+ CTLS is how they can recognize target cells that express only very few cognate pMHC complexes (1, 2). Antigen-presenting cells typically express a high number of pMHC class I complexes, displaying a vast diversity of peptides. Although the majority of these complexes are unable to bind to a given TCR, a fraction does, however with low to very low affinity. These non-cognate pMHC complexes are of considerable physiological significance. On one hand, they mediate the positive selection of T cells (51). On the other hand, they can greatly enhance the sensitivity of antigen recognition by T cells (52). The binary TCR binding mode described here provides for the first time a molecular explanation for this. The dynamic formation of pMHC-TCR dimers allows harmonious integration of diverse weak interactions, including several non-antigen-specific ones, which provides a substantial gain in overall binding avidity and, hence, sensitivity of antigen recognition (Figs. 3–5). Direct proof for this is the observation that a mixed DapS pMHC dimer containing one agonist peptide and one prominent endogenous null peptide significantly binds to and activates CTL, whereas monomeric agonist pMHC does not (Fig. 7).

Our results support a binary binding model in which two TCR engage, in an anti-parallel manner, two pMHC complexes facing each other with their α3 domains (Fig. 8). Receptor dimerization is well established for cytokine and growth factor receptors (5, 6) and has also been proposed for TCR (7–10); however, the evidences provided are controversial (11–13). The binary TCR binding mode presented here provides a new view of antigen recognition by CTL and will help to elucidate outstanding questions, such as how CD8 and CD3 units participate in dimer formation and how TCR dimers associate to yield higher TCR aggregation. Because in this study only soluble pMHC complexes were used, it remains to be established whether it also applies to CTL recognition of target cells. There are indications suggesting that this is the case. For example, it has been shown that dimeric pMHC complexes, obtained by fusing pMHC class I complexes at the N terminus of IgG, efficiently inhibit target cell recognition at nanomolar concentrations (53, 54). The pMHC-pMHC distances in these pMHC-IgG fusion proteins are in the range of our long dimers. In agreement with this is the finding that the long P30 dimer efficiently inhibits target cell killing by SI4 CTL at nanomolar concentrations.\textsuperscript{2} These observations argue that the binding of long pMHC complexes to CTL interferes with pMHC-induced TCR dimer formation, thus stressing its importance for CTL activation.

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