exists between perforin and C9[22,23]. The structural differences at the N and C termini may account for the functional dissimilarity.

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A kinetic intermediate in the reaction of an antigenic peptide and I–E^k

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Helper T cells are triggered by molecular complexes of antigenic peptides and cell surface glycoproteins of the MHC (gene products of the major histocompatibility complex) on antigen-presenting cells[1]. There is now a lot of evidence that the complexes between isolated class II MHC molecules and selected peptides have long half-lives of approximately one day[2,3]. The reported equilibrium binding constants between antigenic peptides and class II MHC molecules however, are only micromolar[4,5], suggesting that the association rate constants are very low. The only reported[2] association rate constant is for a chicken ovalbumin peptide (OVA323–339) binding to I–A^d, and is indeed remarkably low, about 1 litre per mole per second. Prompted by these unusual data, we have used the pigeon cytochrome-*c* peptide pCytc(88–104) and I–E^k reconstituted in planar lipid bilayers on glass slides to investigate further the kinetics of peptide–MHC reactions. We report the formation of two IE^k-pCytc peptide complexes. One complex has slow apparent association and dissociation kinetics, very similar to those reported previously for the chicken ovalbumin peptide and I–A^d. The second complex forms and dissociates about a hundred times more rapidly. The short-lived complex shows peptide–MHC specificity and is a kinetic intermediate in the formation of the long-lived complex; the long-lived complex is recognized by specific T-helper cells.

The helper-T-cell hybridoma 2B4 recognizes the class II MHC molecule, I-E^k and the 17 amino-acid C-terminal peptide of pigeon cytochrome-c, pCytc (88-104)[6]. This hybridoma is triggered to produce interleukin-2(IL^{-2}) when pCytc peptide is presented by I-E^k incorporated in planar lipid bilayers on solid supports[7]. Pigeon-Cytc labelled at the N terminus with fluorescein isothiocyanate FpCytc) binds to I-E^k incorporated into planar membranes. The two peptides, pCytc and FpCytc, are almost equally effective in triggering the 2B4 hybriodma (unpublished results). Two protocols were used to study the kinetics of FpCytc binding to I-E^k in planar membranes. In one, the fluorescence intensity attributed to FpCytc-I-E^k complexes was measured as a function of time. A reaction between FpCytc and I-E^k was essentially complete after ~100 seconds when the initial peptide concentration was 80 μM (see Fig. 1*a*). The binding measurements for times less than 100 s are reproducible but are of uncertain significance because of possible mixing artefacts. To test the kinetic order of the reaction a second protocol was used with series of incubations varying the incubation time (t) and peptide concentration (c) so that the product, (ct) was a constant (Fig. 1*b*). The data are not consistent with a single-step pseudo first-order reaction that goes to completion, as this should lead to a constant amount of complex when (ct) is kept constant.

Table 1 Specificity of the fast reaction between pigeon cytochrome c peptide (88–104) and I-E^k in planar bilayer membranes

Reactants	Photon counts s^{-1}
I-E^k + FpCytc	9,864 ± 625*
I-A^d + FpCytc	<1,000*
Lipid + FpCytc	<1,000*
I-E^k + α I-E^k, then FpCytc	1,100†
I-E^k + α I-A^k, then FpCytc	9,860†
I-E^k + pCytc, then FpCytc	1,300‡

*Incubation was with 80 μM FpCytec for 2 min.
† Preincubation with anti-MHC antibody was for 5 min, then washed and 80 μM FpCytc added.
‡ 80 μM pCytc was incubated with I-E^k in planar membranes for 2 min and FpCytc was then added for another 2 min, then washed with PBS.

The rapid reaction of fluorescent peptide and I-E^k was found to be specific, as (1) fluorescent OVA peptide did not bind to I-E^k, (2) preincubation with an antibody specific for I-E^k inhibited rapid binding of FpCytc to I-E^k whereas the non-specific antibody (anti-I-A^k) did not, and (3) preincubation of unlabelled pCytc with the I-E^k-containing planar membrane inhibited rapid binding of FpCytc peptide (see Tables 1, 2). The number of I-E^k molecules per unit area in the membrane (~3 × 10^{11} cm^{-2}) corresponds to the number of I-E^k molecules used in the preparation of the I-E^k-containing vesicles, and was confirmed using fluorescinated anti-I-E^k antibody. In the experiments reported here the number of fluorescent peptides bound at maximal binding exceeded 50% of the I-E^k in the planar membrane. In a number of other experiments the maximum fluorescent peptide binding was so low (~10%) that it precluded kinetic measurements, because of a reduced signal: background ratio.

In the cellular assay, we measured the extent of T-cell stimulation (IL-2 secretion) as a function of the number of preformed complexes. The number of preformed complexes was established by incubation time and peptide concentration. The planar membranes containing the resulting I-E^k-peptide complexes were then washed, and T cells added (Fig. 1*c*). Again, the

Table 2 Specificity of the kinetic intermediate complex of pigeon cytochrome *c* and I-E^k

Reactants	Photon counts s^{-1}
I-E^k + FpCytc	5,800
I-E^k + FpCytc (2 min) then pCytc*	3,800
I-E^k + FpCytc (1 min) then pCytc	1,600
I-E^k FpCytc (2 min) then OVA	4,600
I-E^k + FpCytc (1 min) then OVA	4,300

Experimental conditions as described in Fig. 1*a* legend.
* Unlabelled peptides were incubated for 2 min at a concentration of 100 μM.

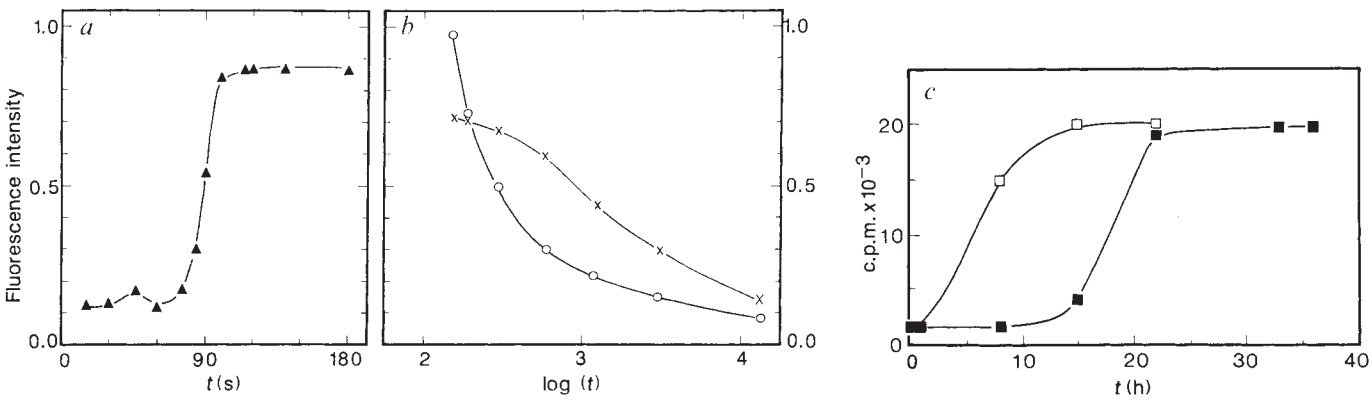

Fig. 1 Kinetics of binding of FpCytc to I-E^k in planar bilayer membranes. *a*, *b*, Fluorescence assays for the binding of FpCytc to I-E^k. *a*, Fixed 80 μM concentration of peptide versus incubation time. Fluorescence intensity is in units of 10^4 photons s^{-1}. Data are corrected for non-specific background, which is 30–40% of total signal. *b*, Fluorescence intensity is relative to the peak intensity. ○, FpCytc at concentration *c* incubated for time *t*, such that $tc = 12 \times 10^3$ μM s. X indicates theoretical points based[15], on estimated values of the rate constants k_{oi}, k_{it}, k_{ti}, k_{io} given in the text, using $k_{it} = 4 \times 10^{-5}$ s^{-1}. The values of X give the sum of the concentrations of C_i and C_t. The maximum value of X corresponds to 71% occupation of the available binding sites. A larger value of $k_{it} = 4 \times 10^{-4}$ significantly increases ($C_i + C_t$) only at the two longest times. *c*, Cellular assay for the kinetics of binding of pCyte to I-E^k. Solutions of 3 μM (■) or 30 μM (□) pCytc were incubated with I-E^k in planar membranes for the indicated times, the membranes washed, 2B4 T cells added, and IL-2 secretion was assayed by [^{3}H]thymidine uptake of the CTLL-2 cell line. The ratio of the two times (5.5 and 18 h) for 50% triggering is used to estimate the equilibrium dissociation constant K_i for the intermediate complex. The absolute value of each half-time can be used to estimate the rate constant k_{it} for the unimolecular transition of the intermediate complex to the terminal complex under the assumption that 50% of maximal T-cell triggering corresponds to 50% occupation of available I-E^k peptide binding sites. Competition experiments show that the half times in these experiments do not depend on the number of available I-E^k binding sites (unpublished results). This analysis is equivalent to drawing first-order binding curves through the mid-points of the experimental data, and assuming that the deviation of the data from these curves is due to non-linearity in T-cell responses. **Methods.** I-E^k was purified by affinity chromatography using monoclonal antibody 14-4-4S as described[9,10]. Lipopolysaccharide (*Escherichia coli* 0.111:84, Difco) stimulated blast cells from spleens of CBA (H-2^k) mice were used as the source of I-E^k. Reconstitution of I-E^k into planar membranes was as described[10]. Vesicle preparations for cellular assays used the lipid mixture of dipalmitoylphosphatidylcholine and dilinoleoyl phosphatidylcholine and cholesterol[7]. In *a*, 80 μM FpCytc was incubated with planar membranes containing I-E^k for the times indicated, then washed for one minute with PBS, all at room temperature (20 °C). Fluorescence intensity was measured with the photon counting system described previously[8]. The incubations were carried out in chambers of 20 μl volume assembled on a quartz microscope slide and covered by a circular 12-mm diameter coverglass using two layers of double sticky tape. Each microscope slide had two chambers. Vesicle preparations with or without I-E^k were deposited in the chambers, allowing planar membrane formation. N-terminal fluorescination of pCytc (88–104) was carried out during solid phase peptide synthesis (Peninsula), and purified by reverse phase HPLC on a Vydac C_{18} column. In *c*, the cell-free reaction took place during time intervals shown. Excess unbound peptide was removed by careful replacement of the medium covering the planar membrane, over a period of 10 min or more. T cells were added at a density of 4×10^5 to every well. The amount of IL-2 secreted in culture wells was assayed by [^{3}H]thymidine uptake of an IL-2-dependent T-cell line CTLL-2.

binding kinetics cannot be accounted for by a single-step pseudo first-order reaction. That is, the times for 50% cell triggering are not inversely proportional to the peptide concentration.

The experiments in Fig. 2 (▲) show the dissociation of the FpCytc-I-E^k complex as a function of time. FpCytc and I-E^k in planar membranes were incubated at room temperature for various lengths of time and were then sequentially rinsed to remove dissociated FpCytc. The wash was repeated at the indicated times for 2.5 days. After many washes the samples lose only 5–10% of the I-E^k as judged by the binding of anti-I-E^k. The dissociation curve is evidently biphasic with fast and slow components. Both reactions follow first-order kinetics with half lives of approximately 10 minutes and of 30 hours. The half life for the dissociation of pCytc from I-E^k at 37 °C obtained from the cellular assay is similar: 34 hours (Fig. 2, ●).

The dissociation data can be understood semiquantitatively in terms of two complexes of FpCytc and I-E^k, a short-lived intermediate complex C_i and a long-lived terminal complex C_t.

$$\text{FpCytc} + \text{I-E}^k \underset{k_{io}}{\overset{k_{oi}}{\rightleftharpoons}} C_i \underset{k_{ti}}{\overset{k_{it}}{\rightleftharpoons}} C_t \qquad (1)$$

The first-order dissociation rate constant for the intermediate of FpCytc complex in Fig. 2*a* is $k_{io} = 1.2 \times 10^{-3}$ s^{-1}. The first-order dissociation rate constant for the long-lived terminal complex is $k_{to} \simeq k_{ti} = 6.3 \times 10^{-6}$ s^{-1}. The relative amplitudes of the slow and fast components of dissociation depend on the time of preincubation before the dissociation measurement. The longer incubation time gives a larger proportion of the slowly dissociating component. Thus a 5-min incubation gives ~15% of the slow component, whereas a 20-min incubation gives ~30% of the slow component, both with 80 μM FpCytc.

These results can be interpreted using a first-order rate constant for the conversion C_i to C_t of $k_{it} = 4 \times 10^{-4}$ s^{-1}. The data in both Fig. 1*c* and Fig. 2 provide evidence that C_i is an intermediate in the formation of C_t and is not some irrelevant dead-end complex. The experiments do not show whether C_i is a complex with only one bound peptide (FpCytc) or two peptides (for example, FpCytc and a second peptide). The slowly decaying component C_t can be identified as biologically active, given the excellent agreement of the low off-rates based on the fluorescence and cellular assays (Fig. 2, ●).

According to reaction (1) the resultant kinetics of the formation of the terminal complex C_t from peptide and I-E^k are pseudo first-order with a rate constant that is non-linear in the peptide concentration, $k_{ot} = (k_{it})/(p + K_i)$, where p is the peptide concentration and K_i is the dissociation constant of the intermediate complex C_i. The data in Fig. 1*c* can be interpreted with $K_i \simeq 10^{-5}$ M and $k_{it} \simeq 4 \times 10^{-5}$ s^{-1}; this value for k_{it} assumes the cellular response is half-maximal at 50% saturation of the I-E^k with pCytc. The disparity between the two estimates of k_{it} is not unreasonable in view of the uncertainties in this assumption. We estimate the equilibrium dissociation constant K_t of the terminal complex from the equation $K_t = (k_{ti}/k_{it})K_i$ and obtain $K_t \sim 10^{-6}$–10^{-7} M, depending on the choice of k_{it} from the above estimates. Complexes of other antigenic peptides and Ia studied by Babbitt and co-workers[4] and Buus and co-workers[5] have dissociation constants $K_t \sim 10^{-6}$ M. Our model reduces mathematically to that of Buus at low peptide concentrations ($p \ll K_i$) where only three constants are observed, K_t, k_{0t} and k_{to}.

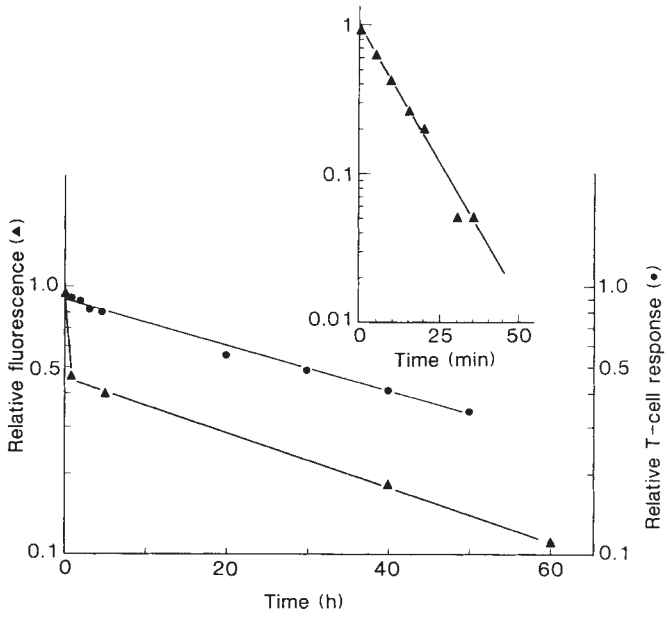

Fig. 2 Kinetics of dissociation of pigeon cytochrome-*c* peptide (88–104)-I-E^k complex. Fluorescence assay, ▲, curve and insert). Following an ~25 min incubation of I-E^k in planar membrane together with 80 μM FpCytc, the sample was washed sequentially at the indicated times. Each wash lasted about 1 min, and involved flowing 10–15 volumes PBS through the sample chamber. The data given in the insert were obtained after a 5-min incubation. Fluorescence intensities in the insert are used to estimate the off-rate constant k_{io} for the intermediate complex, and are corrected for both background fluorescence and the fluorescence intensity of the long-lived complex. After this 5-min incubation the short lived fluorescence (10-min half-life) is about 85% of the total specific fluorescence. The half-time for the dissociation of FpCytc from the terminal complex from the fluorescence data is 30 hours. In the cellular assay, (●) 15–20 μM unlabelled peptide was incubated at 37 °C with planar membranes with I-E^k for 24 h in RPMI medium without serum. Excess unbound peptide was removed. Each data point was obtained by allowing dissociation of peptide from I-E^k-peptide complexes followed by a second rinse before addition of 2B4 T cells in 10% fetal calf serum. The half time obtained from these cell assay data is 34 h. At each time point, the proliferation of CTLL was measured by titrating the supernatant and using data in the linear range.

The kinetic scheme (1) does not account for the observed but uncertain shape of the short time (<100 s) binding curve. A value of $k_{oi} = 1.2 \times 10^2$ l mol^{-1} s^{-1} obtained from the above estimates of K_i and k_{io} leads to a half-time for the formation of C_i of 70 seconds at 80 μM peptide, a result close to the onset time of ~90 seconds seen in Fig. 1*a*. These estimated values of K_i and k_{oi} also lead to the calculated points X in Fig. 1*b*, to be compared with the experimental points O. The calculations used the solution for two sequential first-order reactions given by Moore and Pearson[15]. At short times and high peptide concentrations the fraction of the available I-E^k binding sites occupied by the peptide is approximately $pk_{oi}k_{it}t^2$, so that even at times as short as 100 s as much as 1% terminal complex can form, which may account for some of the very recent observations on the kinetics of peptide–MHC complex formation, as measured by calcium influx in specific T cells[16].

In summary, a variety of kinetic data all point to the formation of an intermediate complex between an antigenic peptide and an MHC molecule. This result is particularly significant as the intermediate complex shows peptide-MHC specificity. The molecular structures of the intermediate and terminal complexes may be very different as the time required for the unimolecular conversion of the intermediate complex to the terminal complex is long, estimated to lie between 30 and 300 min. The slow step may or may not involve the release of a second peptide. Thus, although specific helper T cells presumably recognize the structure of the terminal complex, the selective peptide recognition by MHC may involve a substantially distinct protein-peptide conformation[12–14].

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Identification of specific binding proteins for a nuclear location sequence

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The nuclear envelope is a selective barrier against the movement of macromolecules between the nucleus and cytoplasm[1]. Nuclear proteins larger than relative molecular mass 20,000–40,000 are probably actively transported across the envelope through the nuclear pore complex[2,3] and are directed by specific nuclear location sequences (NLS) in the proteins[4]. NLS mediate the nuclear import of isolated nuclear proteins after microinjection into whole cells[5,6] and the nuclear accumulation of chimaeric proteins[7–9] or of non-nuclear proteins conjugated to synthetic peptides[10–12]. The best-characterized NLS is the simian virus 40 large T-antigen sequence[4,13,14]. We have identified two proteins of rat liver by chemical cross-linking that interact with a synthetic peptide containing this sequence: this interaction is specific for a functional NLS, is saturatable, and high affinity. The binding proteins are present in a post-mitochondrial supernatant, in nuclei and in a nuclear envelope fraction, which is consistent with a role in the transport of nuclear proteins from the cytoplasm to the nucleus.

Chemical cross-linking with disuccinimidyl suberate of a radioiodinated synthetic 34-residue peptide from the SV40 large T antigen containing the NLS was used to identify the proteins in different subcellular fractions of rat liver that interact with the NLS (Fig. 1). A 10-residue synthetic peptide containing the wild-type NLS sequence was used as a competitor to define binding specificity. Two proteins that interact specifically with the NLS, and can be competed out by a 100-fold molar excess of the 10-residue wild-type peptide, are detected in a post-

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