The Efficiency of Antigen Recognition by CD8+ CTL Clones Is Determined by the Frequency of Serial TCR Engagement

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The Efficiency of Antigen Recognition by CD8+ CTL Clones Is Determined by the Frequency of Serial TCR Engagement

Denis Hudrisier,* Benedikt Kessler,* Salvatore Valitutti,† Cloitilde Horvath,* Jean-Charles Cerottini,* and Immanuel F. Luescher2*

Using H-2Kd-restricted CTL clones, which are specific for a photoreactive derivative of the Plasmodium berghei circumsporozoite peptide PbCS252–260 (SYIPSAEKI) and permit assessment of TCR-ligand interactions by TCR photoaffinity labeling, we have previously identified several peptide derivative variants for which TCR-ligand binding and the efficiency of Ag recognition deviated by fivefold or more. Here we report that the functional CTL response (cytotoxicity and IFN-γ production) correlated with the rate of TCR-ligand complex dissociation, but not the avidity of TCR-ligand binding. While peptide antagonists exhibited very rapid TCR-ligand complex dissociation, slightly slower dissociation was observed for strong agonists. Conversely and surprisingly, weak agonists typically displayed slower dissociation than the wild-type agonists. Acceleration of TCR-ligand complex dissociation by blocking CD8 participation in TCR-ligand binding increased the efficiency of Ag recognition in cases where dissociation was slow. In addition, permanent TCR engagement by TCR-ligand photocross-linking completely abolished sustained intracellular calcium mobilization, which is required for T cell activation. These results indicate that the functional CTL response depends on the frequency of serial TCR engagement, which, in turn, is determined by the rate of TCR-ligand complex dissociation. *The Journal of Immunology, 1998, 161: 553–562.

Epitope modification can affect Ag recognition by CTL in a diverse manner, by causing antagonism, an increase or decrease in the efficiency of Ag recognition (strong and weak agonists, respectively), or activation of some, but not other, effector functions (partial agonists) (1–7). Two different concepts have been proposed to explain these phenomena. According to the first, the kinetic proofreading concept, the rate of TCR-ligand complex dissociation is critical (8–10). If the time of TCR engagement is too short, the signaling cascades it elicits are terminated prematurely, resulting in incomplete TCR signaling. Since coreceptor (CD4 or CD8) can bind MHC molecules that interact with TCR, future, resulting in incomplete TCR signaling. Since coreceptor (CD4 or CD8) can bind MHC molecules that interact with TCR, and thus bring the coreceptor-associated Src protein tyrosine kinase (PTK) p56lck to TCR/CD3ζ, Madrenas et al. argued that premature TCR-ligand complex dissociation will limit the phosphorylation of CD3ζ and CD3-associated ZAP-70 by p56lck (11). Alternatively, according to the second concept, epitope modifications provoke conformational changes in TCR that affect the association of TCR with CD3ζ and thus qualitatively alter TCR signaling (12, 13).

In support of the kinetic proofreading concept, Lyons et al. showed that antagonist peptides exhibit faster TCR-ligand complex dissociation than agonists (10). In addition, it has been demonstrated that antagonists elicit only limited phosphorylation of CD3ζ, with a preponderance of pp21-phosphorylated ζ-chain that is unable to bind ZAP-70, a PTK that plays a crucial role in the induction of the NF-AT pathway of T cell activation (14–16). Together these data convincingly explain the observation that peptide antagonists not only fail to elicit a functional T cell response, but can inhibit recognition of an agonist.

However, there exist several observations that seem difficult to explain by the kinetic proofreading concept. For example, it has been reported that cytolytic CTL responses can be antagonized by very low concentrations of altered viral peptides (17, 18). Also, this concept fails to explain how certain altered peptide ligands can induce some, but not other, T cell effector functions. Moreover, we have previously observed that peptide variants that were efficiently recognized by cloned CTL (strong agonists), exhibited faster TCR-ligand complex dissociation than those that were inefficiently recognized (weak agonists) (5). This suggests that when TCR ligation results in integral TCR signaling, the magnitude of the functional CTL response is dependent on the frequency of serial TCR engagement. The serial TCR engagement model of T cell activation proposes that during T cell-APC encounter, ligands on APC are serially engaged by TCR of T cells, which permits sustained TCR signaling, even when the ligand density is low (19–21). For our study we used CTL clones that permit assessment of TCR-ligand interaction by TCR photoaffinity labeling. These clones were H-2Kd restricted and specific for a derivative of the PbCS peptide252–260 (SYIPSAEKI) containing photoreactive iodo-4-azidosalicylic acid (IASA) in place of PbCS S-252 and 4-azidobenzoic acid (ABA) on PbCS K-259 (4, 5, 22). Selective photoactivation of the IASA group permitted cross-linking to ζ, and photoactivation of the ABA group permitted cross-linking to TCR.

In the present study we examined a larger panel of epitope variants and CTL clones to verify this hypothesis. In addition, we
addressed the question of how blocking of CD8 affects Ag recognition and TCR-ligand complex dissociation. CD8 plays various roles in CTL function such as 1) by coordinate binding of TCR-bound MHC molecules, CD8 strengthens TCR-ligand binding, primarily by decreasing TCR-ligand complex dissociation (23, 24); 2) following TCR/CD3 ligation, CD8 acquires an increased affinity for MHC class I molecules and can act as an adhesion molecule (25, 26); and 3) by virtue of the associated p566, CD8 is involved in the phosphorylation of CD3/ζ and ZAP-70 (14, 15). Since anti-CD8 mAb can differentially affect these CD8 functions and hence have diverse effects on functional CTL responses (23), we used in the present study Fab’ fragments of the anti-κ’3 mAb SFI-1.1.1. a reagent that selectively blocks CD8 participation in TCR-ligand binding, without affecting CD8-dependent adhesion and the CD8-mediated signaling it involves (25, 26).

We report that the efficiency of Ag recognition (cytotoxicity and IFN-γ production) is directly related to the rates of TCR-ligand complex dissociation, and that these, in turn, are determined largely by CD8.

Materials and Methods
Peptide and peptide derivative synthesis
Amino acids and other chemicals for peptide and conjugate synthesis were obtained from Bachem (Bubendorf, Switzerland), Sigma (Buchs, Switzerland), and NeoSystmes (Strasbourg, France). Synthesis of peptides and conjugates, in radioactive and nonradioactive forms, was performed as previously described (4, 5, 22). In brief, all peptides were synthesized on an ABI 431 solid phase peptide synthesizer (Applied Biosystems Instruments, Foster City, CA), using Fmoc for transient N-terminal protection. The PbCS 431 solid phase peptide synthesizer (Applied Biosystems Instruments, Foster City, CA), using Fmoc for transient N-terminal protection. The PbCS peptide derivatives were synthesized using Fmoc-Tyr(H2O)-OH as second residue, Fmoc-Lys(ABCA)-OH in position 8, and ASA-ONu for N-capping. Following reverse phase HPLC purification on a C18 column (1 × 25 cm, 5-μm particle size, Marcherey & Nagel, Oensingen, Switzerland), the peptide derivatives were purified in the presence of chloramine-T, followed by dephosphorylation with alkaline phosphatase. All peptide derivatives were characterized by mass spectrometry on an LDI 7000 mass spectrometer (Linear Scientific, Reno, CA) and UV spectrometry on an ABI 1000S diode array spectrometer. After lyophilization, nonradioactive peptides and peptide derivatives were reconstituted in PBS at 1 mM. The radioactive conjugates were immediately used for Kd photoaffinity labeling; their specific radioactivity was approximately 2000 Ci/mmol.

Cytolytic assays
Cloned CTL were obtained from BALB/c × C57BL/6 F1 mice immunized with IASA-YIPSAEK(ABA)1 as previously described (22). They were stimulated weekly with irradiated BALB/c splenocytes and irradiated peptide derivative-pulsed P815 cells in the presence of IL-2 (EL4 supernatant). 51Cr-labeled P815 cells were used as targets in a chromium release assay as previously described (4, 5, 22). In brief, sensitized target cells (5 × 106/well) were incubated in microtiter plates for 1 h at 37°C in medium containing 10-fold dilutions of peptides, followed by UV irradiation (see below). Cloned CTL (1.5 × 105/well) were added, and after 4-h incubation at 37°C, the 51Cr content of the supernatants was determined. In some experiments target cells were preincubated with SFI-1.1.1 Fab’ (20 μg/ml) for 15 min at 37°C. The specific lysis was calculated as 100 × ([experimental – spontaneous release]/[total – spontaneous release]). The relative antigenic activities were calculated by dividing the concentration of IASA-YIPSAEK(ABA)1 required for half-maximal lysis, by that required for the variant peptide derivatives. The relative Kd competitor activity, expressing the Kd binding of the different peptide derivatives (4, 22), was used to normalize the relative antigenic activities by dividing them by the corresponding relative Kd competitor activities. In all experiments used for calculating relative antigenic activities, the slopes of the plots for specific lysis vs peptide concentration were parallel, as shown, for example, in Ref. 4.

IFN-γ production
P815 cells (5 × 105/well) were sensitized with the respective peptide derivatives, as described in the previous section, and incubated with cloned CTL (1.5 × 105/well) for 24 h at 37°C. The IFN-γ content of the supernatants was determined by ELISA, using for absorption anti-IFN-γ mAb R4–6A2 and for detection biotinylated anti-IFN-γ mAb AN18.

The biotinylated Ab, horseradish peroxidase-conjugated streptavidin (Amersham, Arlington Heights, IL) was used, followed by incubation with o-phenylenediamine hydrochloride (Sigma). The color was measured at 490 nm using an ELISA reader (MR7000, Dynatech, Chantilly, VA). The relative antigenic activities were calculated and normalized as described in the previous section. For calculation of relative antigenic activities, only experiments in which the slopes of the titrated functions were parallel were used.

Kd and TCR photoaffinity labeling
All photoaffinity labeling procedures were performed as previously described (4, 5, 22). In brief, for Kd photoaffinity labeling, P815 cells were incubated with cloned CTL (1.5 × 105 cells/well) at 37°C for 2 to 3 h, followed by UV irradiation at ≥350 nm with a 3000-watt SupUVA Sun UV irradiator (Mutzhas, Munich, Germany). After washing (three times) the cells were incubated at 37°C for 1 h with [35S]methionine and used in a cytolytic assays as previously described (5, 22). Alternatively, 125I-labeled peptide derivatives were incubated with soluble Kd (50 μg) in PBS (200 μl) and βγ (2.5 μg/ml; Sigma) for 1 to 2 h at room temperature, followed by UV irradiation at ≥350 nm. The samples were diluted with PBS (300 μl) containing PbCS peptide 52–62 (10 μM) and after ≥12 h of incubation at 20 to 25°C were subjected to gel filtration fast protein liquid chromatography. The purified soluble Kd-peptide derivative complexes had concentrations of 0.7 to 2.5 × 105 cpm/ml and were used within 1 mo. For TCR photoaffinity labeling, cloned CTL were resuspended in DEMEM supplemented with FCS (1%) and HEPES (10 mM) and incubated with 125I-labeled Kd-peptide derivative complexes (0.6–4 × 105 cpm/ml) at 26°C for 1 to 1.5 h. After UV irradiation for 20 s with a 5000-watt UV B irradiator (Dr. Höne, Inc., Munich, Germany), equipped with a shutter as well as an infrared and UV 296-μm filter, the cells were detergent lysed and TCR immunoprecipitated with anti-TCR Cβ mAb H57-597. The immunoprecipitates were analyzed by SDS-PAGE (10%, reducing conditions), and the P815 cells were detergent lysed and TCR immunoprecipitated with anti-TCR Cβ mAb H57-597. The immunoprecipitates were analyzed by SDS-PAGE (10%, reducing conditions), and the immunoprecipitated TCR photoaffinity labeling was assessed as described above. The kinetics of intercellular TCR-ligand binding were determined similarly as described previously (27). Briefly, aliquots of P815 cells (5 × 105), previously 125I photoaffinity labeled with 125I-ASA-YIPSAEK(ABA)1 or variants (1–6 × 105 cpm/ml) in the presence of the absence of SFI-1.1.1 Fab’ (20 μg/ml) for 2 to 3 h at 0 to 4°C. At time zero aliquots were diluted into 10-ml aliquots of DEMEM containing anti-Kd α1 mAb 20-8-4S (10 μg/ml). After the indicated periods of incubation at 26°C, the samples were UV B irradiated, and TCR photoaffinity labeling was determined as described above. Mean values and SDs were calculated from three to six independent experiments. The kinetics of intercellular TCR-ligand binding were determined similarly as described previously (27). Briefly, aliquots of P815 cells (5 × 105), previously 125I photoaffinity labeled with 125I-ASA-YIPSAEK(ABA)1 or variants (1–6 × 105 cpm/ml) in the presence of the absence of SFI-1.1.1 Fab’ (20 μg/ml) for 2 to 3 h at 0 to 4°C. At time zero aliquots were diluted into 10-ml aliquots of DEMEM containing anti-Kd α1 mAb 20-8-4S (10 μg/ml). After the indicated periods of incubation at 26°C, the samples were UV B irradiated, and TCR photoaffinity labeling was assessed as described above. Mean values and SDs were calculated from two to six experiments.

Kd chain phosphorylation
For γ chain phosphorylation, P815 (3 × 105 cells/incubation), previously labeled with 125I photoaffinity labeled with the indicated peptide derivatives, were mixed in the presence or the absence of SFI-1.1.1 Fab’ (20 μg/ml) with cloned CTL (1 × 105 cells/incubation) in DEMEM, supplemented with 5% FCS, centrifuged at 1500 × g for 1 min, and incubated for 8 to 10 min at 37°C. After centrifugation (20 s at 14,000 × g), cells were lysed in 0.5 ml of cold lysis buffer (1% Triton X-100, 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 0 μg/ml aprotinin, and 10 mM DNa WO3). Detergent-insoluble materials were removed by centrifugation (15 min at 14,000 × g) and membrane fraction and subjected to immunoprecipitation using anti-γ chain mAb H146. Immunoprecipitates were washed (three times) in lysis buffer, boiled for 3 min in reducing SDS-PAGE sample buffer, resolved on 15% SDS-polyacrylamide gels, transferred on polyvinyllidene difluoride membranes (New England Nuclear, Boston, MA), and immunoblotted with anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY). The blots were developed by the enhanced
Results

Strong agonists exhibit fast and weak agonists slow TCR-ligand complex dissociation

To compare the efficiency of Ag recognition with TCR-ligand binding, we tested seven variants of the PbCS peptide derivative IASA-YIPSAEK(ABA)I on the CTL clones S4, S14, S17, and T1 (Fig. 1). Three of these variants contained alanine in place of PbCS P255, S256, and E258, respectively, and four others contained K, L, N, or S in place of PbCS P255. The antigenic activities were assessed in a chromium release assay, and TCR-ligand binding was determined by TCR photoaffinity labeling at 26°C: both were normalized relative to values for the wild-type peptide derivative (wt). In 10 cases the normalized antigenic activity and TCR photoaffinity labeling deviated by fivefold or more. Four of these cases were weak agonists, i.e., Ag recognition was less efficient than TCR-ligand binding, and four were strong agonists, i.e., recognition was more efficient than TCR-ligand binding. While weak agonists were observed on all four CTL clones, strong agonists were limited to S17 and T1 CTL. In only one case was TCR-ligand binding observed in the absence of detectable cytotoxicity (P255L on S14 CTL). We have shown previously that this variant is an antagonist for S14 CTL (4). It is noteworthy that in some instances the efficiency of Ag recognition differed depending on whether the peptide derivative was cross-linked to Kd (Fig. 1) or not (4, 5) (e.g., variant E258A on S4 CTL or variants P255L and P255N on S17 CTL).

From Fig. 1, it emerges that the efficiency of Ag recognition did not correlate with TCR-ligand binding. We therefore examined whether it was related to the rates of TCR-ligand complex dissociation. As shown in Figure 2, for strong agonists, TCR-ligand complex dissociation was more rapid than for the wild-type peptide derivative (e.g., variants S256A, P255N, and P255L on S17 CTL). Conversely, for all the weak agonists, TCR-ligand complex dissociation was slower than for the wt. An exception to this rule was variant P255L, for which TCR-ligand complex dissociation on T1 was remarkably slow, but Ag recognition was only slightly less efficient than TCR-ligand binding (Figs. 1 and 2). By contrast, on S14 CTL this variant was an antagonist and exhibited very rapid TCR-ligand complex dissociation, with a half-life of only approximately 15 s. It is noteworthy that all dissociation kinetics in this study were performed at 26°C. This reduced temperature was used to increase the accuracy of measurement, as the dissociation increases significantly with the temperature (5, 23).

Since cytolytic CTL responses are rapid and can occur at very low peptide concentrations (28), we assessed for some of the cases the corresponding IFN-γ response, which requires higher peptide concentrations and sustained TCR signaling for extended periods of time (29). As shown in Figure 3, IFN-γ production showed essentially the same patterns as the cytotoxic response (Fig. 1). In only a few instances were deviations of up to threefold observed (e.g., the IFN-γ production was higher than cytotoxicity on S17 CTL for variant P255S, whereas the opposite was true on S14 for variant S256A). Occasional differences between these two CTL responses have been observed in other systems (30). Thus, despite the different activation requirements, the two CTL responses were generally similar.

Blocking of CD8 increased or abolished functional CTL response

Taken collectively these results suggest that the magnitude of the functional CTL response was determined by the rates of TCR-ligand complex dissociation. To verify this conclusion we repeated these experiments in the presence of SF1-1.1.1 Fab’, which, by blocking participation of CD8 in TCR-ligand binding, accelerates TCR-ligand complex dissociation (22, 23, 27). Blocking of CD8 in several instances, especially on S17 and T1 CTL, either had no significant effect on Ag recognition or even increased it (e.g., wt, P255A, and P255S on S17 or P255L on T1 CTL; Figs. 1 and 3). Since blocking of CD8 always considerably decreased TCR-ligand binding, the efficiency of Ag recognition was significantly increased relative to that of TCR-ligand binding. Thus, agonist or weak agonists were converted to strong agonists (e.g., wt, P255A, S256A, and P255S on S17; P255L and S256A on T1; and E258A on S4 CTL).

In contrast, there were nine cases, mainly on S14 and S4 CTL, where blocking of CD8 dramatically impaired or abolished Ag recognition. For two of these cases (P255A on T1 and S256A on S4) we have previously shown that blocking of CD8 resulted in antagonism (5). Essentially the same findings were obtained for the IFN-γ response; actually in some instances blocking of CD8 increased cytokine production more than cytotoxicity (e.g., wt and P255S on S17 CTL; Figs. 1 and 3). Also, in all cases where blocking of CD8 abolished cytotoxicity, IFN-γ production was undetectable as well, except for variant P255A, which elicited a weak IFN-γ response.

In the presence of SF1-1.1.1 Fab’, TCR-ligand complex dissociation was considerably faster than in its absence (Table I). For all cases where blocking of CD8 abolished detectable Ag recognition, TCR-ligand complex dissociation was very rapid, with half-lives in the range of 10 to 23 s. The only comparably rapid dissociation was observed on S14 CTL for variant P255L, which is an antagonist for this clone (Fig. 2). In contrast, when TCR-ligand complex dissociation was slower than this (t1/2 = >23 s), Ag recognition was typically most efficient (e.g., S17 CTL clone, on which dissociations were slower than on the other clones). Together these findings support the conclusion that the efficiency of Ag recognition increases with the rate of TCR-ligand complex dissociation up to a critical value, beyond which TCR antagonism prevails.

Epitope modifications can alter CD8 participation in TCR-ligand binding

While blocking of CD8 contribution to TCR-ligand binding by SF1-1.1.1 Fab’ always reduced TCR-ligand binding, the degree of reduction varied among CTL clones as well as among epitope variants on given clones. As shown in Figure 4, the ratio of TCR
photoaffinity labeling in the absence divided by the labeling in the presence of SF1-1.1 Fab' for the wild-type ligand was highest for S17 CTL (13.6-fold), lowest for T1 CTL (5.6-fold), and intermediate for the S4 and S14 CTL clones (11.7- and 8.6-fold, respectively). While these differences are explained at least in part by clone-specific differences in CD8 and TCR expression (5), the differences observed on given clones among peptide derivative variants indicate that epitope modifications can...
change the CD8 contribution to TCR ligand binding. For example, on S17 CTL, blocking of CD8 TCR-ligand binding was decreased by 25-fold in variant P255L, but only about 6-fold in variants P255A and P255S.

Correlation between CD3/ζ-chain phosphorylation, Ag recognition, and TCR-ligand complex dissociation

Among the earliest TCR signaling events are PTK-mediated phosphorylations of CD3/ζ (14, 15). We therefore examined the phosphorylation of ζ-chain, namely the pp21 and pp23 phospho forms, that are induced by TCR engagement. While the pp21 form is predominantly elicited by peptide antagonists, the pp23 form is induced by agonists and can bind ZAP-70 (6, 7, 14). As shown in Figure 5, the intensity and ratio of pp21 vs pp23 ζ-chain phosphorylation varied considerably among the different cases. Incubation of S17 CTL with P815 cells expressing covalent Kd-IASA-YIPSAEK(ABA)I complexes resulted in the formation of pp21 and less pp23 ζ-chain phosphorylation. In the presence of SF1-1.1.1 Fab’ ζ-chain phosphorylation increased slightly in variant S256A and remained unchanged for the wt, but decreased significantly, mainly the pp23 ζ-chain phosphorylation pattern is typical for peptide antagonists, which is consistent with the finding that upon blocking of CD8, the variants P255A and P255S were antagonists for T1 CTL (5) (data not shown). On T1 CTL, the wt peptide derivative and variants P255A and S256A induced similar ζ-chain phosphorylation in terms of both overall intensity and pp21 to pp23 ratio (Fig. 5). By contrast, the weak agonist P255S elicited less efficient ζ-chain phosphorylation, mainly of the pp21 form. In the presence of SF1-1.1.1 Fab’ ζ-chain phosphorylation increased slightly in variant S256A and remained unchanged for the wt, but decreased significantly, mainly the pp23 form, in variants P255A and P255S. The latter ζ-chain phosphorylation pattern is typical for peptide antagonists, which is consistent with the finding that upon blocking of CD8, the variants P255A and P255S were antagonists for T1 CTL (5) (data not shown). On S17 CTL, the ζ-chain phosphorylation induced by the wt ligand decreased slightly upon blocking of CD8, especially the pp23 form (Fig. 5). For variant S256A this decrease was more pronounced, which is in accordance with the finding that blocking of CD8 abolished recognition of this agonist (Figs. 1 and 3). This was also true for variant P255K. On S14 CTL extensive ζ-chain phosphorylation, especially of the pp23 form, was observed only in the wt peptide derivative and variants P255A and S256A, compounds that were efficiently recognized (Figs. 1, 3, and 5). For the weak agonist E258A, little ζ-chain phosphorylation, mainly of the pp21

FIGURE 2. TCR-ligand complex dissociation. Aliquots of cloned CTL, preincubated with soluble Kd-peptide derivative complexes, were diluted in medium containing anti-Kd mAb 20-8-4S and UV irradiated after the indicated periods of incubation at 26°C. The TCR were immunoprecipitated from detergent lysates, analyzed by SDS-PAGE (10%, reducing) and quantified by phosphorimaging. TCR photoaffinity labeling at time zero was referred to as 100%. The kinetics for strong agonists are shown in bold, those for weak agonists in dotted lines, and those for antagonists in broken lines. The half-life expresses the time required for 50% dissociation. The mean values and SDs were calculated for three to five independent experiments.
form, was observed. Upon blocking of CD8, ζ-chain phosphorylation, namely of the pp23 form, was significantly reduced in all cases, except for variant P255A. The remaining weak ζ-chain phosphorylation, consisting mainly of the pp21 form, is reminiscent of peptide antagonist, which is consistent with the lack of detectable Ag recognition in these instances (Figs. 1 and 3).

It is noteworthy that on S17 CTL the pp23/pp21 ratio for the wt was lower than that on the other clones. This is likely to be accounted for by slower kinetics of pp23 accumulation on S17 CTL due to remarkably slow TCR-ligand complex dissociation on this clone (Fig. 2).

Permanent TCR engagement abrogates TCR signaling

Based on the observation that weak agonist typically exhibited slow TCR-ligand complex dissociation and elicited inefficient ζ-chain phosphorylation, we next examined what effect permanent TCR engagement has on CTL activation. In our system this can be accomplished by photocross-linking of intercellular TCR-ligand complexes. To this end, indo-1-labeled S4 CTL were incubated with P815 cells expressing covalent Kd-IASA-YIPSAEK(ABA)I complexes and 

\[ \text{[Ca}^{2+}\text{]} \] was monitored in the presence or the absence of TCR-ligand photocross-linking. S4 CTL exhibited significant calcium-dependent fluorescence upon incubation with P815 cells that were sensitized with 0.3 or 30 nM of IASA-YIPSAEK-(ABA)I (Fig. 6, B and D). This calcium mobilization was Ag specific, since it was not observed upon incubation with untreated P815 cells (Fig. 6A). Importantly, following UV irradiation, [Ca²⁺], levels rapidly dropped to background levels regardless of whether the target cells expressed a high or a low ligand density (Fig. 6, C and E). This effect was not due to UV irradiation damage of the CTL, since S4 CTL that were UV irradiated before incubation with sensitized targets exhibited the same calcium mobilization as untreated CTL (Fig. 6F). On the other hand, UV irradiation of sensitized P815 cells before incubation with S4 CTL abolished significant calcium mobilization (G). This is consistent with the finding that the ABA group of IASA-YIPSAEK(ABA)I constitutes an essential part of the epitope recognized by S4 and related CTL.

Table I. TCR-ligand complex dissociation

<table>
<thead>
<tr>
<th>CTL Clone</th>
<th>Epitope</th>
<th>Complex Half-Life (sec)</th>
<th>+ SF1-1.1 Fabα</th>
<th>− SF1-1.1 Fabβ</th>
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<tr>
<td>S17</td>
<td>wt</td>
<td>60 ± 4°C</td>
<td>220 ± 32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P255S</td>
<td>84 ± 4°C</td>
<td>370 ± 27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S256A</td>
<td>30 ± 3.6°C</td>
<td>163 ± 33</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>wt</td>
<td>28 ± 5°C</td>
<td>133 ± 28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P255A</td>
<td>17 ± 3°C</td>
<td>50 ± 32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P255L</td>
<td>34 ± 2°C</td>
<td>324 ± 43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S256A</td>
<td>23 ± 3°C</td>
<td>108 ± 33</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>wt</td>
<td>26 ± 3.4°C</td>
<td>44 ± 14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S256A</td>
<td>17 ± 3°C</td>
<td>91 ± 18</td>
<td></td>
</tr>
<tr>
<td>S14</td>
<td>wt</td>
<td>15 ± 2.5°C</td>
<td>36 ± 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E258A</td>
<td>9 ± 2.6°C</td>
<td>51 ± 16</td>
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α TCR-ligand complex dissociation was assessed as described for Figure 2, except that SF1-1.1 Fab were present during the incubation of CTL with ligand. The mean values and SD were calculated from three to five different experiments, some of which were used in Ref. 5.

β Values are taken from Figure 2.

Strong agonist.

Antagonist (always in presence of SF1-1.1 Fab').
and demonstrates that photolysis of this group destroys nominal ligand for S4 TCR.

To determine whether abrogation of calcium mobilization by TCR cross-linking was due to inefficient TCR-ligand photo-cross-linking, we assessed the kinetics of intercellular TCR-ligand binding. To this end, P815 expressing radiolabeled Kd-IAA-YIP-SEK(ABA)I complexes was incubated with cloned S4 CTL and UV irradiated after different periods of incubation. As shown in Figure 7A, the TCR photoaffinity labeling reached a maximum after approximately 4 min. After longer incubations, TCR-ligand binding decreased gradually by about 20% after 20 min. This decrease was prevented by TCR photocross-linking after 4 min of incubation. These results indicate that in the previous experiment (Fig. 6) the cells that were subjected to TCR photocross-linking had an average TCR engagement that was equal to or higher than that in the experiment without TCR-ligand cross-linking.

Next, we determined the efficiency of TCR photocross-linking, i.e., the fraction of engaged TCR that became cross-linked upon UV irradiation. The amount of TCR-bound ligand was measured by incubating S4 CTL with radiolabeled covalent ligand and by separating cell-associated and free ligand by spinning the cells through an oil gradient. From this value the nonspecific binding, i.e., cell-associated counts per minute in presence of anti-Kdα1 mAb 20-8-4S, which blocks specific TCR-ligand binding (27), was subtracted. The amount of cross-linked TCR was determined in the same way, except that the incubations were followed by UV irradiation and incubation with 20-8-4S mAb at 37°C. By dividing cross-linked by bound ligand, a value of 0.45 was obtained. Thus, for S4 TCR approximately 45% of the bound ligand became cross-linked upon UV irradiation, indicating that intercellular TCR-ligand photocross-linking can diminish TCR engagement by up to 2.2-fold.

In the experiment shown in Figure 6, the same calcium mobilization in S4 CTL was observed when the target cells were sensitized with 0.3 or 30 nM of IASA-YIPSAEK(ABA)I. To assess TCR engagement under these conditions, we used again TCR photoaffinity labeling with P815 cells expressing radiolabeled Kd-IAA-YIPSAEK(ABA)I complexes. As shown in Figure 7C, TCR photoaffinity labeling increased with the ligand density on the target cells. If one defines the intercellular TCR photoaffinity labeling observed with 30

(22, 27) and demonstrates that photolysis of this group destroys nominal ligand for S4 TCR.
nM $^{125}$IASA-YIPSAEK(ABA)I as 100%, the labeling observed with target cells pulsed with 0.3 nM was approximately 11%. Thus, by increasing the peptide concentration by 100-fold, TCR engagement increased by 9-fold. Together these results indicate that the drop in $[Ca^{2+}]_{i}$ shown in Figure 6, C and E, is not accounted for by inefficient TCR-ligand cross-linking.

**Discussion**

Assessment of Ag recognition (cytotoxicity and IFN-γ production), TCR-ligand binding, and TCR-ligand complex dissociation kinetics of seven peptide derivative variants on four CTL clones indicated that the magnitude of the functional CTL response is related to the rate of TCR-ligand complex dissociation and not the avidity of TCR-ligand binding (Figs. 1–3). This relation was bimodal in nature. While the efficiency of Ag recognition was maximal when TCR-ligand complex dissociation was rapid, it decreased when dissociation either further increased and, surprisingly, also when it decreased. The former results indicate that the drop in $[Ca^{2+}]_{i}$ shown in Figure 6, C and E, is not accounted for by inefficient TCR-ligand cross-linking.

FIGURE 6. Permanent TCR engagement abolishes calcium mobilization on S4 CTL. Cloned S4 CTL, previously labeled with indo-1, were incubated with P815 cells that were either untreated (A) or Kd photoaffinity labeled with IASA-YIPSAEK(ABA)I using 0.3-nM (B and C) or 30-nM (D, E, G, and H) concentrations at an E:T cell ratio of 1:3. After 3 min of incubation at 37°C, the cultures were UV irradiated (C and E) or not (A–B and D). Alternatively, as controls, either the S4 CTL (F) or the target cells (G) were UV irradiated before incubation. Calcium-dependent fluorescence of indo-1 was assessed by flow cytometry over a period of 15 min. A representative experiment of four is shown.

FIGURE 7. Effect of TCR-ligand photocross-linking on TCR-ligand engagement. A, The kinetics of intercellular S4 TCR-ligand binding were assessed by TCR photoaffinity labeling. C, P815 cells P815 cells previously Kd photoaffinity labeled with $^{125}$IASA-YIPSAEK(ABA)I using a 1-nM concentration or as specified were incubated for the indicated periods of time at 37°C with cloned S4 CTL at an E:T cell ratio of 1:3. After UV irradiation, the immunoprecipitated TCR were analyzed by SDS-PAGE and phosphorimaging. One hundred percent corresponds to the maximal TCR photoaffinity labeling. B, Alternatively S4 CTL were incubated with soluble monomer Kd-$^{125}$IASA-YIPSAEK(ABA)I complexes and UV irradiated (cross-linked) or not (bound) in the absence or the presence of anti-Kd mAb 20–8–4S.

gagement results in incomplete TCR signaling and no or very limited activation of T cell effector functions (9, 10, 31). It is interesting to note that for all the cases we have examined, antagonism was observed when the half-lives of the TCR-ligand complexes were in the range of 9 to 23 s. Since this was true for different CTL clones and ligand variants as well as in situations in which CD8 was blocked or not, it appears that there exists a critical threshold below which TCR engagement results in aberrant TCR signaling and antagonism.

Moreover, our results revealed that different CTL clones have different tendencies for TCR antagonism. When testing a larger panel of peptide derivative variants, antagonists were observed only for S14 CTL (4). Similarly, acceleration of TCR-ligand complex dissociation by blocking of CD8 participation in TCR-ligand binding on S14 CTL converted agonists into antagonists in all but one case (Figs. 1 and 3). Conversely, on the S17 CTL clone, antagonism was never observed, and blocking of CD8 often increased the efficiency of Ag recognition (Figs. 1 and 3). From kinetic experiments we know that TCR-ligand complex dissociation on S14 CTL typically was considerably faster than that on S17 CTL (Fig. 2). It thus appears that the clone-specific susceptibilities for antagonism are related to clone-specific differences in TCR-ligand complex dissociation rates.
A main finding of the present study is that peptide derivative variants that were recognized less efficiently than expected from their TCR-ligand binding (weak agonists) exhibited slower TCR-ligand complex dissociation than those that were recognized efficiently (strong agonists). Consistent with this, we observed that weak agonists induced less efficient ζ-chain phosphorylation and typically less of the pp23 form than strong agonists (Figs. 1, 3, and 5). The finding that brief TCR engagement resulted in more efficient TCR signaling than prolonged engagement is not consistent with the kinetic proofreading concept (8 –10). Instead, this observation is in agreement with the concept of serial TCR engagement, according to which TCR sequentially engage ligand on APC (19 – 21). Indeed, one expects that when TCR-ligand complex dissociation is rapid, the rate of serial TCR engagement will be higher than when dissociation is slow. Our data are consistent with the view that in the absence of antagonism, the magnitude of the functional T cell response is directly related to the frequency of serial TCR engagement.

This conclusion is supported by the finding that blocking of CD8 can increase the efficiency of Ag recognition (Figs. 1–3). While blocking of coreceptor participation in TCR-ligand binding always accelerated TCR-ligand complex dissociation, it had different effects on Ag recognition. If dissociation became too rapid, blocking of CD8 can convert agonists and weak agonists into antagonists. This is consistent with studies showing that blocking of CD4 can convert agonists and weak agonists into antagonists on Th cells (11, 32). In the study by Madrenas et al. blocking of CD4 has been shown to result in the same pattern of CD3/ζ phosphorylation as elicited by peptide antagonist, i.e., a decrease in phosphorylation and a preponderance of pp21 phospho-ζ (11). While most of our data are in accordance with this, others suggest more complex relations in some cases. For example, for variants P255A and P255S on T1 CTL, blocking of CD8 dramatically reduced Ag recognition, but only moderately reduced the pp23/pp21 ratio and the overall intensity of phospho-ζ (Figs. 1 and 5). Also, on S17 CTL, in cases where blocking of CD8 augmented the efficiency of Ag recognition, the overall ζ-chain phosphorylation was increased rather than the pp23/pp21 ratio. These patterns suggest that CD8 may influence TCR signaling in a quantitative as well as qualitative manner (see below). On the other hand, if TCR-ligand complex dissociation remained above a critical limit, blocking of CD8 enhanced the functional CTL response, either absolutely or relative to TCR-ligand binding (Figs. 1–3). Since in our experiments, artifacts that anti-CD8 mAb may cause are ruled out, this observation argues in favor of the concept of serial TCR engagement.

Another observation that supports this conclusion is that blocking of serial TCR engagement by photocross-linking of TCR-ligand complexes rapidly abolished sustained intracellular calcium mobilization in S4 CTL, an obligatory early event of T cell activation (14, 31) (Fig. 6). Consistent with this, we observed that irreversible TCR engagement also abrogated Ag-specific IFN-γ production by S4 CTL and provoked barely detectable ζ-chain phosphorylation (unpublished observations). Together, these findings strongly suggest that serial or sequential TCR engagement (our data do not distinguish whether single ligands serially engage many TCR or whether single TCR sequentially engage different ligands) is a requirement for TCR signaling and that its frequency determines the magnitude of CTL activation.

It has been recently shown that incubation of TCR and ligand at concentrations close to the equilibrium constant leads to the formation of TCR-ligand aggregates, suggesting that TCR aggregation may be critical for T cell activation, as has been observed for other receptor systems (33). Our finding that permanent TCR engagement abrogates TCR signaling does not rule out this possibility, but stipulates that TCR aggregation would be a dynamic event, taking place in the course of serial TCR engagement.

In terms of TCR signaling it is not clear how permanent TCR engagement abolishes CTL activation. In view of reports showing that hyperphosphorylation of ZAP-70 results in binding of the phosphatase SHP-1 to ZAP-70, which then dephosphorylates ZAP-70 (34, 35), it is tempting to speculate that permanent TCR ligation activates a phosphatase-mediated extinction of TCR signaling. One would indeed expect that upon permanent TCR ligation, CD8-associated p56(CD3ζ) remains in the vicinity of CD3ζ and CD3ζζ-associated ZAP-70 and hence hyperphosphorylates these molecules (11). Since weak agonists typically exhibit slow TCR-ligand complex dissociation, they may partially activate the same inhibitory mechanism. This would explain the inefficient CD3ζζ phosphorylation observed for weak agonist (Fig. 5).

A surprising finding was that blocking of CD8 reduced the avidity of TCR-ligand binding to different degrees (Fig. 4). While such variations among different CTL clones are explained at least in part by clone-specific differences in CD8 and TCR expression (5), those observed among epitope variants on given clones strongly suggest that epitope modifications can alter the CD8 contribution to TCR-ligand binding. Since similar changes were also observed at 0 to 4°C, i.e., in the absence of metabolically active cellular processes (our unpublished observations), it appears that epitope modifications may induce either conformational changes in TCR or ligand or slightly alter the orientation in which they interact, as a result of which CD8 can more or less avidly participate in TCR-ligand binding. This view is consistent with the observation that epitope modifications can provoke changes in the ratio of TCR α vs β-chain photofinity labeling (5).

The finding that epitope modifications can alter the participation of CD8 in TCR-ligand binding is likely to have implications for Ag recognition by CTL. For example, it will influence the rates of TCR-ligand complex dissociation. Since these, in turn, determine the efficiency of Ag recognition (see above), this implies that epitope modifications can affect functional CTL responses by altering the avidity of CD8 contribution to TCR-ligand binding. This differential involvement of CD8 in TCR-ligand binding probably also explains at least in part why the functional phenotype of epitope variants cannot be predicted from the avidity of TCR-ligand binding (Figs. 1 and 3).

Moreover, since CD8 brings p56(CD3ζ) to CD3ζζ, it is conceivable that topologic changes in the docking of CD8 to TCR-ligand complex alters the relative extents by which p56(CD3ζ) phosphorylates the different ITAM of CD3ζζ. Since ITAM are not identical in terms of initiating downstream signaling cascades (36 –38), this may explain how epitope modifications can result in selective activation of CTL functions, such as activation of Fas, but not perforin-dependent cytotoxicity or differentially activate IFN-γ production and cytotoxicity (3, 30, 38) (Figs. 1 and 3). It is interesting to note that blocking of CD8 generally had more dramatic effects on Ag recognition and ζ-chain phosphorylation on S14 and S4 CTL than on S17 and T1 CTL (Figs. 1 and 5 and our unpublished data). Interestingly, the latter CTL clones are CD8 independent (i.e., their cytolytic responses are not affected or are little affected by anti-CD8 mAb), whereas the former are CD8 dependent (5). It is thus conceivable that for CD8-independent clones CD8-associated p56(CD3ζ) is less critical for cell activation than it is for CD8-dependent clones.

Our preferred interpretation of the results presented in this study is schematically shown in Figure 8. The main points we would like to make are 1) that serial TCR engagement is a prerequisite for CTL activation; 2) that the magnitude of the functional CTL response is dependent on the frequency of serial TCR engagement;
and 3) that its frequency is essentially determined by the rate of TCR-ligand complex dissociation. Although this scheme explains most of our data, it is not meant to be a unifying concept for T cell activation; rather, it suggests that aberrant T cell function is probably best explained by a combination of several principles and that the coreceptor plays an important role.

**References**


**FIGURE 8.** Hypothetical scheme showing how epitope modification may affect functional CTL response. Δ stands for a change in, i.e., a change in TCR-ligand complex dissociation.

- Epitope modification
- Δ TCR conformation or TCR-ligand orientation
- Δ CDB participation in TCR-ligand binding
- Δ TCR-ligand complex dissociation
- Δ Frequency of serial TCR engagement
- Δ Functional CTL response