Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12862–12866, December 1994
Immunology

Kinetics of T-cell receptor binding to peptide/I-E^k complexes: Correlation of the dissociation rate with T-cell responsiveness

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Contributed by Mark M. Davis, August 19, 1994

ABSTRACT **Recognition by T-cell antigen receptors (TCRs) of processed peptides bound to major histocompatibility complex (MHC) molecules is required for the initiation of most T-lymphocyte responses. Despite the availability of soluble forms of TCRs and MHC heterodimers, this interaction has proven difficult to study directly due to the very low affinity. We report here on the kinetics of TCR binding to peptide/MHC complexes in a cell-free system using surface plasmon resonance. The apparent association rates for the interactions of related peptide/MHC complexes to one such TCR are relatively slow (900–3000 $M^{-1}\cdot s^{-1}$) and dissociation rates are very fast (0.3–0.06 s^{-1}) with $t_{1/2}$ of 2–12 s at 25°C. The calculated affinity of the engineered soluble molecules compares well with previously reported competition data for native TCRs or competition data reported here for native peptide/MHC complexes, indicating that these soluble heterodimers bind in the same manner as the original molecules expressed on cells. We also find that the peptide variants which give weaker T-cell stimulatory responses have similar affinities but distinctly faster dissociation rates compared with the original peptide (when loaded onto the MHC molecule) and that this later property may be responsible for their lower activity. This has implications for both downstream signaling events and models of TCR-peptide antagonists.**

A primary event in the generation of a cytotoxic or helper T-cell response is the recognition by $\alpha\beta$-type T-cell antigen receptors (TCRs) of processed peptides bound to molecules encoded by the major histocompatibility complex (MHC). While similar to immunoglobulins with respect to sequence homology and gene rearrangement (1), the binding characteristics of TCRs have been difficult to study. One reason for this has been the lack, until recently, of soluble forms of this normally integral membrane heterodimer, and a second has been their relatively low affinity. With respect to the first problem, a number of groups have now engineered soluble forms of TCR and MHC molecules such that they can be expressed in large quantities (reviewed in ref. 2).

With respect to the affinity of TCRs for their ligands, two studies have used soluble peptide/MHC complexes to block a labeled anti-TCR antibody binding to native TCRs on T cells to obtain equilibrium dissociation constants (K_d) of 4–6 $\times 10^{-5}$ M (3) in one case and 10^{-4}–10^{-7} M in another (4). Alternatively, Weber *et al.* (5) used a soluble TCR–immunoglobulin chimera ($V_\alpha C_\alpha C_\kappa \cdot V_\beta C_\beta C_\kappa$, where V_α and V_β are the variable regions of the TCR α and β chains and C_κ is the constant region of the immunoglobulin κ light chain) to inhibit a T-cell response and estimated a K_d of 10^{-5} M. However, none of these studies give direct information about the kinetics of the molecular interactions and are dependent on live cells, thus greatly limiting the range of conditions (temperature, ionic strength, etc.) that can be assessed.

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Recently a new instrument for measuring protein–protein interactions has been introduced (BIAcore from Pharmacia Biosensor) which uses biosensor technology to measure ligand binding (6). In this system, one molecule is immobilized on a dextran matrix coating a gold surface, and a putative ligand is passed over the surface in solution. The biosensor is very sensitive to changes in local protein concentration, and the specific binding of molecules in solution with those on the matrix is detected by an optical phenomenon known as surface plasmon resonance. Using this approach we are able to measure the kinetics of the interaction of soluble cytochrome *c* peptide/I-E^k complexes with the 2B4 TCR heterodimer. These results yield an affinity measurement which agrees well with previous competition data (3) and, furthermore, indicate that the low affinity of this particular peptide/MHC/TCR interaction derives from some unusual kinetic parameters—notably, a slow association rate combined with a fast dissociation rate. Data obtained with different peptides suggest that small differences in the dissociation rate may lead to much larger shifts in the dose–response curve for T-cell activation. Thus the dissociation rate of the TCR from peptide/MHC complexes may be a critical parameter in the activation cascade. We have used soluble TCR to inhibit the binding of an anti-peptide/MHC complex antibody to its ligand on antigen-presenting cells and obtained a K_d value (3×10^{-5} M) very similar to that obtained previously with soluble peptide/MHC complexes ($4–6 \times 10^{-5}$ M) (3). Thus there do not appear to be significant effects due to abnormalities in soluble molecules.

MATERIALS AND METHODS

Synthetic Peptides. Peptides were synthesized by the Stanford University PAN facility by standard fluorenylmethoxycarbonyl chemistry. Synthetic peptides had the following amino acid sequences: ANERADLIAYLKQATK [moth cytochrome *c* (MCC) peptide], ANERADLIAYLKQASK [MCC(102S)], ANERADLIAYLKQATAK [pigeon cytochrome *c* (PCC) peptide], and ANERADLIAYLEQATK [MCC(99E)].

Soluble TCR and MHC. The soluble 2B4 TCR and I-E^k were obtained as described (7, 8), by expression as inositol-phospholipid-linked proteins in CHO cells which were grown to high density in hollow-fiber bioreactors. Protein was recovered by continuous flow of medium containing phosphatidylinositol-specific phospholipase C (40 μg/day in 400 ml of RPMI 1640/1% fetal bovine serum) into the cell side of

Abbreviations: IL-2, interleukin 2; MCC, moth cytochrome *c*; PCC, pigeon cytochrome *c*; MHC, major histocompatibility complex; TCR, T-cell antigen receptor; RU, resonance units.

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the bioreactors. Each protein was purified from medium by immunoaffinity chromatography (7, 8).

Preparation of Peptide/I-E^k Complexes. Peptides were bound to soluble I-E^k as described (3). Protein was diluted to 0.1 mg/ml in citrate/phosphate buffer (pH 5.1)/100 mM NaCl containing a 20- to 50-fold molar excess of peptide. Reactions were allowed to go for 2–3 days at 37°C. To remove aggregated material peptide/I-E^k complexes were purified by gel filtration (Superdex 200 HR or Sephacryl S300; Pharmacia) prior to BIAcore or competition analysis (3, 9, 10). This results in a highly homogeneous preparation that is loaded to 80–100% with antigenic peptide (3) and can efficiently stimulate T cells when bound to a plate (8).

T-Cell Activation Assay. Stimulation of 2B4.11 T cells was determined by measuring interleukin 2 (IL-2) production (8).

BIAcore Analysis. Soluble 2B4 TCR was immobilized in a BIAcore flow cell by standard amine-targeted chemistry (11) by first activating the carboxy-dextran layer with a mixture of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 0.025 M and 0.1 M, respectively. TCR was then coupled via the active esters by injection into the flow cell in 10 mM acetate (pH 4.0), until 6000–8000 resonance units (RU) were bound. The surface was inactivated with 1 M ethanolamine (pH 8.5). The amount of immobilized TCR was optimized so that surfaces with binding capacities of only a few hundred RU were obtained. This was in order to minimize mass transport as a binding-rate limiting factor (12), while still having an acceptable signal/noise ratio. Typically, this surface was allowed to stabilize by an overnight wash. Binding of peptide/I-E^k complexes to immobilized TCR was performed in phosphate-buffered saline with 20-μl injections of protein and a flow rate of 15 μl/min.

Competitive Binding Assays. Affinity estimates were obtained from competitive binding involving specific competition of an ^{125}I-labeled antibody Fab′ fragment (KJ25 or D4) with soluble peptide/MHC complexes (3) or soluble 2B4 TCR, respectively. For competitive binding to T cells, 2B4.11 cells (2.5×10^5) were incubated with 2.5 nM ^{125}I-KJ25 Fab′ and various concentrations of unlabeled KJ25 Fab′, MCC/I-E^k, MCC(102S)/I-E^k, or PCC/I-E^k for 1 hr at room temperature as described (3). For the determination of the K_d of soluble TCR for native I-E^k, CHO cells (1×10^4) transfected with native I-E^k were incubated with MCC, washed twice in phosphate-buffered saline, and incubated with 1 nM ^{125}I-D4 Fab′ and either unlabeled D4 Fab′ or soluble TCR for 90 min at 4°C in RPMI 1640/5% fetal bovine serum. After incubation, bound and free ligands were separated by centrifugation through a layer of fetal bovine serum.

The affinities of KJ25 and the D4 Fab′ were determined by Scatchard analysis (13) to be 2×10^{-9} M and 3×10^{-9} M, respectively (ref. 13 and data not shown), and the K_d values of the unknowns were determined by the Cheng–Prusoff relationship (15):

$$K_d(\text{unknown}) = \frac{IC_{50}}{1 + \left(\frac{[\text{standard}]}{K_d(\text{standard})}\right)}.$$

RESULTS

The 2B4 T-cell hybridoma is specific for the PCC- or MCC-(88–103) peptide bound to the class II MHC molecule I-E^k (16, 17). As is typical of T cells it gives varying responses to different analogs of its peptide antigen, usually seen as a shift in the concentration of peptide required to achieve maximal IL-2 production (17). Thus, as shown in Fig. 1, PCC is required at a 3-fold higher concentration than MCC to elicit the same amount of IL-2 (8, 17), whereas the response to the MCC(102S) variant is shifted by 100- to 1000-fold relative to

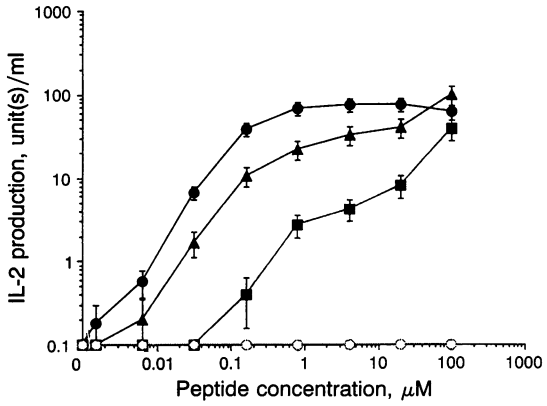

FIG. 1. T-cell activation by peptide/MHC complexes on antigen-presenting cells. 2B4.11 T cells were incubated with various concentrations of MCC (●), MCC(102S) (■), PCC (▲), or MCC(99E) (○) presented by the native I-E^k-transfected CHO cells (8). Stimulation of 2B4.11 T cells was determined by measuring IL-2 production, as described (8). Error bars indicate the range obtained in duplicate assays.

MCC (as originally noted in ref. 18). Lys^{99} of the MCC peptide is critical for recognition (19), and thus substitutions such as glutamic acid (99E) abolish reactivity and were used as a negative control. As all of these peptides bind I-E^k equivalently (14, 20), these differences in dose–response are likely to reflect different affinities or kinetics for TCR interactions.

To investigate this further, we prepared a soluble form of the 2B4 TCR heterodimer from a glycolipid chimera (7) and immobilized it onto a BIAcore sensor chip by standard amine coupling chemistry. We have also made glycolipid forms of I-E^k (8) and it appears to be "empty" of bound peptides (21). Soluble I-E^k alone at 1.1 mg/ml showed no evidence of binding, whereas the same material loaded with the MCC-(88–103) peptide bound the TCR on the sensor chip (Fig. 2*A*). I-E^k loaded with the MCC(99E) peptide behaved the same as I-E^k alone (data not shown). The binding of MCC/I-E^k at different concentrations showed a concentration-dependent increase in the slope of the initial binding phase (Fig. 2*B*), allowing us to calculate an association rate constant (k_{on}) (plotted in Fig. 2*C*). The k_{on} plot [−slope(dR/dt vs. R) vs. concentration] is linear (r^2 value of 9.8%) for the concentration range of 0.28–2.26 mg/ml (Fig. 2*C*). In this case, the apparent k_{on} is 900 $M^{-1}\cdot s^{-1}$, a very slow rate compared with the value for many molecules of 10^4–10^6 $M^{-1}\cdot s^{-1}$. The k_{on} value never varied by more than 2-fold in over six experiments. When the injection of analyte ends, buffer alone washes through the flow cell and the dissociation of MCC/I-E^k from the surface becomes directly visible (Fig. 2 *A* and *B*). The dissociation phase was used to calculate a first-order dissociation rate constant (Fig. 2*D*). The bulk of bound material dissociates with an apparent rate constant of 0.057 s^{-1}. This is a very fast off-rate, with a $t_{1/2} \approx 12$ s. As with k_{on}, k_{off} always fell within the range of 0.03–0.065 s^{-1} in multiple experiments. Some of this variation may have been due to carbohydrate heterogeneity in different MHC and TCR preparations. BIAcore analysis of *Escherichia coli*-expressed I-E^k loaded with the MCC peptide (and thus free of carbohydrates) gave very similar rates to those presented here (J. Altman, personal communication). The affinity (K_d) of the MCC/I-E^k complex–TCR interaction can be calculated from these results as k_{off}/k_{on} and equals 6×10^{-5} M. This affinity compares very well with our previous estimate of 4–6 $\times 10^{-5}$ M (3).

We also made I-E^k complexes with the PCC- and MCC(102S) peptides and compared them with MCC/I-E^k

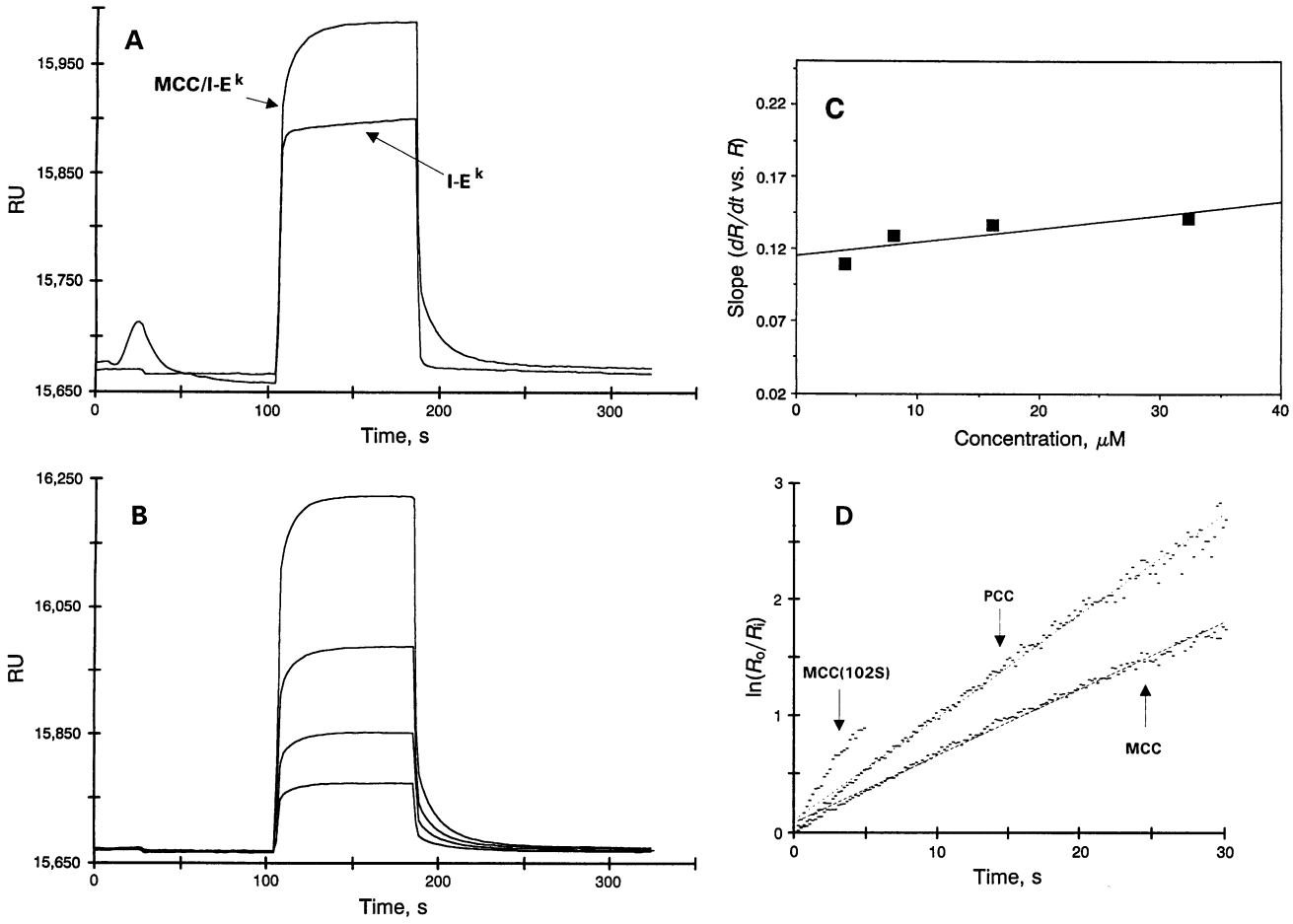

FIG. 2. Kinetic analysis of MCC/I-E^k binding to TCR. (*A*) Comparison of MCC/I-E^k and I-E^k binding at 1.13 mg/ml to TCR. (*B*) Concentration dependence for MCC/I-E^k binding at 0.28, 0.57, 1.13, and 2.26 mg/ml to TCR. (*C*) Slope of dR/dt vs. R (where R is resonance) as a function of concentration of MCC/I-E^k complex (derived from data in *B*). The slope of this curve gives the association rate constant (k_{on}) of 900 $M^{-1}\cdot s^{-1}$ [details of this linear transformation are described elsewhere (22)]. (*D*) Dissociation kinetics of MCC/I-E^k, PCC/I-E^k, and MCC(102S)/I-E^k complexes from immobilized soluble TCR, plotted as $\ln(R_o/R_i)$ against $(t_i - t_o)$, where subscript i and o indicate a given time point and the initial time, respectively. The slope of these plots gives estimates for first-order dissociation rate constants of 0.057, 0.09, and 0.1–0.3 s^{-1}, respectively.

results. We found that PCC/I-E^k was almost twice as fast as MCC/I-E^k in both its association (1700 $M^{-1}\cdot s^{-1}$) and dissociation (0.090 s^{-1}) rates (Table 1; Fig. 2*D*). Interestingly, for the 102S/I-E^k complex we were unable to detect an association phase, even at 18 mg/ml, but we did observe a slight dissociation phase above background (I-E^k alone) and in three experiments obtained a range of dissociation rates from 0.10 to 0.30 s^{-1}, which correspond to $t_{1/2}$ values of 2–5 s. Plots of the PCC- and MCC(102S) dissociation curves are shown together with that of MCC in Fig. 2*D*.

We also obtained estimates of the affinity of the PCC/I-E^k and MCC(102S)/I-E^k complexes for the 2B4 TCR by the competitive binding assay discussed above (3). Both complexes were less able to inhibit KJ25 Fab′ binding than MCC/I-E^k (Fig. 3). Normalizing each to the MCC/I-E^k curve yielded a $K_d \approx 10^{-4}$ M for each (Fig. 3; Table 1). This agrees to within a factor of 2 for the BIAcore estimate for PCC/I-E^k (5×10^{-5} M) and agrees well with other solution estimates for MCC(102S)/I-E^k binding to the 2B4 TCR (7×10^{-5} M; D. Lyons, J.J.B., and M.M.D., unpublished work; also see below). Thus the association rate for the 102S complex must be comparable to, if not higher than, those of the PCC/I-E^k and MCC/I-E^k complexes. That is, if the affinity rate of the 102S complex is 10^{-4} M and the dissociation rate is 0.10–0.30 $M^{-1}\cdot s^{-1}$, then the association rate should be 1000–3000 $M^{-1}\cdot s^{-1}$. Thus if we take the BIAcore data together with the affinities derived from the competition curves, it seems that the only variable that could account for the decreased T-cell stimulation with MCC and PCC is the faster dissociation rates with those peptide/MHC complexes.

Table 1. Interaction of 2B4 TCR with peptide/I-E^k complexes

Complex	BIAcore analysis (2B4 soluble TCR) k_{on}, $M^{-1}\cdot s^{-1}$	k_{off}, s^{-1}	K_d, M	Competitive binding assay K_d, M
MCC/I-E^k	900	0.057	6.0×10^{-5}	$4–6 \times 10^{-5}$
PCC/I-E^k	1700	0.090	5.0×10^{-5}	$\approx 10^{-4}$
MCC(102S)/I-E^k	—	0.1–0.3	—	$\approx 10^{-4}$
Soluble TCR (D4 antibody)	—	—	—	3.0×10^{-5}

One possible caveat with all these measurements is the possibility that the soluble I-E^k molecule used here gives artificially low affinities due to some structural alteration that may have occurred due to its expression as a glycosylphosphatidylinositol-linked chimera in a heterologous cell system. To test this possibility, we utilized an antibody specific for the MCC/I-E^k complexes (D4; P.A.R. and M.M.D., unpublished work) to measure the affinity of native peptide/I-E^k complexes for the soluble 2B4 TCR. Soluble TCR inhibited the binding of ^{125}I-labeled D4 Fab′ fragments (Fig. 4). Using the Cheng–Prusoff relationship (15), we estimated the K_d to

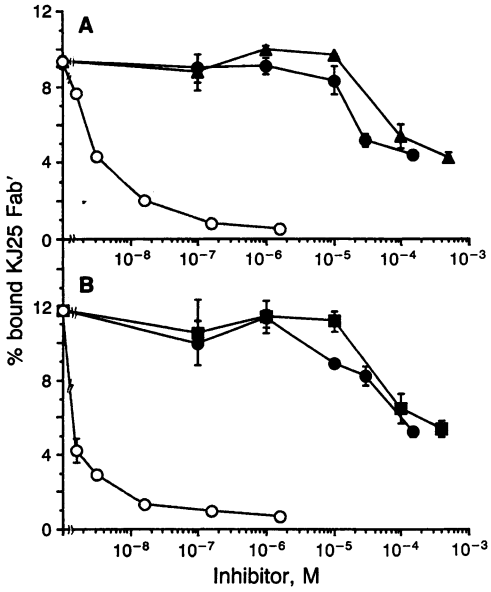

FIG. 3. Competitive binding of soluble peptide/I-E^k complexes to T cells. (*A*) Inhibition of KJ25 Fab' binding to 2B4.11 T cells by unlabeled KJ25 Fab' (○), MCC/I-E^k (●), or PCC/I-E^k (▲). (*B*) Inhibition of KJ25 Fab' binding to 2B4.11 T cells by unlabeled KJ25 Fab' (○), MCC/I-E^k (●), or MCC(102S)/I-E^k (■). Errors bars indicate the range of duplicate assays.

be 3.0×10^{-5} M. This agrees well with the data discussed above and indicates that measurements of the interactions of these chimeric TCR and class II MHC molecules accurately reflect those of the native molecules on their respective cell surfaces. We have also performed the same assay with the MCC(102S) peptide and obtained a K_d of 6.0×10^{-5} M, or 2-fold higher, similar to the values obtained above (K.M., P.A.R., and M.M.D., data not shown).

DISCUSSION

The data presented here show that we can measure the interaction kinetics of TCR and peptide/MHC molecules in a completely cell-free, aqueous system using the BIAcore instrument. While O'Shannessy (12) has pointed out the potential importance of differential mass transport at the interface of the BIAcore chip surface and solvent, we chose the conditions to specifically limit such effects (see *Materials and Methods*), and computer simulations using the data here do not indicate any significant mass-transport limitation (P.S., unpublished observations). O'Shannessy also questions the use of linear transformations of BIAcore data, which is no doubt a potential problem, but these fit the data presented here well and in our experience thus far. Another indication of veracity is that the calculated affinity based on the BIAcore data presented here supports previous competition studies (3), both indicating a K_d value of 5×10^{-5} M for the MCC/I-E^k complex and the 2B4 TCR. Such low affinities may prove to be the rule, rather than the exception, for those interactions occurring between adjacent lipid bilayers of interacting cells, as shown by recent measurements for CD2 (9.0×10^{-5} M; ref. 9) and LFA-1 (1.0×10^{-4} M to perhaps 1.0×10^{-6} M on activated cells; ref. 23), both of which are T-cell surface molecules that recognize ligands on the surfaces of other cells. While the low serum concentration of many soluble ligands necessitates affinities in the nanomolar range (2), the large effective concentration of ligands within opposing lipid bilayers may allow for affinities several orders of magnitude lower. Interestingly, Sykulev *et al.* (4) have reported a much broader range of affinities (1000-fold) for the interaction of a cytotoxic T-lymphocyte line with a class I protein complexed with different peptides. The upper limit of K_d values in their system is about 0.5 μM, which is significantly higher than any measurement reported here. It will be interesting to determine to what extent this range of affinities might relate to their function [i.e., the cytotoxic, alloreactive T cell of Sykulev *et al.* (4) versus the low-affinity foreign peptide-specific helper T-cell models studied here, by Weber *et al.* (5), and more recently by Seth *et al.* (24)].

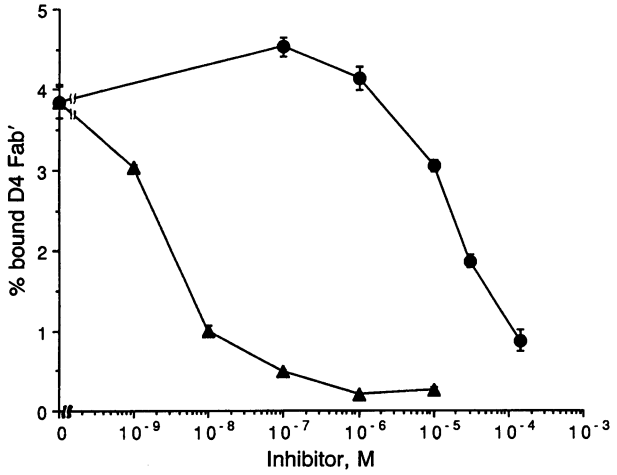

FIG. 4. Measuring the K_d of soluble TCR for native I-E^k on CHO cells. The binding of ^{125}I-D4 Fab' to native I-E^k on CHO cells was inhibited by either unlabeled D4 Fab' (▲) or by soluble TCR (●) at the concentrations indicated. The affinity of the D4 Fab' for native I-E^k was determined by Scatchard analysis and the K_d of the unknown competitor (soluble TCR) was then determined by the Cheng–Prusoff relationship (15) as described (3).

With respect to kinetic parameters, we find that TCR ligand interactions examined here have very slow association rates (900–3000 $M^{-1}\cdot s^{-1}$ for the various MCC peptide/I-E^k complexes and the 2B4 TCR) and very fast off-rates (0.30–0.06 s^{-1}). These slow association rates indicate that the binding of the TCR to its ligand is in some way intrinsically limited. One explanation is that it is dependent on a conformational change in either the MHC molecule (25) or the TCR (26) or both. A related hypothesis would be that the peptide side chains which have to bind to the TCR must be oriented in a specific way. The rapid dissociation rates indicate that any single interaction will have a $t_{1/2} \ll 1$ min. Additionally, we find that the dissociation rate may have a dominant effect on the T-cell stimulatory ability of a given peptide/MHC complex, at least within the very narrow affinity range that we find in this system. This is in contrast to the results of Sykulev *et al.* (4), who found a strict correlation between affinity and the efficiency of specific T-cell killing. This apparent contradiction may be explained by noting that Sykulev *et al.* started with a much higher affinity TCR and their lowest detectable activity occurred in the affinity range that we deal with here. Thus, TCR affinities of 10^{-4} M might be the weakest capable of giving any T-cell response, and near that range dissociation rates may become critically important. The striking differences that we see in biological activity between the different peptide/MHC complexes (Fig. 1), which vary in their $t_{1/2}$ values from only 2 to 12 s (Fig. 2*D*), must therefore reflect some critical (and unidirectional) later step that follows TCR engagement within this time period. This later step could be CD3 ζ-chain phosphorylation, as this is the earliest known covalent change in response to antigen (27), or some other event or sequence of events. This finding also suggests a specific mechanism for some peptide antagonists of TCRs (28, 29), particularly those which require a large excess of the nonstimulatory peptide over the stimulatory one. That is, they may have association rates similar to,

or faster than, those of the peptides described here but have even shorter half-lives. Thus they could compete effectively for TCRs but be unable to bind long enough to trigger T-cell activation. For antagonists that can effectively block T-cell responses when equimolar with agonist peptides, one would have to postulate a much faster association rate than the stimulatory peptide (in order to out-compete it) and again a half-life that is too short to be stimulatory (but could result in an altered signal in some cases as per ref. 29).

In summary, we have utilized surface plasmon resonance to directly measure the kinetics of TCR binding to peptide/MHC complexes. The slow association–fast dissociation kinetics that we see contrast with most antibody–antigen interactions yet are extremely specific, at least in their peptide antigen requirements. We find good agreement with affinity measurements of native versus chimeric molecules and find evidence that relatively small changes in these kinetics, particularly changes in the dissociation rate, can yield very dramatic changes in the dose–response characteristics.

Note Added in Proof. Corr *et al.* (30) reported on the kinetics of the 2C TCR using the BIAcore. Interestingly, the dissociation rate they obtained (0.026 s^{-1}; $t_{1/2}$ of 27 s) is very similar to the range reported here, but the association rate is much faster (2×10^5 $M^{-1} \cdot s^{-1}$ versus $1–3 \times 10^3$ $M^{-1} \cdot s^{-1}$). This difference may represent the typical range of TCR kinetics or it reflect the requirements of different types of T cells (2C is an alloreactive, cytotoxic T-cell line that recognizes a very short-lived peptide/MHC complex, whereas 2B4 is a foreign-antigen-specific helper T-cell line that recognizes a very stable complex).

We thank the Howard Hughes Medical Institute for funding, A. Townsend and S. Coughlin for suggesting the use of the BIAcore instrument, A. Goldstein and Y. Chien for helpful discussions, D. Lyons and J. Altman for discussions and the communication of unpublished data, and J. Wells and H. McConnell for comments on the manuscript. J.J.B. is a Fellow of the Irvington Institute.

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