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A TCR Binds to Antagonist Ligands with Lower Affinities and Faster Dissociation Rates Than to Agonists

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Summary

T lymphocyte activation is mediated by the interaction of specific TCR with antigenic peptides bound to MHC molecules. Single amino acid substitutions are often capable of changing the effect of a peptide from stimulatory to antagonistic. Using surface plasmon resonance, we have analyzed the interaction between a complex consisting of variants of the MCC peptide bound to a mouse class II MHC (E^k) and a specific TCR. Using both an improved direct binding method as well as a novel inhibition assay, we show that the affinities of three different antagonist peptide–E^k complexes are ~10–50 times lower than that of the wild-type MCC–E^k complex for the TCR, largely due to an increased off-rate. These results suggest that the biological effects of peptide antagonists and partial agonists may be largely based on kinetic parameters.

Introduction

In the past several years, many variants of T cell antigenic peptides have been found that can change an agonist ligand into one capable of either inhibiting a wild-type response (antagonism) or causing only partial activation of T cells. Such variant peptides may provide important insights into the mechanics of TCR engagement and early events in T cell activation. They may also help shape immune responses by blocking autoimmune diseases, providing a means by which viruses escape from immunosurveilance, and promoting positive selection of thymocytes in vitro (reviewed by Sette et al. 1994; Jameson and Bevan, 1995; Sloan-Lancaster and Allen, 1996).

The differences in signaling through the T cell antigen receptor (TCR) by a major histocompatibility complex (MHC) molecule complexed with agonist versus antagonist peptides could be of a qualitative or a quantitative nature. In the former category, it has been suggested that differences in TCR conformation induced by a peptide–MHC complex could determine an agonist or antagonist response (Yoon et al., 1994). In the latter, differences in the kinetics of TCR binding to peptide–MHC might be responsible for a positive or negative signal being propagated (Rabinowitz et al., 1996; Jameson and Bevan, 1995; Kersh and Allen, 1996). The kinetics of binding between agonist peptide–MHC complexes and TCR have now been characterized by several groups (Corr et al., 1994; Sykulev et al., 1994a, 1994b; Matsui et al., 1994). Of particular relevance here is the report of Matsui et al. (1994), which employed surface plasmon resonance to analyze the interaction of a soluble form of the 2B4 TCR (Lin et al., 1990) with its ligand, a moth cytochrome c (MCC) peptide bound to the mouse class II MHC molecule E^k (Wettstein et al., 1991). It was found that the most stimulatory ligand (MCC–E^k) had the longest half-life of binding to the TCR (~12 s), while the least stimulatory ligand (T102S–E^k) had the shortest half-life (~2–4 s); thus, agonist activity correlated with the dissociation rate of TCR from the peptide–E^k complex. Matsui et al. (1994) speculated that antagonist peptide–E^k and TCR complexes might have even shorter half-lives, and that this parameter might determine the whole range of T cell responsiveness. Here, we report binding measurements of three antagonist peptide–E^k complexes with the 2B4 TCR. We find that all three complexes show significantly lower binding affinity to the 2B4 TCR as compared with agonist and weak agonist/peptide–MHC complexes. This decrease in affinity is largely due to an increase in the off-rate of the peptide–MHC complex from the TCR. This suggests that the stability of the ternary complex between TCR and a given peptide–MHC is a major determinant of T cell responsiveness.

Results

Biological Activity of MCC Peptide Analogs

The 2B4 T cell hybridoma recognizes a MCC peptide in association with the mouse class II MHC molecule I-E^k. In this system, the variant peptides T102S (Matsui et al., 1994) and T102N (Reay et al., 1994) are weak agonists and K99Q is a weak antagonist (Spain et al., 1994). As originally suggested by surveys with other cytochrome-reactive T cells (Page et al., 1994; P. A. Reay et al., unpublished data), we find that K99R and T102G are even more robust antagonists in this system (Figures 1A and 1B). All of the peptides studied here, including the null peptides K99A and K99T (Figures 1A and 1B), bind to E^k with similar affinity (Reay et al., 1994), indicating that the observed differences in their ability to elicit T cell responses are due to alterations solely in the interactions between the TCR and peptide–MHC complex.

The question of how these antagonist peptide–MHC complexes interact with the 2B4 TCR was then addressed in two different ways: by direct measurements of peptide–MHC binding to immobilized TCR and by a newly developed competitive binding assay.

Binding of MHC–Peptide Complexes to TCR as Measured Directly with Cysteine-Coupled TCR

Our initial attempts to measure antagonist peptide–E^k complexes binding to TCR-coated biosensor surfaces

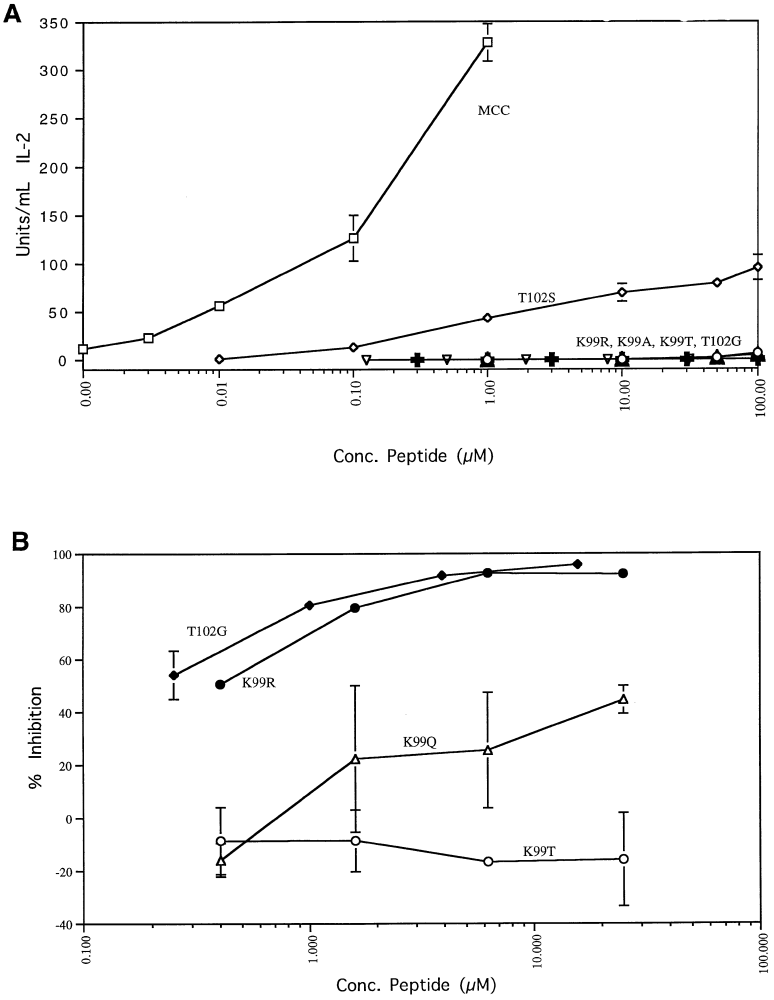

Figure 1. Activation and Antagonism of the 2B4 Hybridoma by MCC Peptide Analogs

(A) 2B4 hybridoma cells were stimulated with E^k expressing CHO cells and the indicated concentrations of either wild-type MCC (open squares), T102S (open diamonds), K99T (open circles), K99A (closed triangles), K99R (pluses), or T102G (open inverted triangles) peptide, and IL-2 levels were measured. K99Q (data not shown) is also nonstimulatory. (B) Antagonist activity of analogs K99R (closed circles), T102G (closed diamonds), K99Q (open triangles), or K99T (open circles) was assessed by adding increasing concentrations of each peptide and 2B4 hybridoma cells to E^k expressing CHO cells prepulsed with 0.005 μM MCC peptide. The percent inhibition with each analog peptide is shown, and similar results were obtained in at least five independent experiments, some of which were done by mixing MCC and the antagonist peptides instead of prepulsing.

resulted in no detectable signal. Reasoning that a substantial amount of TCR was being inactivated by the NHS/EDC coupling of free amine groups, we decided to attempt an oriented coupling of the 2B4 TCR through the exposed thiol in the constant region of the β chain (Bentley et al., 1995). This should allow a much larger percentage of the surface to be accessible for binding by peptide–MHC.

Immobilizing the TCR in this way resulted in approximately a 2- to 4-fold increase in signal compared with the amine-coupling method when similar concentrations of MCC–E^k were injected over the surfaces. Clear binding was also detectable at much lower concentrations of MCC–E^k as is seen in the dose response of Figure 2A in comparison to that of Matsui et al. (1994). Fitting of the association phase (Figure 2B) and dissociation phase (Figure 2C) using the nonlinear fitting routines in BIAEvaluation 2.1 (Pharmacia Biosensor) gives equilibrium dissociation constant (K_D) values that are somewhat lower than those reported previously (Matsui et al., 1994). The major source of the difference in affinity (from 90 μM to around 40 μM) between amine-coupled and cysteine-coupled TCR is the on-rate, which changes from 900 $M^{-1}s^{-1}$ to 1600 $M^{-1}s^{-1}$. This difference may reflect an increased availability of the complete binding epitope in the cysteine-coupled TCR. One other difference between the two systems was that the concentrations of MCC–E^k used in experiments with cysteine-coupled TCR in general were lower than those used with amine-coupled TCR (Matsui et al., 1994), because of the increased sensitivity. We noted that on-rates decreased slightly as concentrations of peptide–MHC were increased in the dose reponse (Figure 2A), and thus the observed K_D value may be dependent upon the concentration of peptide–MHC in solution during binding. The observation that immobilization chemistry can alter the kinetics has also been observed in other systems, such as in MHC class I binding to immobilized peptides (Khilko et al., 1993).

Binding to the cysteine-coupled TCR was highly specific, as K99A–E^k (a null peptide–MHC complex which is neither an agonist nor an antagonist) elicited no detectable signal (Figure 3A) at a concentration similar to that of MCC–E^k used in the dose response. We also tested the weak agonist T102S–E^k (Figure 3A) and found that while the on-rate of binding to 2B4 TCR (Figure 3B) is almost identical to that of MCC–E^k, the off-rate (Figure 3C) is about 6-fold faster, consistent with previous observations (Matsui et al., 1994). The association phase is now clearly observed, which was not possible using

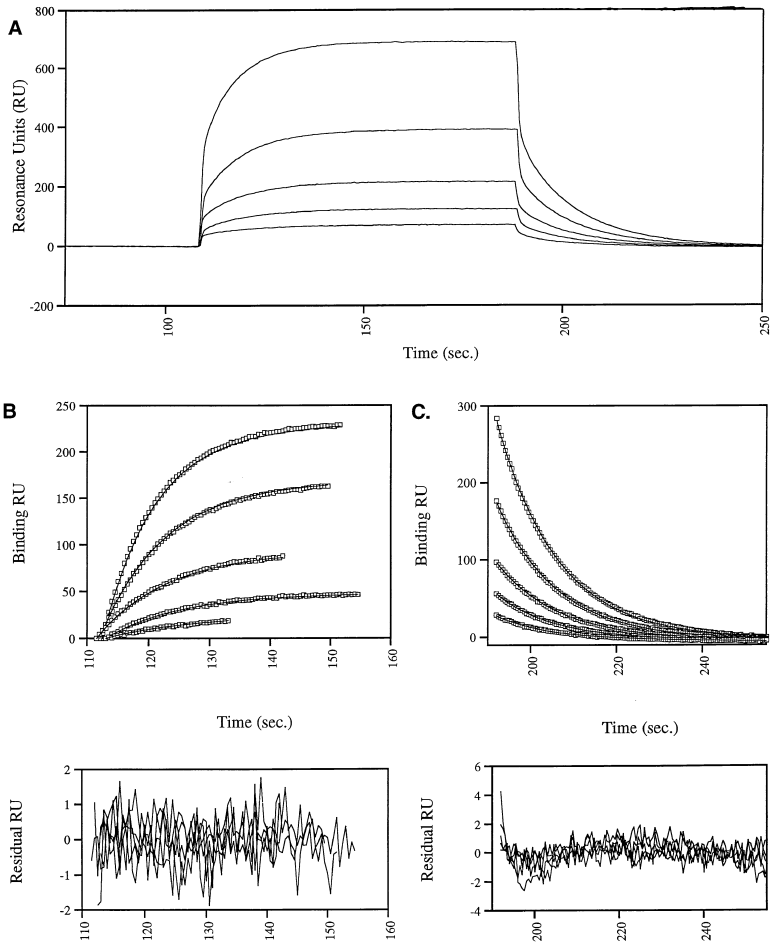

Figure 2. MCC–E^k Binding to Cysteine-Coupled TCR

(A) MCC–E^k complexes were passed over TCR coupled to the sensor chip (see Experimental Procedures) via the free cysteine in the β chain constant region. MCC–E^k was injected at concentrations of 0.19, 0.38, 0.77, 1.5, and 3.1 mg/ml. The association (B) and dissociation phases (C) were fit using the single-site binding model of BIAEvaluation 2.1 (Pharmacia Biosensor). Data points are displayed (open squares) with the fitted curves overlaid (lines). The residuals (the difference between fitted curve and data curve at each timepoint) are plotted below each curve as an estimate of the error. These plots are representative of five independent experiments.

the amine-coupled surface. In addition, another weak agonist, T102N, which is slightly less stimulatory than T102S (Reay et al., 1994), exhibited similar kinetics, with a slightly faster off-rate of 0.44 s^{-1} (Table 1).

This improved sensitivity of the cysteine-coupled TCR suggested that specific binding might be measurable with antagonist peptide–MHC complexes. Therefore, K99R–E^k (Figure 4A) and T102G–E^k (Figure 4B) were tested for binding to the TCR surface at varying concentrations, using the null peptide (K99A–E^k) complex as a control. These two antagonist–E^k complexes consistently showed higher levels of signal at equilibrium in comparison to K99A–E^k, indicative of specific binding with rapid kinetics. Taking the difference between the null peptide K99A–E^k signal (arising from nonspecific changes in refractive index upon injection of protein) and that of the antagonist peptide–E^k complexes allowed us to calculate the amount bound at equilibrium. Fitting a plot of this amount bound versus the concentration of peptide–E^k complex (Figure 4C) to an equilibrium binding model gave K_D values of 500 μM for K99R and 1500 μM for T102G, as summarized in Table 2. By the same approach, K_D values of 70 μM and 150 μM were determined for MCC–E^k and T102S–E^k, respectively (Table 2). The close agreement between these values and those determined earlier provides further evidence for the validity of this equilibrium method. As an additional control for specificity, a monoclonal antibody (MAb) D-4, which recognizes particular cytochrome c peptide–E^k complexes (P. A. Reay and M. M. D., unpublished data), was able to reduce binding down to the background level found with K99A–E^k for both K99R–E^k and T102G–E^k (data not shown). Because this antibody covers the binding domain of the peptide–MHC complex, it serves as an effective inhibitor.

The dissociation rate was first estimated by fitting the dissociation phase of the binding curve to a single exponential. This gives an off-rate in the range of 1.0–3.0 s^{-1} for K99R–E^k and T102G–E^k at flow rates of 15 or 30 μl/min, but the absolute accuracy of these numbers is questionable, as there is always a time at the beginning of the dissociation phase when the flowcell is not completely exchanged into running buffer. This time is directly proportional to the flow rate. Therefore, to improve the accuracy of these measurements, we used the technique of van der Merwe et al. (1994), in which initial off-rates are determined over a series of increasing flow rates. This allows us to extrapolate to an infinite flow rate, where the time of washout approaches zero. A plot of the apparent dissociation time versus the inverse of the flow rate gives a linear curve with the y intercept equivalent to the dissociation time at infinite flow rate. Only the first 1–4 s of the dissociation curve were fit to a single exponential to calculate the dissociation time

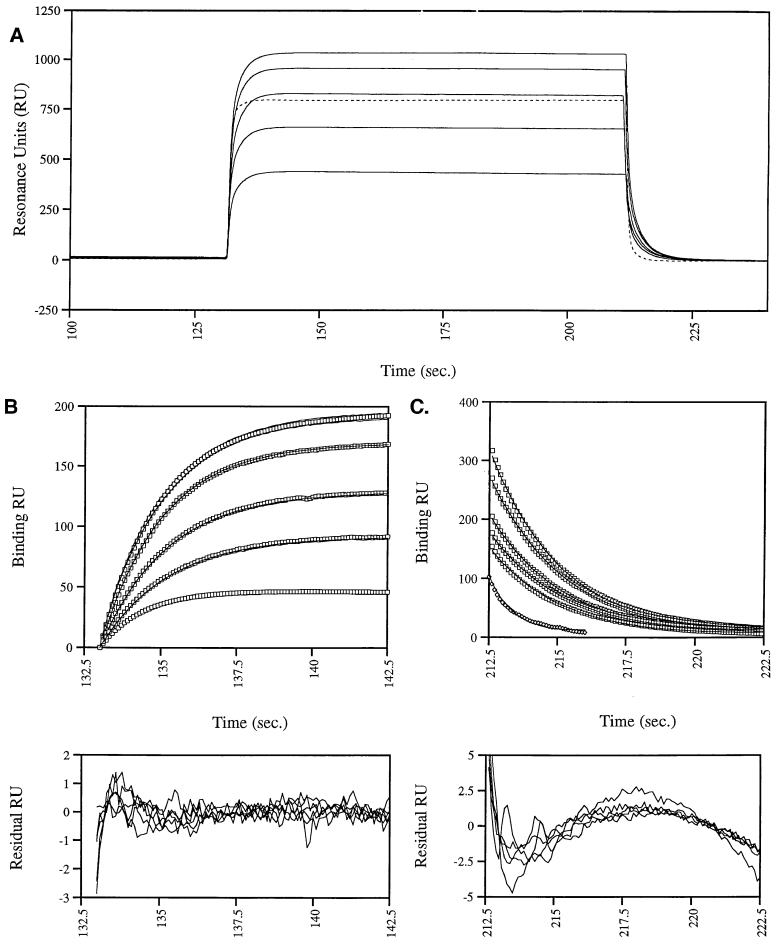

Figure 3. T102S–E^k Binding to Cysteine-Coupled TCR

(A) T102S/E^k (solid line) was passed over cysteine-coupled 2B4 TCR at concentrations of 3.1, 4.6, 6.1, 7.07, and 8.0 mg/ml. The null peptide K99A/E^k (dotted line) was also injected at 8.0 mg/ml as a reference for the background level of signal. Association (B) and dissociation (C) curve fitting is displayed as in Figure 2. These results are representative data from two independent experiments.

(which is equivalent to the inverse of the k_{off}) using BIAEvaluation 2.1 (Pharmacia Biosensor) for either bovine serum albumin (BSA), K99R–E^k, or T102G–E^k (fitting not shown). A comparison of the y intercept values gave k_{off} values of 4.8 s^{-1} for K99R–E^k and 5.1 s^{-1} for T102G–E^k (Figure 4D). By comparison BSA exhibited a k_{off} value of 7.5 $\pm$ 0.9 s^{-1}. The association rate constant (k_{on}) is difficult to measure directly due to the rapid dissociation, but as $k_{on} = (k_{off} / K_D)$, we can estimate this as 9600 $M^{-1}s^{-1}$ for K99R and 3400 $M^{-1}s^{-1}$ for T102G, respectively (Table 2).

The K99Q–E^k complex showed no interaction with the 2B4 TCR above the level of the null complex K99A–E^k, even at concentrations as high as 18.5 mg/ml. Formally, this could be due to a slower association rate or a faster dissociation rate or some combination of the two. In this case, however, it is most likely due to a much faster off-rate, for the following reasons. First, as seen in Table 1, all of the cytochrome c–E^k complexes have on-rates no slower than 600 M^{-1} s^{-1}, and it seems likely that the K99Q–E^k complex would fall into this range as well. By this argument, the lack of binding is unlikely to be due to a slowing of the on-rate. Second, if the off-rate was the same or slower than the other two antagonists, we should have detected at least some specific dissociation from the TCR. Because we cannot detect this even at the higher concentrations and the affinity of K99Q–E^k differs from T102G–E^k by a factor of only 2 (see below, Table 3) the dissociation rate is probably even faster than that of K99R–E^k and T102G–E^k (> 5.1 s^{-1}).

Table 1. Direct Binding to Immobilized 2B4 TCR

Peptide	Coupling	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (μM)
MCC (agonist)	amine	600	0.057	90
PCC (agonist)	amine	1105	0.09	80
MCC (agonist)	cysteine	1600 $\pm$ 810	0.063 $\pm$ 0.009	40 $\pm$ 21
MCC (T102S) (weak agonist)	cysteine	1500 $\pm$ 360	0.36 $\pm$ 0.018	240 $\pm$ 60
MCC (T102N) (weak agonist)	cysteine	1400 $\pm$ 730	0.44 $\pm$ 0.020	320 $\pm$ 90

2B4 and peptide–E^k apparent binding constants. The data on MCC and PCC–E^k binding to amine-coupled 2B4 TCR are from Matsui et al. (1994), after correction for the change in extinction coefficient (see text). All values represent the mean of at least three independent experiments, with the exception of T102S and T102N, which are from two independent experiments.

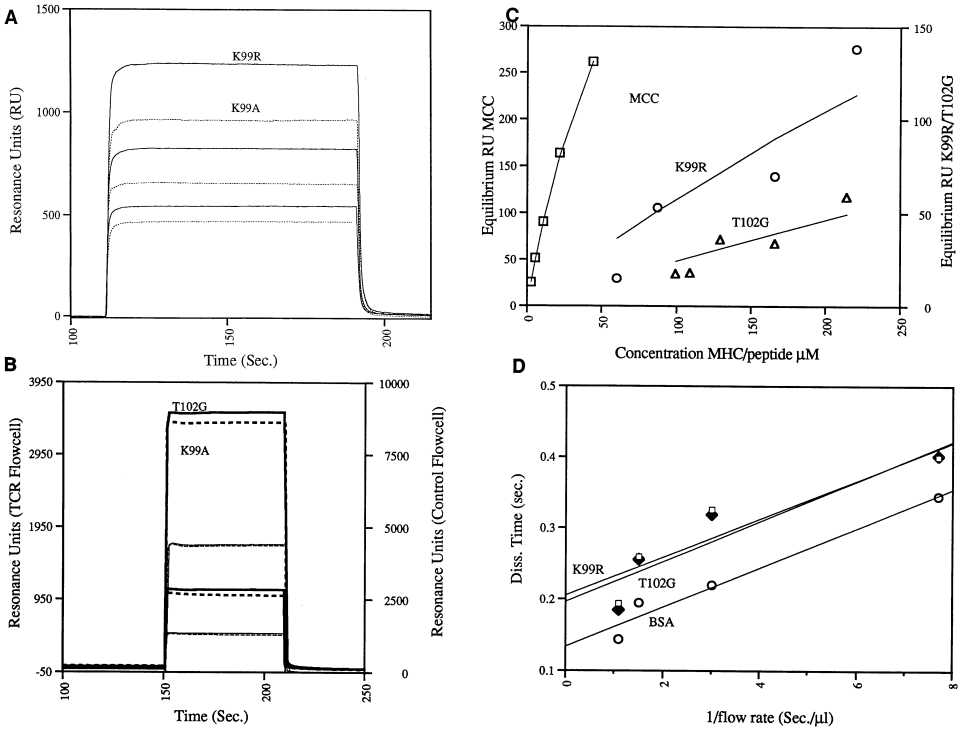

Figure 4. Direct Binding of Peptide–MHC Complexes to Immobilized TCR

(A) K99A (dotted line) and K99R–E^k (solid line) complexes were injected over the 2B4 TCR surface at concentrations of 5.1, 7.7, and 11.5 mg/ml.

(B) T102G and K99A–E^k complexes were injected over either a control blank flow cell (thin lines) or over the cysteine coupled TCR (thick lines for T102G) and (broken lines for K99A) as in (A) at concentrations of either 29 or 9.2 mg/ml.

(C) The observed equilibrium level of bound peptide–MHC (obtained by taking the difference between the MCC, K99R, or T102G signal at equilibrium and the K99A signal) is plotted against concentration of either MCC (open squares), K99R (open circles), or T102G/E^k (open triangles) complexes. The fit of these curves to an equilibrium binding model (steady-state fit model in BIAEvaluation 2.1) is also displayed (thin lines). This figure is representative data from three independent equilibrium binding experiments.

(D) The calculated dissociation times ($1/k_{off}$) for the K99R and T102G–E^k complexes and for BSA are plotted against 1/flow rate. After linear fitting of each curve, the y intercept gives the dissociation time. The observed fitting is averaged from two independent determinations. The fits give intercepts of 0.206, 0.197, 0.134, which yield k_{off} values of 4.8, 5.1, and 7.5 s^{-1} for K99R–E^k, T102G–E^k, and BSA, respectively.

A Competitive Binding Assay for Measuring TCR Affinities

Another approach to measure the affinity of weak interactions using the BIAcore (Pharmacia Biosensor) instrument is to utilize a competition assay. This should extend the sensitivity of the instrument even further, as was seen by Takemoto et al. (1996) in studying the interactions of hemagglutinin with its cell surface receptor. To develop such an assay, we took advantage of the recent results of J. H. et al. (unpublished data), who have shown that detergent-solubilized native E^k complexed with peptide can be coupled to a biosensor chip in a manner that allows efficient binding to soluble TCR in a peptide-specific manner.

In this application, detergent-solubilized MCC-loaded E^k was immobilized on the sensor chip, and soluble 2B4 TCR was then passed over the surface either alone or with competitor peptide–MHC complexes. A clear dose-dependent decrease in the amount of TCR bound was observed when TCR was preincubated with soluble E^k complexed with MCC (Figure 5A), T102S, K99R (Figure 5B), K99Q (Figure 5C), and T102G peptides. In contrast,

Table 2. Steady-State Analysis

Peptide	Coupling	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (μM)
MCC (agonist)	cysteine	N/A	N/A	70 ± 5.0
MCC (T102S) (weak agonist)	cysteine	N/A	N/A	150
MCC (K99R) (antagonist)	cysteine	(9600)	4.8 ± 0.80	500 ± 260
MCC (T102G) (antagonist)	cysteine	(3400)	5.1 ± 0.90	1500 ± 800
MCC (K99Q) (weak antagonist)	cysteine	—	> 5.1	—

Values for k_{off} were obtained from fitting the data in Figure 6D (from two independent experiments), and the K_D values were obtained from steady-state fitting of equilibrium binding curves (Figure 6C) from three independent experiments as described in Experimental Procedures. The values for k_{on} are in brackets to indicate that they were calculated based on the experimentally determined K_D and k_{off} values.

Table 3. Solution Inhibition Assay

Peptide	c0	c1	c2	IC_{50} (μM)	K_D (μM)
MCC	83.5 ± 1.1	−77.9 ± 0.004	0.10 ± 0.004	8.1	N/A
T102S	67.6 ± 4.0	−59.0 ± 0.005	0.025 ± 0.005	48	240
K99R	70.3 ± 4.0	−63.1 ± 3.7	0.02 ± 0.003	66	330
T102G	65.8 ± 6.1	−56.3 ± 6.7	0.005 ± 0.002	240	~880–1200
K99Q	60.3 ± 16	−62.1 ± 12	0.004 ± 0.003	420	2080

The inhibition curves in Figure 5 were fit to a single exponential equation of the form: percent inhibition = c0 + c1 * exp (−c2*(concentration peptide–MHC). Estimates of the error in the competitive binding experiment may be made from the standard error in the fitting parameters c0, c1, c2. The K_D values were calculated from the observed IC_{50} as described in Experimental Procedures. The K_D value for T102G–E^k shows a range of possible values, dependent upon whether its inhibition curve was extrapolated to 65% or 100%.

the null peptide K99A complexed with E^k (Figure 5D) and empty E^k molecules (data not shown) exhibited no inhibition. The degree of inhibition depended on the concentration of competitor peptide–E^k complex, with 50% inhibition (IC_{50}) values estimated from the fit of the curves in Figure 5E (see Experimental Procedures). As shown in Table 3, these measurements yield equilibrium dissociation constants of 330 μm for T102S, 330 mM for K99R, 880–1200 μM for T102G (which seems to aggregate at lower concentrations than any of the other complexes), and 2080 μM for K99Q. These values are all in reasonably good agreement with the direct binding experiments, where applicable. The ability to measure a K_D value for K99Q–E^k shows that this competition method can significantly extend the range of the BIAcore (Pharmacia Biosensor) instrument beyond the direct binding method, where this complex showed no detectable binding.

Discussion

Thus, as summarized in Tables 1, 2, and 3, it appears that the agonist peptide–MHC complexes in general have a higher affinity (or lower K_D) for the 2B4 TCR and slower

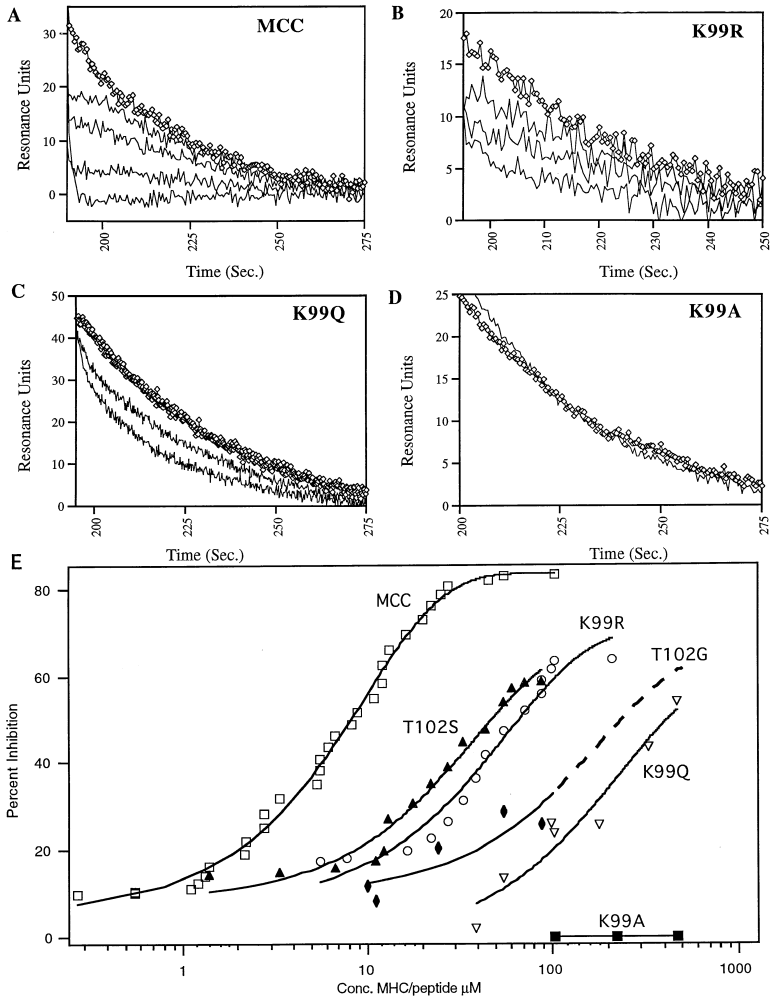

Figure 5. Solution Inhibition of TCR Binding to Immobilized MCC/E^k

The dissociation phase is plotted for 2B4 TCR binding to detergent-solubilized MCC–E^k. RU are plotted against time for TCR binding in the presence of varying concentrations of either (A) MCC, (B) K99R, (C) K99Q, or (D) K99A complexed to soluble GPI-linked E^k. The baselines are corrected such that the end of the sensorgram is 0 RU, and levels of inhibition are calculated by comparing the number of RU bound at a given timepoint versus the number of RU bound without inhibitor. Each plot contains the TCR alone curve (open diamonds) with each lower curve showing a decreasing amount of binding as the concentration of competitor peptide–MHC is increased. The concentrations are 0.09, 0.23, 0.85, and 3.2 mg/ml in (A), 1.15, 2.3, 14.8 mg/ml in (B), 6.9 and 32.6 mg/ml in (C), and 15.5 mg/ml in (D). (E) The percentage inhibition is plotted against the concentration of inhibitor to obtain an IC_{50} value for MCC (open squares), T102S (closed triangles), K99R (open circles), T102G (closed diamonds), and K99Q (open inverted triangles).K99A/E^k is shown (closed squares) as a negative control. An exponential fit to the data is shown (thin line) for each peptide, and for T102G this fit was extrapolated to 65% inhibition from early data points (broken line).

off-rates than antagonist complexes. In this subline of the 2B4 hybridoma, the CD4 coreceptor is not present to any significant extent, allowing us to concentrate on the interactions between the TCR and peptide–MHC alone.

The approximate range of K_D values for partial agonist peptide–E^k complexes appears to be around 100–250 μM (very similar to the lower limit for stimulation reported in another T cell recognition system by Sykulev et al. [1994a]). The range for antagonist peptide–E^k complexes begins around 250–500 μM for K99R–E^k and extends to at least 2100 μM for K99Q–E^k. The dissociation rates for K99R, K99Q, and T102G of $\sim$5.0 s^{-1} or more are much faster than those of the agonist ligands, which range from 0.06–0.44 s^{-1}. That there is only a 2-fold difference in affinity between the weak agonist and the highest affinity antagonist suggests that dissociation rate is a better predictor of a T cell response. Similarly, the finding that T102G and K99R have almost the same antagonist activities (Figure 1) and nearly identical dissociation rates, but differ $\sim$3-fold in affinity (Table 2), also supports this conclusion. Several mechanisms can be invoked to explain this dependence on dissociation rate. One suggests that peptide–MHC ligands that bind to the TCR less stably would disrupt the formation of TCR oligomers, which appears to be necessary for T cell activation (Sette et al., 1994; Brown et al., 1994; Koenig et al., 1995). Another possibility is that rapid dissociation could change the nature of the signal delivered by the T cell receptor. Rapid dissociation might allow only unproductive initial phosphorylation reactions of the CD3 complex, as observed by Sloan-Lancaster et al. (1994) and Madrenas et al. (1995). Long-lived complexes, on the other hand, would allow the entire signal transduction pathway to be completed, resulting in T cell activation. This is consistent with the kinetic proofreading model proposed by McKeithan (1995) and extended by Rabinowitz et al. (1996). This model suggests that the multiple reactions in the signal transduction machinery of the T cell could amplify differences in affinity between agonist and antagonist ligands for the TCR, increasing the specificity of the system for agonist ligands, and that the fast off-rates of antagonist ligands may be critical for their function. We would further suggest a "substrate depletion" model, in which some intermediate required for normal T cell activation is depleted by the engagement of a TCR by antagonist peptide–MHC complexes, thus weakening the ability of agonist peptides to trigger a response. This critical substrate could be CD3ζ-associated molecules or one or more of the other intermediates in TCR activation. The faster off-rates of the antagonist and weak agonist peptide–E^k complexes in comparison to strong agonists would allow them to engage more TCR molecules via serial engagement (Vallitutti et al., 1995) and thus more rapidly deplete a limiting substrate. Although the generality of the progression seen here may differ in other systems (for example see an anomalous agonist peptide described by Al-Ramadi et al. [1995]), our results indicate that within this particular T cell recognition model there is a direct correlation between the stability of TCR–ligand binding and the range of biological activities observed.

Experimental Procedures

T Cell Activation Assay

The 2B4 hybridoma (Hedrick et al., 1982) was used for T cell activation assays in which 5×10^4 2B4 hybridoma cells were cultured with 5×10^4 E^k-expressing Chinese hamster ovary (CHO) cells and varying concentrations of the MCC peptide (88–103) (sequence ANE RADLIAYLKQATK) or the indicated peptide analogs. Antagonism assays were done either by mixing the peptides as described in Spain et al. (1994) or by prepulsing the antigen-presenting cells for 2 hr with 0.005 μM MCC peptide, washing, and then exposing them to varying concentrations of peptide analogs with 2B4 hybridoma cells. Both methods gave identical results. After 24 hr of culture, supernatants from duplicate cultures were harvested, and interleukin-2 (IL-2) was quantitated by titration on HT-2 indicator cells (Watson, 1979). One U/ml corresponds to one-tenth maximal proliferation of the indicator cells. For antagonist assays, the percent inhibition was calculated as: 100 − 100*(IL-2 concentration in the presence / IL-2 concentration in the absence) of the MCC peptide analog.

Peptides

MCC (88–103) and K99R (93–103), peptides were synthesized by the Tufts University Medical School Microchemistry Facility. The peptides T102S (88–103), K99T (93–103), and K99A(88–103) were obtained from the Protein and Nucleic Acid (PAN) Facility at Stanford University. The T102G MCC (88–103) peptide was synthesized in the laboratory of Dr. H. McConnell. Initial experiments with K99R (88–103) were done with peptide given by Dr. A. Sette and gave identical results when compared with the later material. All peptides were high pressure liquid chromatography purified prior to use. For BIAcore binding studies the following additional peptides K99R (88–103 acetylated N terminus), K99Q (88–103), MCC (88–103), T102G (88–103), K99A (88–103 plus 2 D alanine residues at C terminus), and T102N (88–103) were synthesized by the PAN facility at Stanford University.

Protein Purification

Native E^k was prepared by detergent lysis of CH27 B cell lymphomas or from transfected E^k–CHO cells and purified by affinity chromatography on a 14-4-4 MAb column as described (Buus et al., 1986; Schild et al., 1994). Fractions containing E^k were pooled and peptides were loaded according to modifications of published procedures (Matsui et al., 1994; Boniface and Davis, 1996). Purity and biological activity of the prepared peptide–E^k complexes was determined by nonreducing SDS–PAGE, ELISA utilizing the MAbs 14-4-4 (anti-E^k) and D-4 (anti-E^k/MCC88–103) (P. Reay and M. M. D., unpublished data), and T cell activation assays with plate bound MCC–E^k complex (Kane et al., 1989; Schild et al., 1994). The efficiency of peptide loading was estimated to be around 25% for the MCC–E^k complex as determined by ELISA with 14-4-4 and D-4 (data not shown).

Soluble GPI-linked 2B4 TCR was prepared according to Lin et al. (1990) with the following modifications. Following cleavage from CHO cells expressing GPI-linked TCR, harvests were affinity purified on both an A2B4 (anti-Cα) antibody followed by an H57 (anti-Cβ) antibody (Kubo et al., 1989) column. The resulting TCR was then size purified on a Superdex-200 size exclusion column (Pharmacia). The protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.3 $(mg/ml)^{-1}cm^{-1}$.

Soluble GPI-linked E^k was prepared as described by Wettstein et al. (1991). GPI-linked E^k complexes for all BIAcore experiments were prepared as described earlier (Matsui et al., 1994; Boniface and Davis, 1996). Protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.3 $(mg/ml)^{-1}cm^{-1}$ derived from amino acid composition analysis. It should be noted that this is a correction from the previous estimate of 2.0 $(mg/ml)^{-1}cm^{-1}$ (Matsui et al., 1994).

Binding Analysis Using Immobilized TCR

2B4 TCR was immobilized using 2-(2-pyridinyldithio)ethaneamine HCl (PDEA) chemistry to the sensor chip. The surface was activated with a 35 μl pulse of NHS/EDC mixture at 5 μl/min, a 35μl pulse of 180 mM PDEA in 0.1 M borate buffer (pH 8.5), and a 35 μl pulse of

ethanolamine. Flow was then slowed to 3 μl/min and TCR at 150 μg/ml in 10 mM (pH 4.0) sodium acetate was added. Finally, a mixture of 50 mM cysteine and 1 M sodium chloride was used to block the surface. All SPR measurements were conducted at 25°C. Binding kinetics of GPI-linked peptide–E^k complexes were obtained at 15 μl/min, 20 μl injection, and 10 Hz data collection rate on a BIAcore (Pharmacia Biosensor) system, and at 30 μl/min, 30 μl injection, and 10 Hz data collection rate for K99R, K99A, and T102G–E^k complexes on a BIAcore 2000 (Pharmacia Biosensor). Binding data was fit using the Marquardt–Levenberg nonlinear least squares algorithm in BIAEvaluation 2.1 (Pharmacia Biosensor) to obtain on- and off-rates. For steady-state fitting, the equilibrium number of resonance units (relative signal on the BIAcore, referred to as RU) from K99A–E^k was plotted against its concentration to calibrate for the linear background level of signal due to refractive index changes associated with the injected protein (Stenberg et al., 1991). Linear fitting of this curve allowed the calculation of the background level of RU at every concentration of either K99R or T102G–E^k. The concentration of K99A–E^k was also corrected by normalizing the level of signal in the control blank (PDEA-activated and cysteine-deactivated) flowcell between K99A and T102G or K99R–E^k complexes (to assure that all of the signal seen with antagonist peptide–E^k versus K99A–E^k over the TCR surface was due to real binding and not to differences in concentration or nonspecific interactions with the surface). The steady-state equilibrium model in BIAEvaluation 2.1 (Pharmacia Biosensor) was used to fit the equilibrium level of bound RU of MCC, T102G, or K99R after subtraction of the K99A–E^k signal to obtain a K_D. For fitting the antagonist peptide–E^k complexes, the maximal response, RUmax, was set to 550 RU, estimated to be the maximal level based on several independent fittings with both MCC and T102S–E^k complexes. By calculating RUmax with these higher affinity complexes, the difficulties associated with fitting only the lower data values on an equilibrium binding curve with the antagonist peptide–E^k complexes could be reduced.

Binding Analysis Using Immobilized E^k

Approximately 5,000 RU of detergent-solubilized MCC-bound E^k was immobilized on a BIAcore CM5 chip (Pharmacia Biosensor) using standard NHS/EDC coupling chemistry (surface activation by NHS/EDC, injection at 5 μl/min of 35 μL of MCC–E^k diluted in 10 mM acetate [pH 4] buffer, and termination by ethanolamine). Injection of the MAb 14-4-4, which recognizes an epitope on the α1 domains of the E^k molecule shows 70%–75% reactivity (data not shown), suggesting that the E^k immobilizes on the CM5 chip in an oriented way, with its peptide binding domain facing away from the gold surface.

For competitive binding experiments, early data points in the dissociation phase (10 s after buffer exchange) were used to quantitate the amount of specifically bound TCR. Analysis during this phase eliminated the need to correct for the nonspecific changes in signal due to the different protein concentrations of competitor peptide–MHC complexes in solution. The association phases are not shown because these nonspecific signals mask the dissociation phase when displayed on the same scale. The observed off-rate was calculated using BIAEvaluation version 2.1 (Pharmacia Biosensor) and found to vary from 0.02–0.04 s^{-1} for bound TCR dissociating from surface MCC–E^k in all data sets, indicating that the competitor peptide–MHC complexes did not affect the observed off-rate, only the amount bound. For calculation of IC_{50} values, inhibition data was first smoothed using the Savitzky–Golay routine and then fit to a single exponential (Igor Pro, Wavemetrics) to solve for the concentration of peptide–MHC that gives 50% inhibition. For T102G, this fit was extrapolated from early data points to either 65% or 100% maximal inhibition to give a range of possible values for the IC_{50}, as higher concentrations failed to demonstrate any increase in inhibition.

Affinity values from the solution inhibition experiments were calculated by converting the observed IC_{50} value into a K_D value using an equation that includes both the equilibrium between TCR and competitor peptide–MHC in solution as well as the equilibrium between TCR and surface bound MCC–E^k. Direct conversion from IC_{50} to K_D using the Cheng–Prusoff equation (Cheng and Prusoff, 1973) is not possible due to ligand depletion (Goldstein and Barrett, 1987), and measuring initial on-rates in the presence of inhibitor (Karlsson 1994) is also inappropriate because in this system it is not possible to generate mass transport-limited conditions. Therefore, as the concentration of bound peptide–MHC and TCR in solution (x) is governed by the law of mass action for competition equilibrium:

$$(1)\quad (([0 - x][t_0 - x] - K_D x)(K_D x + K_D[m_0 - x]) = SK_M(m_0 - x)x,$$

where m_0 equals (peptide–MHC)solution; t_0 equals $(TCR)_{initial}$ equals 1.73 μM; K_M equals solution equilibrium dissociation constant for peptide–MHC and TCR; K_D equals surface equilibrium dissociation constant for MCC–E^k and TCR; and S equals surface concentration of MCC–E^k equals 179 μM.

The surface concentration, S, of immobilized MCC–E^k was based on the conversion factor 100 RU = 1 g/L (Stenberg et al., 1991), the molecular weight of the immobilized MCC–E^k of 70,000 g/mol, and the approximately 5,000 RU immobilized. In addition, this concentration was multiplied by the percent of the immobilized material that had MCC peptide available for binding. This figure was estimated to be 25% based upon recognition by 14-4-4 and D-4. At 50% inhibition ($m_0 = IC_{50}$), equation 1 simplifies in terms of r_2, or the half-maximal fractional occupancy of the surface immobilized MCC–E^k by TCR:

$$(2)\quad (1 - r_2)(t_0[K_M[1 - r_2] + ([K_D r_2] - [IC_{50}]K_D r_2]) = (K_D r_2 + S r_2[1 - r_2])(K_M[1 - r_2] + K_D r_2).$$

Using the observed IC_{50} for MCC–E^k in solution of 8.1 μM and setting K_D and K_M equal to 40 μM for MCC–E^k the equation for r_2 can be solved to give $r_2 = 0.0076$. Using the IC_{50} values for K99R, T102G, and K99QE^k complexes (determined from at least two independent experiments) in equation 2 then allows the determination of their equilibrium dissociation constants.

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