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Kinetic proofreading in T-cell receptor signal transduction

(protein-tyrosine kinase/major histocompatibility complex/mathematical model)

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ABSTRACT Like other cell-surface receptors with intrinsic or associated protein-tyrosine kinase activity, the T-cell receptor complex undergoes a number of modifications, including tyrosine phosphorylation steps, after ligand binding but before transmitting a signal. The requirement for these modifications introduces a temporal lag between ligand binding and receptor signaling. A model for the T-cell receptor is proposed in which this feature greatly enhances the receptor's ability to discriminate between a foreign antigen and self-antigens with only moderately lower affinity. The proposed scheme is a form of kinetic proofreading, known to be essential for the fidelity of protein and DNA synthesis. A variant of this scheme is also described in which a requirement for formation of large aggregates may lead to a further enhancement of the specificity of T-cell activation. Through these mechanisms, ligands of different affinity potentially may elicit qualitatively different signals.

T cells are sensitive to antigens that are present even in very low abundance on the antigen-presenting cell (APC). Experimentally, 60–200 molecules of the specific peptide–major histocompatibility complex (MHC) on the APC are sufficient for a T-cell response; this represents as few as 0.03% of the MHC molecules on the APC (1, 2). The T-cell receptor (TCR), however, must also have some affinity for self-peptide–MHCs for maturation in the thymus. In addition, since the peptide bound by the MHC is short, the few specific interactions may be insufficient to cause dramatic differences in affinity between a foreign antigen and the gamut of self-antigens.

If this is true, the number of TCRs engaged at any given time by weak interactions with self-antigens may possibly equal or exceed the number of receptors required for "correct" activation by rare foreign antigens with high-affinity interactions. Thus, it is not immediately obvious how T cells simultaneously achieve the necessary high sensitivity and high selectivity for antigen recognition.

Here I outline a model for TCR activation based on "kinetic proofreading" (3–5). Initially developed to explain the remarkable accuracy of DNA replication and protein synthesis, kinetic proofreading models posit that the mechanistic complexity of these processes, which superficially appears unnecessary and even wasteful, is in fact responsible for their accuracy. In each model, two or more independent substrate-recognition events combine to enhance fidelity beyond that which would result from the relatively small difference in binding energy between a correct and incorrect interaction. The increase in fidelity results from the more frequent use by incorrect substrates of nonproductive, but energy-consuming, "discard" pathways. An essentially equivalent way of looking at such models is that the presence of energy-utilizing intermediate steps introduces a delay between substrate binding and the enzymatic reaction. As a result, incorrect substrates, with a high off rate, will only rarely remain bound long enough to react.

General Model

As in the classic examples of kinetic proofreading, a signal is not immediately generated when a ligand binds to a receptor that utilizes a tyrosine kinase in signaling. Instead, several intermediate steps ensue, typically beginning with receptor dimerization. Each receptor next phosphorylates its partner on several tyrosine residues (6, 7), which then provide docking sites for proteins containing *src* homology 2 (SH2) domains. Typically, such proteins will then be phosphorylated themselves, potentially leading to binding by additional SH2-containing proteins. A large signal transduction complex can thus form at the plasma membrane. Tyrosine phosphorylation itself, allosteric effects of binding, or membrane localization may be responsible for functional activation of its component enzymes (8).

Thus, a time delay separates initial binding from the output, as several enzymatic steps must occur at such receptors before second messengers are generated and disseminated to the rest of the cell. As a consequence, short-lived nonspecific complexes should usually fail to signal before dissociating. The total quantity of signal generated from nonspecific complexes, which rapidly turn over, would be expected to be much less than from the same steady-state concentration of more stable, specific complexes. According to the hypothesis presented here, a reduction in the basal level of activation and an increase in selectivity result from the requirement for several thermodynamically irreversible steps between ligand binding and generation of a signal.

The hypothesis proposes the following. (*i*) The initial specific or nonspecific ligand–receptor complex C_0 is converted through a series of intermediates C_i to an active complex C_N; many of these steps are energy-requiring and typically involve tyrosine phosphorylation. Other steps may involve recruitment of additional components to the complex. (*ii*) Dissociation of the complex leads to reversal of the modifications, for example, through the action of phosphatases. A cycle of association and dissociation therefore results in the "waste" of metabolic energy. (*iii*) The rate of dissociation of nonspecific complexes is sufficiently high that dissociation almost always occurs before the nonspecific complex can be activated and generate signals.

Conversion of the components of the complex back to their unmodified forms (the second assumption above) requires dissociation of phosphotyrosine–SH2 interactions; consistent with the model, these interactions have a high dissociation rate *in vitro* (9, 10). Receptors not bound in a complex may be preferentially accessible to enzymes (e.g., phosphatases) that reverse the modifications. However, receptors that have dissociated from a nonspecific complex are unlikely to reassociate rapidly. Thus, the rates of the enzymatic reactions that reverse

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Abbreviations: APC, antigen-presenting cell; TCR, T-cell receptor; SH2, *src* homology 2; MHC, major histocompatibility complex.

the activation steps need not differ between bound and free receptor molecules and may be relatively slow. Thus, of the three assumptions above, the last is the most uncertain.

To provide a quantitative illustration of this effect, a large number of assumptions and simplifications must be made since the relevant rate constants are not known; in fact, not all of the proteins involved in TCR signaling have been identified. For mathematical simplicity, let us assume that the intermediate steps are of equal rate and that they occur in an obligatory order (see Fig. 1). Let us initially also assume that the dissociation constant is the same for complexes at all stages of modification. Let k_1 = the association rate constant; k_{-1}, the dissociation rate constant; k_P, the rate constant for each of the steps of phosphorylation or other modification. We assume that only the fully modified complex can generate the most important signaling molecules. The possibility that certain intermediates may generate a distinctive signal is discussed below. Let $\alpha \equiv k_P/(k_P + k_{-1})$. α equals the likelihood that a given modification step will occur before the complex dissociates. At steady state, $[C_i] = [C_{i-1}]\alpha = [C_0]\alpha^i$ for $i < N$. $C_N = [C_0]k_P\alpha^{N-1}/k_{-1}$. Let C_{total} be the total concentration of a particular complex C.

$$C_{total} = [C_0]\left(\alpha^{N-1}\frac{k_P}{k_{-1}} + \sum_{i=0}^{N-1}\alpha^i\right) = [C_0]\left(1 + \frac{k_P}{k_{-1}}\right).$$

Thus, the fraction of complexes in the active form equals

$$\frac{[C_N]}{C_{total}} = \alpha^N. \qquad [1]$$

In the case of the TCR–MHC interaction, we will assume here and throughout that the association constant is independent of the nature of the peptide but that the dissociation constant varies. The term "affinity" will be used somewhat broadly to include the influence of other proteins, such as coreceptors, on the stability of MHC–TCR complexes. Let us temporarily assume that activation involves interaction of a single peptide–MHC with a TCR complex. Let [T] be the concentration of free TCR. In this example, let $N = 4$ and $[T] = k_P/k_1$. Suppose that $k_{-1} = 10 \cdot k_P$ for a moderate-affinity self-peptide. Under these conditions, a fraction, 0.091, of the peptide–MHC will be bound, but only $1/(11)^5 = 0.0000062$ will be bound to *active* complexes at steady state. Suppose that for the specific foreign antigenic peptide $k_{-1} = 0.1 \cdot k_P$. Then 0.91 of peptide–MHC molecules will be bound to TCRs and $1/(1.1)^5 = 0.62$ will be bound to active complexes. Thus, under these conditions only a 10-fold difference between specific and nonspecific peptides would be found in the fraction of peptide–MHC bound to the TCR, but a 10,000-fold difference in activation is achieved at the cost of a small loss of sensitivity for the specific ligand.

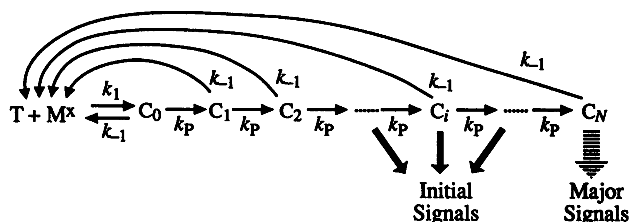

FIG. 1. Proofreading scheme. Nascent complexes C_0, formed from the TCR complex (T) and peptide x-MHC (M^x), must undergo N modifications, each with rate constant k_P, before generating the active complex C_N. At every step, the complex may dissociate with rate k_{-1}, leading to complete reversion of the subunits to their unmodified forms. For full activation, signals must be generated from the final complex C_N. Other signals may be generated from earlier complexes. The relative quantity of these signals will be determined in part by the dissociation constant k_{-1}. In a more elaborate model, the dissociation rate constant varies with the stage of activation, as described in the text.

This scheme is very similar to kinetic proofreading as Hopfield (4) formulated it for protein synthesis and DNA polymerization. As in his model, the requirement for energy-utilizing intermediate steps introduces a time delay before the final reaction; thus, only relatively stable complexes are productive (Fig. 2). The model differs slightly from Hopfield's: (*i*) the final result is an activated complex of enzymes rather than a synthetic product, and (*ii*) since even complete activation is assumed to be reversible, the model is formulated in terms of steady-state values rather than rates of formation.

While the model in theory allows an unlimited increase in selectivity, large increases in cases in which the affinity difference is small would be gained only at the expense of an unacceptably low level of activation from specific stimuli. This problem is alleviated if the dissociation constant k_{-N} for the active form C_N is less than the dissociation constants for the initial and early intermediate stages of the complex. This has the effect of allowing active complexes to accumulate even if a newly formed specific complex has only a small likelihood of receiving the necessary N phosphorylations before dissociating. Let f^x be the fraction of peptide x–MHC molecules bound to fully active TCR complexes. Then

$$f^x = [T]\frac{k_1}{k_{-N}}\alpha^N\left(1 + [T]\frac{k_1}{k_{-N}}\alpha^N + [T]\frac{k_1}{k_P}\sum_{i=0}^{N-1}\alpha^{i+1}\right)^{-1}$$

$$= \frac{[T]k_1k_{-1}\alpha^N}{k_1k_{-N} + [T]k_1[k_{-N} + (k_{-1} - k_{-N})\alpha^N]}. \qquad [2]$$

As a numerical example, let $N = 6$, $k_{-N} = 0.05 \cdot k_{-1}$, and $[T] = k_P/k_1$. If $k_{-1} = 0.5 \cdot k_P$, then the fraction of MHC–peptide in active complexes is 0.554. If $k_{-1} = 5 \cdot k_P$, then the fraction in active complexes is 0.000071. Thus, a 10-fold difference in affinity is manifested as a >7500-fold difference in response while still allowing the majority of specific complexes to propagate a signal. The log–log plots of Fig. 3 illustrate the increased selectivity resulting from the kinetic proofreading model. According to the model, an MHC–peptide with a moderate dissociation constant will spend most of its time bound to a rapid succession of TCRs, many of which will be partially modified before dissociating. The fraction of MHC–

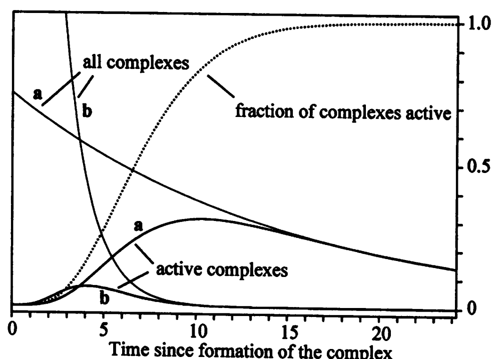

FIG. 2. Activation in relation to the "age" of the complex, the time since its formation, based on Eq. 1. The age distribution of complexes is determined by the dissociation constant k_{-1}. The dotted line shows the fraction of complexes of a given age that are active, based on the assumption that five successive steps are required for activation. Each interval on the abscissa represents one half-life for each modification step ($\ln 2/k_P$). The thin lines represent the age distribution (in arbitrary units) of complexes with either (a) $k_{-1} = 0.1 \cdot k_P$ or (b) $k_{-1} = k_P$. The two thick lines represent the corresponding age distributions for active complexes. Integrating over the age distribution, 62% (a) or 3.1% (b) of complexes are active.

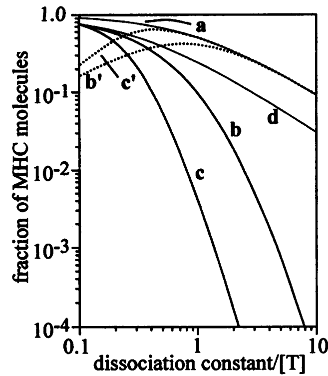

FIG. 3. Calculation of the active fraction f^x with specific values for the parameters, based on Eq. 2; [T] is the concentration of the TCR. k_1 is held constant while k_{-1} varies. The graph begins on the left with the values for specific complexes, for which $k_{-1} = 0.1{\cdot}k_1[T]$. Curve a is the fraction of peptide–MHC bound in the absence of stabilization; for the specific peptide, a fraction 0.909 is bound. In curves b and c, f^x is constrained to be 0.75 for specific interactions, and the number of modification steps $N = 5$. In curve b, $k_{-N} = k_{-1}$ while for curve c, there is 10-fold stabilization of the fully active complex ($k_{-N} = 0.1{\cdot}k_{-1}$). The values of $k_P/(k_1[T])$ that yield these curves are 2.549 and 0.317 for curves b and c, respectively. The corresponding curves b′ and c′ (dotted lines) show the fraction of peptide–MHC bound to TCRs that are not fully active. Curve d is identical to curve a, but shifted over to intersect curves b and c; it is perhaps a more appropriate curve for comparison.

peptide molecules bound to TCRs but not fully activated is quite high for a significant range of values of the dissociation constant (Fig. 3).

If forms bound but not fully activated can send a signal that differs from that generated by the fully active complex, then ligands of different affinities could yield qualitatively different effects through a single receptor. For example, if intermediate stages of modification can send a positive signal, but the most highly modified complexes send a negative signal, activation might be restricted to interactions of moderate affinity. This possibility may be relevant to thymocyte selection.

Mechanisms of TCR Activation

Receptor–Coreceptor Interaction. The ligand for the TCR on the interacting APC consists of a MHC heterodimer and the antigenic peptide that it binds. T-cell activation typically involves juxtaposition of the TCR itself and a CD4 or CD8 coreceptor that binds the same MHC molecule; the tyrosine kinase Lck is associated with the coreceptor. Lck then phosphorylates the accessory molecules of the TCR (the γ, δ, and ε chains of the CD3 complex and the ζ dimer) within a conserved motif called (among other names) the "antigen recognition activation motif" (ARAM) (11–14). After phosphorylation of both tyrosines within an ARAM, a second tyrosine kinase, ZAP-70, containing two SH2 domains, binds to the phosphotyrosines and is subsequently activated through phosphorylation by Lck. Although the remaining steps are not understood in detail, they appear to require ZAP-70 and lead to activation of some of the enzymes involved in signaling through the classical receptor tyrosine kinases. In particular, phospholipase C-γ1, which is of major importance in T-cell activation through the TCR (11), requires phosphorylation at two sites for full activation (15).

Given the large number of energy-utilizing steps in TCR activation, the model predicts that highly selective activation is possible despite a modest difference in dissociation constant between specific and nonspecific peptide–MHC. As described above, this is especially true if the dissociation rate of the fully active complex is decreased in comparison to the initial complex. During the signaling process, Lck may bind through its SH2 domain to phosphotyrosines present on the TCR complex or on recruited proteins, thereby stabilizing the receptor–coreceptor interaction and increasing the avidity for peptide–MHC (16). There is evidence for binding between Lck and tyrosine-phosphorylated ZAP-70 (17).

Receptor Clustering. Signaling is even more complex than described so far since it appears to require clustering of TCR complexes. Because the interface between the T cell and the APC represents only a fraction of the surface of each cell, MHC–peptide molecules with high affinity for the TCR will accumulate at the interface and lead to a corresponding increase in the local concentration of TCRs. Lateral interactions between TCR complexes may then lead to activation (38). However, models in which this effect has a central role in T-cell activation cannot readily explain the sensitivity of the T cell to small numbers of specific peptide–MHC molecules unless the T-cell–APC interface is very small and the affinity for specific antigen is much greater than for nonspecific self peptides.

The mechanism proposed here dovetails with a recent model of receptor signaling based on the MHC class II molecule's crystallographic structure, which revealed an interaction between pairs of heterodimers ("superdimers") (18, 19). In this model, the MHC and the TCR each have a weak tendency to homodimerize. Through cooperativity, stable MHC–TCR binding promotes dimerization of each of the component complexes on the two interacting cells. It was also proposed that CD4 may bind the MHC at the superdimer interface; the associated Lck kinases may be activated by transphosphorylation. Evidence from antibody crosslinking suggests that even TCR dimerization may be insufficient for activation, which may require formation of larger aggregates (20).

In physiological T-cell signaling, receptor–coreceptor interactions and receptor clustering presumably act in concert. In one possible scenario, an initial phase of TCR–MHC interaction (perhaps involving superdimer formation) leads to covalent modifications that stabilize the receptor–coreceptor complex and thereby decrease the off rate of the associated MHC molecule. Additional covalent modifications make the complexes prone to aggregate into larger clusters, which generate the major downstream signals.

The effect of stability of complexes on their state of aggregation is explored in Fig. 4. The curves are based on a simple model in which the rate of dissociation (turnover) of complexes is independent of their state of aggregation. In this model, for A_n, an aggregate of n complexes ($n > 1$), $d[A_n]/dt =$

$$k_{-1}((n+1)[A_{n+1}] - n[A_n]) + \left(\sum_{i=1}^{n/2} k_{\text{on}}^{i,n-i}[A_i][A_{n-i}]\right) - [A_n]\left([A_n]\, k_{\text{on}}^{n,n} + \sum_{i=1}^{\infty} k_{\text{on}}^{i,n}[A_i]\right). \quad [3]$$

$k_{\text{on}}^{i,j}$ is the rate constant for aggregation between clusters of sizes i and j, and k_{-1} is the rate of dissociation of an individual MHC–TCR complex. As can be seen in Fig. 4, the fraction of complexes in large aggregates is highly dependent on the rate of turnover. Complexes containing moderate-affinity peptide ligands would be less likely to sustain the modifications required to make them prone to aggregate and, even if they did, would turn over rapidly and therefore form large aggregates inefficiently. Aggregation in this nonequilibrium model would therefore also involve a form of kinetic proofreading. Small clusters of TCR–MHC complexes (or single superdimers) are predicted to generate signals distinct from those of larger aggregates.

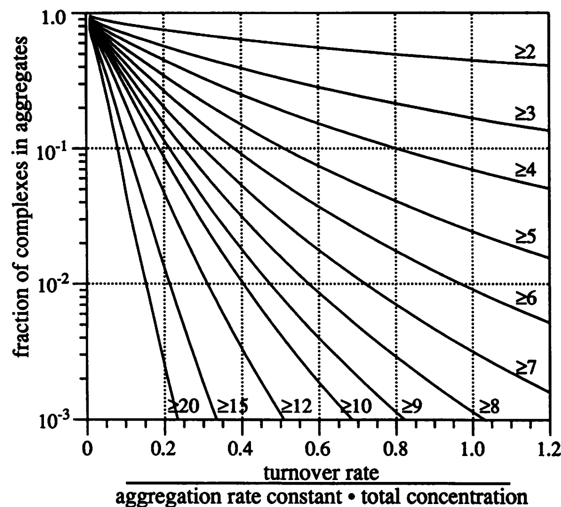

FIG. 4. Dependence of the steady-state degree of aggregation of MHC–TCR complexes on their rate of turnover. Each curve represents the predicted fraction of complexes in aggregates equal to or greater than a particular size. Modeling was performed, based on Eq. 3, with several different formulas for $k_{on}^{i,j}$, the rate constants for aggregation; similar results were found. Let $k_{on} = k_{on}^{1,1}$. In the graphs shown, $k_{on}^{i,j} = k_{on}\frac{\sqrt{i}+\sqrt{j}}{2\sqrt{2}}\sqrt{\frac{1}{i}+\frac{1}{j}}$, where the middle term is intended to approximate the increased "cross section" with increased size of complexes and the last term, the decrease in diffusion rate. The dimensionless ratio $k_{-1}/(k_{on}A_{total})$, where A_{total} is the total concentration of TCR–MHC complexes, was varied in 1.25-fold increments. To permit computation, the maximum size of aggregates was set at 100.

Antagonist Peptides. Several groups have demonstrated that signaling through the TCR is not an all-or-none switch; instead, minor changes in the antigenic peptide can result in a gradient of T-cell responses (21). While peptides unrelated to the normal ligand are without effect, some variants antagonize responses to the normal antigen or have properties of a mixed agonist/antagonist or a partial agonist (18, 22–24). Antagonist activity has been demonstrated from a range of variant peptides; such peptides may retain as few as two of the amino acids involved in specific interaction with the TCR (25) or may differ at only a single residue (26). While the affinities of variant peptide analogs have not been directly measured, correlation of the properties of peptides with the nature and number of the modifications suggests that affinity may be the primary determinant of antagonist or agonist activity.

To explain the ability of some peptides to interfere with a response from agonists, one can envision mechanisms by which antagonist peptides may interfere with the aggregation of TCR–MHC complexes containing higher-affinity peptides, as suggested by others (20). For example, if superdimers exist, their total number would be increased by the addition of a receptor antagonist to cells displaying an agonist peptide, but relatively short-lived superdimers containing only a single high-affinity peptide would form at the expense of superdimers containing two MHC–high-affinity peptide complexes. Such superdimers would turn over rapidly and therefore fail to form large aggregates.

Evidence that small and large aggregates can send opposing signals (27) suggests an alternative or additional explanation for the phenomenon. Addition of a moderate-affinity antagonist peptide to an APC with bound agonist peptide may result in greater TCR binding, but the major increase would be in small clusters (or single superdimers) that send a negative signal or locally interfere with the generation of a positive signal. This effect is highly concentration dependent, so that some variant peptides can signal when present in large quantities. Nevertheless, the existence of negative signaling should enhance T-cell selectivity by reducing the response to moderate affinity ligands, which would generate many small clusters, but few large ones.

Selection in the Thymus. Thymocyte maturation involves positive selection, in which thymocyte survival requires TCR recognition of MHC molecules on thymic epithelial cells, and negative selection, which eliminates cells with high affinity for self-antigens (28). To explain the apparent paradox that TCR engagement can result in such different outcomes, some investigators have proposed a "differential avidity" model, in which the number of occupied receptors determines the response (29). In support of this model, positive or negative thymic selection can be induced by different concentrations of a single peptide acting on a defined transgenic TCR (29, 30). However, the ability of a single peptide to select either positively or negatively appears to be due to variability in the level of expression of coreceptors, and, in fact, thymocytes previously positively selected by a peptide are specifically unresponsive to it (31). Such models also do not offer an explanation for the existence of TCR antagonists.

Others argue that the affinity of the TCR for antigen plays a role in signaling over and above its effect on receptor occupancy (25, 28, 32). For the most part, mechanistically explicit models have not been offered; however, some have suggested that only ligands of high affinity may induce a TCR conformational change required for negative selection (33). Such models do not obviously explain results demonstrating that the level of coreceptor expression can determine whether positive or negative selection occurs (34, 35). The present scheme provides possible mechanisms for an affinity/avidity model, in which thymic selection is determined not only by the total number of bound TCRs but also by the affinity of their interactions.

A number of investigators have emphasized the similarities between negative selection in the thymus and full activation in mature T cells and between positive selection in the thymus and responses of mature T cells to variant peptides that act as antagonists or partial agonists (21, 26, 36). In the hypothesis described here, the mechanisms involved in thymocyte signaling are assumed to parallel those outlined above for mature T cells.

The existence of partial agonist activity in mature T cells implies that certain responses do not require formation of a completely activated complex (37). Thymocytes may require such partial responses for survival but die if significant numbers of TCR molecules are fully activated. Recent evidence suggests that TCR dimers may generate a signal for thymocyte survival, but larger aggregates interfere with this signal or induce negative selection (27). The mechanisms described above may explain how small differences in the stability of TCR–MHC complexes may lead to differing degrees of aggregation and result in qualitatively different signals, with opposite effects on thymocyte survival.

Predictions of the Model. The T-cell's ability to detect very small quantities of short foreign peptides in a sea of self-peptides suggests that the TCR may have a discriminatory ability that is difficult to explain by the level of receptor occupancy alone. The model predicts that a much greater degree of downstream signaling will be found with low concentrations of a high-affinity peptide–MHC than with higher concentrations of a weakly binding peptide–MHC that yields comparable numbers of TCR–MHC complexes. Results with variant peptide antigens are consistent with this prediction. A related prediction, for which there is little current information, is that the difference in affinity of the TCR toward specific peptides versus some self-peptides may be relatively low.

An additional prediction is that MHC binding leads to receptor activation only very slowly, perhaps on the order of seconds. Moderate-affinity, nonspecific TCR–MHC complexes are predicted to have a relatively rapid off rate, giving a half-life also on the order of seconds or less. Thus, the features of this model critical for optimal discrimination include both the complexity of T-cell signaling, tending to slow the rate of formation of an active complex, and low to moderate TCR–MHC affinity, resulting in rapid dissociation of nonspecific complexes. Experimental manipulations that affect the rates of the steps in TCR signal transduction (e.g., partial kinase or phosphatase inhibition or changes in protein expression levels) are predicted to influence whether individual peptides function as agonists or antagonists.

While formulated for T-cell activation, the model may be relevant to other circumstances in which fine discriminations are made between self and nonself, as, for example, by B cells, natural killer cells, and phagocytes. In addition, aspects of the model should apply more generally to other receptors with intrinsic or associated tyrosine kinase activity.

Summary

Specific interaction with <0.1% of the MHC molecules of the presenting cell can activate T cells. The affinity differences toward specific and nonspecific peptide–MHCs may nevertheless be relatively small. T-cell antigen recognition is also quite versatile, for an enormous range of potential foreign peptide–MHC complexes is recognizable by the repertoire of TCRs, which are themselves extremely diverse. The remarkable sensitivity and discriminatory ability of the TCR are obviously of central importance for its role in immunological defense.

Mechanistic complexity is not an inherent feature of cell-surface signaling; the nicotinic acetylcholine receptor, for instance, directly opens an ion channel upon ligand binding, without the necessity of any intermediate energy-utilizing steps. According to the model proposed here, highly selective signaling demands a large number of intermediate steps if specific and nonspecific ligands differ little in dissociation rates. The elaborate construction of a signaling complex at the TCR, involving the sequential recruitment of at least two protein-tyrosine kinases and a large number of enzymatic steps, may enable the T cell to discriminate very precisely between foreign and self-antigens.

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