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Intrinsic disorder in the T cell receptor creates cooperativity and controls ZAP70 binding

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ABSTRACT Many immunoreceptors have cytoplasmic domains that are intrinsically disordered (i.e., have high configurational entropy), have multiple sites of post-translational modification (e.g., tyrosine phosphorylation), and participate in nonlinear signaling pathways (e.g., exhibiting switch-like behavior). Several hypotheses to explain the origin of these nonlinearities fall under the broad hypothesis that modification at one site changes the immunoreceptor's entropy, which in turn changes further modification dynamics. Here we use coarse-grain simulation to study three scenarios, all related to the chains that comprise the T Cell Receptor. We find that, first, if phosphorylation induces local changes in the flexibility of the TCR ζ-chain, this naturally leads to rate enhancements and cooperativity. Second, we find that TCR CD3ϵ can provide a switch by modulating its residence in the plasma membrane. By constraining our model to be consistent with the previous observation that both basic residues and phosphorylation control membrane residence, we find that there is only a moderate rate enhancement of 10% between first and subsequent phosphorylation events. And third, we find that volume constraints do not limit the number of ZAP70s that can bind the TCR, but that entropic penalties lead to a 200-fold decrease in binding rate by the seventh ZAP70, potentially explaining the observation that each TCR has around six ZAP70 molecules bound following receptor triggering. In all three scenarios, our results demonstrate that phenomena that change an immunoreceptor chain's entropy (stiffening, confinement to a membrane, and multiple simultaneous binding) can lead to nonlinearities (rate enhancement, switching, and negative cooperativity) in how the receptor participates in signaling. These polymer-entropy-driven nonlinearities may augment the nonlinearities that arise from, e.g., kinetic proofreading and cluster formation. They also suggest different design strategies for engineered receptors, e.g., whether or not to put signaling modules on one chain or multiple clustered chains.

STATEMENT OF SIGNIFICANCE

Many of the proteins involved in signal processing are both mechanically flexible and have multiple sites of interaction, leading to a combinatorial complexity making them challenging to study. One example is the T Cell Receptor, a key player in immunological decision making. It consists of 6 flexible chains with 20 interaction sites, and exhibits nonlinear responses to signal inputs, although the mechanisms are elusive. By using polymer physics to simulate the T Cell Receptor's chains, this work demonstrates that several of the nonlinear responses observed experimentally emerge naturally due to constraints on the chains that change their entropy. This work points to new avenues to modulate signaling proteins for therapeutics by modulating their mechanical flexibility and spatial extent.

INTRODUCTION

Challenging the tenet that "structure determines function", more than 40% of human proteins contain intrinsically disordered regions longer than 30 amino acids (1, 2). Intrinsically disordered regions appear as linkers between globular domains (3), associated with the cytoskeleton (4, 5), or in signaling networks (6, 7). These regions often include sites for binding and post-translational modification, suggestions they serve a purpose beyond as passive tethers (3, 8, 9). Additionally, the length of the domains themselves influences their interactions, affecting binding kinetics and catalytic performance (10–13).

One example is offered by the T Cell Receptor (TCR) (14–16). TCR has eight subunits, six of which contain intrinsically disordered cytoplasmic tails: ζ (2 per TCR), ϵ (2 per TCR), δ, and γ. The tyrosines on these tails are organized in pairs called ITAMs (Immuno-Tyrsoine Activatory Motifs) and become phosphorylated by a kinase (LCK) upon extracellular ligand binding to the TCR. Phosphorylation of the ITAMs allows a cytosolic signaling molecule, ZAP70, to bind to the phospho-tyrosines (17) and propagate the activation signal (18). Despite lacking structure in the intracellular cytoplasmic tails, the TCR itself endows the signaling pathway with what we term nonlinearities, raising specific questions:

1) The ζ chains exhibit cooperative phosphorylation (19, 20), which can endow systems with sensitivity (21). A hypothesis for this nonlinearity is in local changes in flexibility upon phosphorylation, as shown in Fig. 1Bii and as observed for other intrinsically-disordered proteins (8, 22). Would this local structuring be sufficient to explain the cooperativity? If so, this may explain why many immune receptors have disordered cytoplasmic tails containing multiple phosphorylation sites (10, 23).

2) The ϵ chain is known to associate with the inner leaflet of the membrane (24, 25), as are many other immune receptor chains including ζ (26), CD28 (27) and BCR (28). This has been hypothesized to sequester the tyrosines to guard against phosphorylation (Fig. 1Biii) before a signal is initiated by an antigen. But, given that tyrosine phosphorylation is one of the first steps of T cell activation, how do the first tyrosines become phosphorylated in order to induce membrane dissociation?

3) Finally, although the full TCR complex has 10 ITAMs, only 6 ZAP70 molecules associate with the receptor at one time (29). What property sets the limits of simultaneous occupation (Fig. 1Biv)?

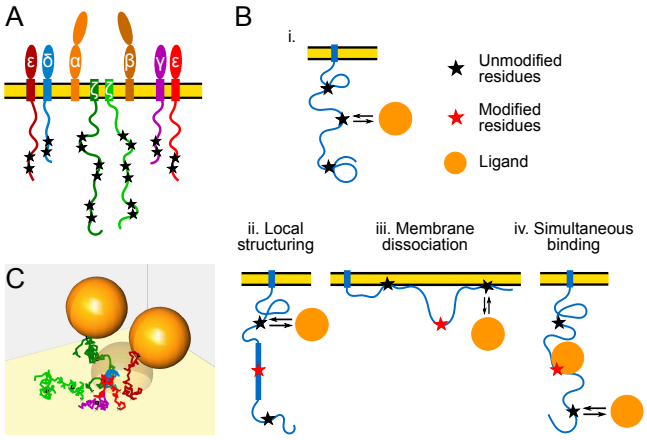

Figure 1: **Possible consequences of membrane-bound disordered protein interacting with ligand.** (A) Cartoon of T cell receptor complex. (B) (i) Interaction of ligand (orange circle) with membrane-bound disordered protein with multiple binding sites (black stars) where (ii) binding causes local stiffening near post-translational modification (e.g. phosphorylation; red stars), (iii) phosphorylation reduces membrane-association of the polymer or (iv) the ligand remains bound while more ligands attempt to bind simultaneously. (C) Snapshot of residue-scale computational model of T cell receptor.

Computational study of the TCR is challenging, not only because it is large and membrane-bound, but the intrinsic disorder introduces a large configuration space that is explored on microseconds timescales, making atomistic molecular dynamics methods computationally expensive. An alternative modeling approach is to use coarse-grain models from polymer physics in which the disordered regions are represented as ideal chains, with each residue represented by a particle. Despite the simplicity of the approach, "residue-scale" models have proven valuable (11, 30, 31), including for formins (12, 32) and kinesins (33). Here we use coarse-grain, residue-scale models to simulate the intrinsically-disordered regions of the T Cell Receptor, exploring the consequences of post-translational modification to answer the above questions.

We find that, first, in agreement with previous theoretical calculations (19), if phosphorylation induces local changes in the flexibility of the chain, this naturally leads to rate enhancements and cooperativity. We find the rate enhancement for a single chain with properties of ζ is around two-fold, with Hill coefficient around 1.8. However, the ultrasensitivity seen is only strong if the kinase is large in molecular size compared to the phosphatase.

Second, we find that CD3ϵ can exhibit a switch-like response to phosphorylation by modulating its residence in the membrane. The observation that both tyrosine phosphorylation and basic residue deletion change membrane residency, under the assumptions of our model, sets a requirement that there is a rate enhancement of at least 10% between first and second phosphorylation events.

And third, we ask how many ZAP70 molecules can simultaneously bind the 10 ITAMs on the 6 chains of a TCR. We find that volume constraints do not limit the number (i.e. there exists a configuration where 10 can fit), but that the entropic penalty needed to bind subsequent ZAP70s leads to a 200-fold decrease in binding rate by the seventh ZAP70, potentially explaining the observation that each TCR has around six ZAP70 molecules bound following receptor triggering. We also explore how binding rates are affected if the 10 ITAMs are distributed on different numbers of chains, a question which may be relevant for artificially engineered receptors. We find that, for parameters similar to the TCR, neighbor-chain interference dominates self-chain interference, so receptors with signaling modules on the same chain would allow faster simultaneous binding.

In all three scenarios, our results demonstrate that phenomena that change an immunoreceptor chain's entropy (stiffening, confinement to a membrane, and multiple simultaneous binding) can lead to emergent behavior with consequences for how the receptor participates in signaling. These polymer-entropy-driven nonlinearities may augment the nonlinearities that arise from, for example, kinetic proofreading and cluster formation. They also suggest different design strategies for engineered receptors, e.g., whether or not to have one chain or multiple clustered chains.

METHODS

Polymer model of disordered protein and globular binding partner

We represent the TCR cytoplasmic tails as ideal disordered proteins using a θ-solvent freely-jointed chain model in which each particle represents a single residue (31). The Kuhn length is $\delta = 0.3$ nm, a parameter value consistent by previous work (30) including on TCR specifically (25). The number of segments N is therefore the number of residues in the tail, i.e., 113, 55, 47, and 45 for $\zeta,\epsilon,\delta,\gamma$, respectively.

The chains interact with LCK and ZAP70, which for generality we refer to as the ligand. This is modeled as an idealized sphere which interacts with the chain. For each ligand, we estimate the volume of the domain of interest and calculate the radius for a sphere with the specified volume. Volume estimates are made using the molecular mass of the domains and an estimated protein density of 1.41 g/cm^3 (34). For comparison, we also estimate volumes from measurements of crystal structures of the domains (LCK - PDB 3LCK; ZAP70 - PDB 2OQ1) in PyMol (35–37). For LCK, we represent the kinase domain as a sphere with radius 2.1 nm (7 Kuhn lengths). For ZAP70, we represent the tandem SH2 domains as a sphere with radius 2.7 nm (9 Kuhn lengths).

For simulations of the full TCR, the relative location of the membrane-anchor for each cytoplasmic domain is estimated from Dong et al. (38) (PDB 6JXR).

We compute quasi-equilibrium statistics of the chain and its ligands using a Monte Carlo (Metropolis-Hastings) Algorithm that generates a distribution of configurations from the canonical ensemble. At each proposed configuration, the Metropolis algorithm computes the energy of the system and accepts or rejects based on energetic preference (e.g., affinity between basic residues and the membrane) and hard constraints (e.g., ligands cannot pass through the polymer).

Calculation of ligand binding rates from occlusion statistics

The dissociation constant of a binding reaction $K_D \equiv k_{\text{off}}/k_{\text{on}}$ is influenced by changes in entropy. If a binding reaction limits the configurational freedom of the binding partners, the entropy, and thus the free energy, is reduced. We can compute the change in K_D between two states (e.g., fully phosphorylated compared to dephosphorylated) as (22, 39):

$$\frac{K_{D_1}}{K_{D_2}} = \exp\left(\frac{\Delta G_1 - \Delta G_2}{k_B T}\right) \tag{1}$$

$$= \exp\left(\frac{(E_1 - TS_1) - (E_2 - TS_2)}{k_B T}\right) \tag{2}$$

$$= \exp\left(\frac{(k_B \ln(\frac{\Omega_2 P_2}{\Omega_2})) - (k_B \ln(\frac{\Omega_1 P_1}{\Omega_1})}{k_B}\right) \tag{3}$$

$$= P_2/P_1 \tag{4}$$

where $G_j = E_j - TS_j$ is the free energy of binding in a given state, $S_j = k_B \ln\Omega_j$ is the entropy of binding, Ω_j is the number of microstates and P_j is the probability in the canonical ensemble that the configuration allows for binding. Since our interest in this work is the influence of entropic changes, we assume $\Delta E_1 = \Delta E_2$, i.e., the change in energy due to ligand binding is the same, regardless of conformation. Define P_{occ} as the probability that the region of space needed by the ligand is occupied by some of the polymer, or another steric barrier. Then, $P_{\text{occ}_j} = 1 - P_j$, and $K_{D_1}/K_{D_2} = \left(1 - P_{\text{occ}_2}\right)/\left(1 - P_{\text{occ}_1}\right)$. Although entropic forces could also impact unbinding of the polymer, we assume this influence to be negligible compared to the change

in k_{on}. Therefore, we assume that the change in K_D manifests as a change in k_{on}. This leads to the final simple equation for attachment rates, $k_{\text{on}} = P_{\text{occ}} k^0_{\text{on}}$, where we refer to k^0_{on} as the free-space (i.e., no occlusion) binding rate.

Simulation of multi-step processes from individual rates

Given the binding rates k_{on} from above, we use a multi-step Gillespie algorithm to investigate the rate-dependent behaviors of both irreversible and reversible (de)phosphorylation reactions. A matrix of occlusion probabilities, to each site in each phosphorylation state, is created from the Metropolis simulations. Catalysis of the phosphorylation or dephosphorylation event is assumed to be fast compared to binding.

For single-direction simulations, e.g., just phosphorylation, we make two calculations: 1) the probability of a specific sequence of irreversible (de)phosphorylation, and 2) the sequence-weighted average binding rate to transition between phosphostates (e.g. from one to two total phosphorylations). At the end of each run of the multi-step process, when all sites are modified, we record the event sequence and the times to transition between each step. Probabilities of each sequence are computed based on the total iterations of the algorithm. The path-weighted average binding rates are calculated as the inverse of the average transition time for a specific step. In other words, transitions between two states that are more likely are weighted higher. In the Supplemental Material, we show average binding rates that are not weighted by the probability of their path.

For simulations of reversible reactions, we do the same, except we run until the system reaches a steady state ($\sim 10^6$ events), and then calculate the average number of phosphorylated sites.

Membrane interactions

Polymer-membrane interactions are modeled as a potential acting on each segment of the chain. We consider three groups of residues: tyrosines, phosphotyrosines, and basic residues. Each experiences a potential summarized in Fig. 5A.

Basic residues are needed to create membrane-association (26). This implies an attractive force, which we model with a piecewise parabolic potential with depth E_{B0} (40, 41),

$$E_{\text{Basic}}(z_i) = \begin{cases} k_{PC} z_i^2 - E_{B0} & z_i < \sqrt{\frac{E_{B0}}{k_{PC}}} \\ 0 & z_i \geq \sqrt{\frac{E_{B0}}{k_{PC}}} \end{cases} \tag{5}$$

Tyrosine phosphorylation is sufficient to dissociate the polymer from the membrane (26). We therefore model phosphorylated tyrosines as having a repulsive interaction with the membrane,

$$E_{Y_p}(z_i) = E_{P0} e^{-z_i/z_{\text{Debye}}}, \tag{6}$$

where the Debye length describes the length scale of electrostatic interactions. For cytoplasm, $z_{Debye} = 1$ nm and $\sqrt{E_{B0}/k_{PC}} = 1$ nm consistent with (31). A soft wall constraint

$$E_S(z_j) = \begin{cases} k_S z_j^2 & z_j < 0 \\ 0 & z_j \geq 0, \end{cases} \tag{7}$$

with $k_S = 0.05 k_B T$, is applied to the unphosphorylated tyrosines and remaining amino acids.

RESULTS

Site difference driven by position along chain

The residue-scale polymer model we developed simulates the T Cell Receptor as it explores its configurations, along with binding partners that we generically refer to as ligands. An example is shown in Fig. 1C and Supporting Movie SM1.

In the present section, we simulate only the ζ-chain. We first assume that phosphorylation does not change the polymer properties of the chain. We calculate the binding rate to each of the six tyrosines. The entropy calculations result in a relative rate from every possible reaction phosphostate, shown as the left columns in Supporting Material Fig. S1, to every phosphostate with one more phosphorylation, in the right column. From these, we obtain the phosphorylation sequences shown in Fig. 2 (green bars, left most bar in each set) and Fig. 3 (pink curve, lowest in both panels).

Rate differences imply emergent preference of phosphorylation sequence

There are six binding sites on the ζ-chain, offering 720 (6 factorial) possible sequences for phosphorylation. Using a Gillespie algorithm in conjunction with the binding rates found above, we compute the probability of (de)phosphorylating in each

sequence. For simplicity, Fig. 2 A.i,B.i (green bars) shows only two extreme sequences: where (de)phosphorylation occurs membrane proximal-to-distal (123456) and membrane distal-to-proximal (654321). For membrane-bound ζ-chains, there is an approximately 10-fold increase in the probability of binding membrane distal-to-proximal compared to membrane proximal-to-distal for both the kinase and phosphatase. This suggested to us that disordered domains in the presence of a membrane can have an emergent preference of phosphorylation sequence.

To test this hypothesis, we simulate an unphosphorylated, cytosolic (i.e., no membrane) ζ-chain. Surprisingly, we found rate differences also persist without the presence of a membrane. Simulations of a cytosolic ζ show reduced preference for phosphorylating membrane distal-to-proximal, in agreement with intuition. However, phosphorylating membrane distal-to-proximal is still 2-fold more likely than phosphorylating proximal-to-distal (Fig. 2 A.ii,B.ii, green bars). When we re-simulate cytosolic ζ-chain, but now with its six tyrosines spaced evenly along the amino acid sequence, there is no preference for phosphorylating membrane distal-to-proximal compared to proximal-to-distal (Fig. 2A.iii,B.iii, green bars). Thus, the emergent preference arises solely from the locations of the tyrosines in ζ-chain. The membrane-distal tyrosine is twelve amino acids away from the C-terminal while the membrane proximal tyrosine is twenty-one amino acids away from the membrane. The extra amino acids between the first tyrosine and the membrane-proximal end increases the steric hindrance that binding site experiences. These results suggest that changing binding site locations by as little as 10 amino acids is sufficient to induce a preferential binding sequence.

Local stiffening

One way in which disordered proteins can participate in signaling cascades is to undergo a disorder-to-order transition (8, 22) upon post-translational modification (e.g., tyrosine phosphorylation), becoming locally stiff, as shown schematically in Fig. 1Bii.

Previous experimental-modeling work (19, 20) suggest that the tyrosines on the ζ-chain experience phosphorylation rates that were enhanced by previous phosphorylations. Can this rate enhancement be explained by phosphorylation-induced local stiffening?

Local stiffening modulates binding rates to multi-site disordered domain

In Fig. 3A, we compute the average binding rate of a kinase to the membrane-bound ζ domain in its unphosphorylated state. Here, each average binding rate is weighted by the most likely (de)phosphorylation sequences. (Average binding rates unweighted by their path's probability are shown in Supporting Material Fig. S2.) Without disordered-to-ordered transitions, the average kinase binding rate decreases as more phosphorylations occur. Because there is a natural preference to phosphorylate membrane distal-to-proximal, it is most likely that the membrane-proximal tyrosine will be the last tyrosine phosphorylated. This tyrosine has a lower binding rate, bringing down the average binding rate of the last phosphorylation compared to the first event, when the faster, membrane-distal sites dominate.

However, if phosphorylation introduces enough local stiffening, then a single phosphorylation event creates an overall increase in the average binding rate of the kinase to another tyrosine. This effect increases with total phosphorylations and degree of local stiffening per phosphorylation. For example, if each phosphorylation locally stiffens 11 amino acids ($\sim$1/12 of the polymer length) then the sixth kinase binding event will occur almost two times times faster than the first. (In Supporting Material Fig. S3 we show the results for larger ranges of stiffening.) In agreement with on-lattice simulations (22), local stiffening causes the polymer to be more elongated on average and sample more configurations where the remaining tyrosines are kinase-accessible. Through this mechanism, disordered-to-ordered transitions can increase the binding rate of a kinase to the remaining unphosphorylated tyrosines.

Interestingly, there is a decrease in the average binding rate between the fourth and fifth binding event. To understand this effect, we examine the probability of the identity of the second-to-last binding event in Supporting Material Fig. S4. For membrane-bound ζ-chains, the fifth binding event is most likely to occur at the second tyrosine from from the membrane, Y83. Therefore, the average binding rate is most heavily influenced by the binding rate at the Y83. The dip in binding rate seen there marks a competition between disordered-to-ordered transitions, which tends to make all sites more accessible, versus the increasing difficulty of phosphorylating sites that are highly occluded. Although occlusion by the chain is reduced by disordered-to-ordered transitions, it is not enough to overcome the high membrane occlusion that Y83 experiences. Therefore, since the fifth binding event is dominated by the low binding rate that Y83, the average rate decreases.

While local stiffening at phosphorylated tyrosines enhances the binding rate of kinases to unphosphorylated tyrosines, it could also enhance the binding rate of phosphatases to phosphorylated tyrosines. We therefore explore how dephosphorylation (i.e., loss of local stiffening) impacts the binding rate of phosphatases to the domain in Fig. 3B. For example, if each phosphorylation locally stiffens 11 amino acids, then we assume each dephosphorylation similarly relaxes 11 amino acids. As dephosphorylations occur, entropic flexibility is returned to the polymer. By the sixth dephosphorylation event, the average phosphatase binding rate

is decreased by half.

Binding rate cooperativity creates ultrasensitivity, even in reversible symmetric phosphorylation cycles

To simulate reversible phosphorylation cycles, we explore two models of dephosphorylation: 1) "constant dephosphorylation", in which the phosphatase enzymatic domain is assumed to be small enough that steric effects are insignificant, and 2) "steric dephosphorylation", in which the phosphatase enzymatic domain is large enough that the steric effects we report above significantly influence dephosphorylation.

In the first model, the phosphatase is assumed to be of negligible size, and therefore dephosphorylation occurs at a constant rate at each site. Using the site-specific binding rates determined above for membrane-bound ζ, we calculate the steady state fraction of sites phosphorylated through a Gillespie algorithm, with dose-response curves shown in Fig. 4A. As expected, when no local stiffening occurs, reversible phosphorylation occurs with approximately Michaelis-Menten kinetics. However, as the number of amino acids stiffened per phosphorylation increases, the dose-response curves become steeper. This ultrasensitivity can be quantified as a Hill coefficient, here defined as the maximum logarithmic slope of each curve (see Supporting Material text and Fig. S5 for definition of Hill coefficient and alternative definitions), attaining a Hill coefficient of 1.8 when approximately 1/12 of the chain is stiffened per phosphorylation event. Therefore, the binding rate cooperativity introduced by local disordered-to-ordered transitions can lead to ultrasensitivity in a signaling network.

In the second model, the phosphatase is assumed to experience the same steric constraints as the kinase. We find dose-response curves shown in Fig. 4B. At 1/12 chain local stiffening per phosphorylation, the Hill coefficient is 1.3. Therefore, even under steric dephosphorylation, a reversible system with local disordered-to-ordered transitions is still capable of creating mild ultrasensitivity.

Membrane affinity

Recent evidence (24, 42) suggests a model for T Cell signaling in which, before receptor triggering, the ϵ- and ζ-chains are unphosphorylated and membrane-associated, protecting them from phosphorylation by kinases (Fig. 1Biii), only dissociating from the membrane after triggering, at which time they can become phosphorylated. But TCR phosphorylation is one of the first steps in triggering (23), raising a "chicken-and-egg" the question: how are the first chains phosphorylated?

A possible hypothesis (27, 43–45) to resolve this puzzle is that, prior to triggering, the chain is biased towards the membrane but spends a small-yet-significant time in the cytoplasm, accessible to kinases. Then, upon initial phosphorylation, the bias is shifted towards the cytoplasm, allowing further phosphorylation. This hypothesis suggests a delicate balance between membrane affinity and phosphorylation. In this section, we ask, if phosphorylation controls membrane association, what are the quantitative constraints on the interaction strengths between the chain, the membrane, and the kinase? And what proportion of states are accessible to the kinase before and after initial phosphorylation?

Simplified membrane interaction model can explain both basic residue and phosphorylation effects

Experimental data suggests that ϵ membrane-association prior to TCR triggering has two features: first, that the basic residues in ϵ are required for membrane association, and second, that fully phosphorylated ϵ does not associate with the membrane. We create a simplified electrostatic interaction model, where phosphorylated tyrosines feel a repulsion from the cell membrane and basic residues feel an attraction, shown in Fig. 5B and described by Eq. 5-7. Each potential is parameterized to reproduce the two experimental phenomena. We first look at how strong the basic residue-membrane attraction, E_{B0}, needs to be in order to confine the tyrosines to the membrane. Here, we define tyrosine confinement as when its positional variance is below ((1/e)(range(variance))+min(variance)), as shown in Fig. 5C, inset. We find that the minimum E_{B0} for basic residues to achieve this is 0.5 $k_{\mathrm{B}}T$. In other words, this is the energetic cost to overcome the chain's entropic pull away from the membrane.

We next tune the strength of the phosphorylation potential, E_{P0}, such that tyrosine phosphorylation compensates for the basic residues (i.e. to match the distribution where $E_{\mathrm{B0}} = 0$), shown in Fig. 5D. A priori, we expected that in order for full phosphorylation to be enough to counteract the membrane association created by the basic residues, we would require that

$$E_{\mathrm{P0}} \geq n_B/n_Y \cdot E_{\mathrm{B0}}, \tag{8}$$

where n_B and n_Y are the numbers of basic residues and tyrosines, respectively. Indeed, in Supporting Material Fig. S6, we find that for the ζ chain, this is consistent with simulation. However, for ϵ, our simulations indicate that $E_{\mathrm{P0}} \sim 2k_{\mathrm{B}}T$ is sufficient for its two tyrosines to compensate for the basic residues (Fig. 5D), in contrast to Eq. 8, which predicts a minimum E_{P0} of $3.5k_{\mathrm{B}}T$. We can explain this by examining the distribution of basic residues compared to tyrosines. For ζ, there are both tyrosines and basic residues along the full length of the domain. In ϵ, the two tyrosines are clumped at the membrane-distal end of the domain, with fewer basic residues nearby.

Phosphorylation-modulated membrane association allows some early phosphorylation, and at least a small acceleration for late phosphorylation

Using the parameter constraints found above, we examine how phosphorylation influences binding kinetics for ϵ, shown in Fig. 6A. At the minimum $E_{P0} = 2k_BT$, the average kinase binding rate increases approximately 10% between the first and the second phosphorylation. When E_{P0} is stronger ($E_{P0} \geq 2k_BT$), the average kinase binding rate increases significantly for future phosphorylation events (up to 50% for $E_{P0} = 10k_BT$).

We next investigate dephosphorylation ϵ in Fig. 6B. Interestingly, there is a weak increase in dephosphorylation rates, in contrast to the stiffening model above. We understand this as follows. As more residues become dephosphorylated, more sections of the chain become restricted to the membrane. The remaining phosphorylated tyrosines remain far from the membrane by the repulsive phosphate-membrane interaction, and therefore more accessible to the phosphatase. The cooperative effect of dephosphorylation only produces a small increase (1.15-fold) in binding rate since the membrane still creates a large steric barrier to phosphatase binding.

Investigation of the reversible system, shown in Supporting Material Fig. 8, shows that stronger phosphate-membrane interactions ($E_{P0} > 2k_BT$) cause the dose-response curve to diverge slightly from Michaelis-Menten kinetics under both constant and sterically-influenced dephosphorylation.

Multiple simultaneous binding

One of the first molecular participants in signaling downstream of TCR is ZAP70, a molecule with two SH2 domains that bind phosphorylated tyrosines (18, 19, 29, 46, 47). A recent in vivo experimental study found that approximately six ZAP70 molecules are bound per TCR (29), despite having ten possible binding sites. This raises two questions: Do entropic constraints arising from polymer flexibility prevent more ZAP70 ligands from binding (in addition to possible low occupancy set by dissociation constant)? And, if so, from a design viewpoint, are there advantages to the cell in not using the full array of possible ZAP70 binding sites? To address this question, we simulate binding of a ligand, ZAP70, to the full TCR, when other ligands are already bound, shown schematically in Fig. 1Biv.

Multiple binding to multi-site disordered domain gives rise to negative cooperativity

We calculate the average binding rates of ligands to all six membrane-bound chains of TCR, assuming a given number of previously bound ligands. In this section, rather than a single chain, we simulate all 6 chains anchored to the membrane according to Dong et al. (38), and rather than represent all tyrosines as individual sites, we place a binding site at the center of each ITAM. In other words, in the previous sections, we independently treated Lck and CD45 enzymatic domains interacting with single tyrosines, whereas here we treat ZAP70 interacting with two tyrosines on an ITAM.

First, we ran simulations to see if there is a hard limit to the number of ZAP70 molecules that can bind to the TCR. We find that at our estimate for the size of ZAP70, there are configurations that allow binding a full ten ZAP70 molecules to the 6 chains. Indeed, ten molecules with radius of up to 6.9nm (larger than our estimate of the size of ZAP70) can still fit, as shown in Fig. 7B. Given that the steric limit is not being reached, is there a limit set by entropic effects?

As more ligands are simultaneously bound to the TCR, the average binding rate decreases. For a ligand with size of ZAP70, approximately 2.7 nm radius, the binding rate for the seventh ligand is approximately 200 times lower than the binding rate of the first ligand (Fig. 7Ci). Simulations where the chains are anchored further apart, as shown in Fig. 7Ciii, exhibit significantly less hindrance: the seventh ligand binds at an approximately 10 times lower rate than the first ligand.

In both cases, the rate decrease implies a "negative cooperativity" effect, which could allow TCR to recruit a regulated number of ZAP70 molecules while still maintaining high binding rate. The first few ZAP70 molecules are able to bind to the domain relatively easily, but it quickly becomes prohibitive to bind more. The alternative way of regulating a limit around 6 ZAP70 molecules would be to have only 6 binding sites, but then the total binding rate would be lower (since there would be 4 fewer opportunities to bind).

Optimal distribution of binding sites on multiple nearby chains

The above finding suggests that the chain's entropy influences how readily a multi-chain receptor can become loaded with signaling molecules. This led us to ask: Given ten binding sites, if the goal is fast loading, is it preferable to have 10 different chains with 1 binding site each, or 1 chain with all 10 binding sites (or, perhaps, have the 10 sites distributed on 6 chains, as they are for TCR)? A priori, it could be that the two effects of a nearby membrane and ligands bound to neighboring chains "in trans", together, prohibit binding, leading to a preference for concentrating the sites on fewer chains. Alternatively, it could be that other molecules bound "in cis" on the same chain prohibit binding, leading to a preference for sites that are distributed

across many chains.

We simulate 10 sites (for a ligand approximately the size of ZAP70) distributed on 1, 2, 3, 5, 9 or 10 chains, as shown in Fig. 8A. The chains are assumed to each be anchored to the membrane on the perimeter of a circle. The radius of the circle is either 1.5nm, similar to the spacing found in the crystal structure of the TCR (38), or wider at 5nm. We report the average rate of the 6th binding event.

We find that, if the chains are in the narrow configuration, fastest binding occurs when the sites are all on a single chain (Fig. 8Bi). This result holds over a wide range of spacings between the sites on the chain. When the chains are anchored further apart, we find that the fastest binding is achieved when the ten sites are distributed across ten chains (Fig. 8Bii), in agreement with intuition. These results suggest a consideration for engineered receptors: There are significant rate differences obtained by having the same signaling modules on multiple versus single chains. Specifically, for the base separation distance estimated for TCR, we find that it is beneficial to have the sites on the same chain to minimize neighbor interference.

For a receptor with 10 binding sites, the sixth binding event can happen in 1260 ways (= $5 \times$ (10choose5)). Interestingly, we find that the rates of these 1260 configurations can have multimodal distributions. In Supporting Material Fig. S10, we explore how this multimodality arises.

DISCUSSION

Multisite modification of signaling molecules leads to a high combinatorial complexity that is challenging to study. Since there are 10 ITAMs on a TCR, to study the dynamics of its phosphorylation, we were required to simulate ~1000 (= 2^{10}) binding states and ~3.6 million (= 10 factorial) possible sequences of phosphorylation. Other explorations in this work have similar combinatorial complexity, for example, the six tyrosines on CD3ζ imply 64 binding states and 720 sequences. Furthermore, a major opportunity of computational simulation is its ability to simulate counter-factual parameters, for example larger or smaller ligand radii, and ITAMs arranged on different numbers of chains, since these counter-factuals provide insight into the real parameters. The coarse-grain modeling approach we use here — representing structured domains as simple rigid bodies and disordered regions as simple polymers, and membrane interactions with simple potentials — are computationally efficient enough for us to perform such parameter exploration.

While this granularity of modeling omits many details, such as sequence-dependent persistence lengths (48), we join a growing body of modeling efforts (49, 50) at the granularity between particles representing individual proteins and particles representing individual atoms. Many previous studies have used differential equations or Markov chains to model binding to multisite molecules (51–54). Our work provides information on the rate constants that can go into these models.

Multi-site reactions can be classified in two categories: sequential or random. For CD3ζ, evidence for sequential phosphorylation is mixed (52, 55, 56). Our work offers a possible resolution. As opposed to obligate sequential modification, meaning the previous event is required before the next event can occur, we find that multi-site disordered domains can display preferential sequential modification, meaning that previous events reduce the probability of future sequences, but do not prohibit them. Note that the extensive theoretical studies of the consequences of sequential binding (21, 51–54) assumed obligate sequences. In some cases, the sequence is assumed, and the mechanism enforcing it is unclear. In contrast, in our current model, the sequence is an emergent property of the distribution of sites along the chain. We find that the membrane increases the differences in binding rate and therefore amplifies the preference, but it is the positions of the tyrosines along the chain that are the primary drivers.

Previous results suggested that the ζ-chains exhibit cooperative phosphorylation (19). We demonstrate that phosphorylation-induced local structuring of ζ naturally leads to cooperativity, offering a possible explanation for these results. Alternative models to explain the TCR ultrasensitivity include TCR clustering (57) and changes in lipid composition (45, 58, 59). To distinguish these, our model makes the following prediction: If it is local structuring that drives cooperativity, adding extra residues or changing the spacing between ITAMs would alter ultransensitivity.

Prior to T cell triggering, the CD3ϵ domains of the TCR associate with the cell membrane, with the tyrosines primarily embedded in the bilayer (24–26). Post-triggering, they dissociate from the membrane, revealing the tyrosines for phosphorylation (26). Thus, membrane association could act as a switch controlling the activity state of the CD3ϵ chain. However, given that tyrosine phosphorylation is one of the first events in TCR triggering, this hypothesis features a "chicken-and-egg" paradox. A plausible resolution (26) is that the unphosphorylated chain is exploring an ensemble of configurations in which its accessibility to kinases is limited but not totally forbidden. The question, then, is whether this accessibility changes enough between unphosphorylated and semi-phosphorylated states to provide a switch. We find that, to be consistent with the two observations that both basic residues and phosphostate control membrane proximity, phosphotyrosines in CD3ϵ must experience a repulsive potential of approximately $E_{P0} \approx 2k_BT$. However, at this repulsion energy, the semi-phosphorylated state is only $\sim$ 10% more accessible than the unphosphorylated state. To get an increase in accessibility of even 50% would require $E_{P0} \approx 10k_BT$, which is several times larger than typical residue-membrane energies (40). Our results do not preclude the membrane association

switch hypothesis, but do set a requirement for large interaction energies in order for it to hold. One possible mechanism for altering membrane interaction energies includes changes in lipid composition.

As shown in Fig. 7, we find that ten ZAP70 molecules readily fit on a TCR, but that the seventh ZAP70 molecule binds at a rate ~ 200-fold slower than the first, potentially explaining the 6-ZAP70-per-TCR stoichiometry previously reported (29). To clarify the difference between these two forms of steric occlusion, in the first case, we ask whether all microstates (polymer configurations) in the fully-bound macrostate are forbidden, while in the second case, we ask what proportion of microstates are forbidden. Our finding is that a significant proportion of microstates are forbidden, even at ligand sizes where the fully bound macrostate is not forbidden. In other words, entropy limits binding much before volume exclusion forbids it.

These steric effects imply negative cooperativity. Negative cooperativity can endow signaling systems with two features: high turnover of ligands even in high ligand concentration (which might be advantageous if ligands are involved in other reactions), and constant signaling activity in low ligand concentration. Similarly, this might reduce the impact of inhibitors, requiring much higher concentrations of inhibitor to completely turn off signaling (60).

Our model predicts that multiple ITAMs on the same chain will bind to ZAP70 faster than the same number of ITAMs on multiple clustered chains, provided the chains have membrane domains with similar relative spacing as the TCR. For larger relative spacing of chains, the relationship flips, and it is better to have ITAMs on multiple chains (Fig. 8B). This is a testable prediction. Furthermore, even without experiments that modify the spacing, our model predicts the fold-change reduction in binding rates for subsequent ZAP70 molecules. The rate of a single ZAP70 binding to an ITAM has been measured (17). We predict that, for example, the 2nd ZAP70 will bind at rate 20% slower (Fig. 7). If realized, this would add to a growing body of work demonstrating the importance of the disordered regions of amino acids *between* catalytically active or post-translationally modified parts of proteins (10, 13).

AUTHOR CONTRIBUTIONS

LC wrote simulation code, carried out simulations, analyzed the data and prepared the manuscript. OD helped design the research and prepare manuscript. JA designed the research, supervised the project and helped prepare the manuscript.

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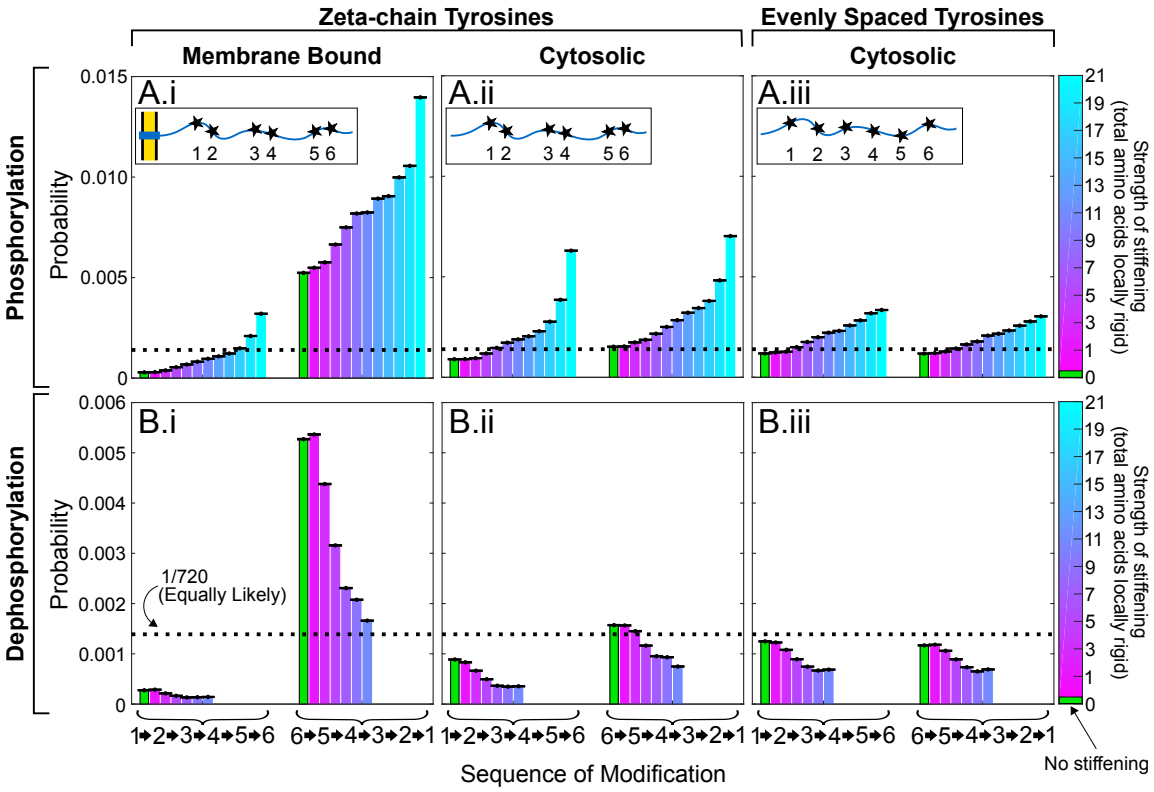

Figure 2: **Binding rate differences imply emergent preference of (de)phosphorylation sequence.** Probability of (A) phosphorylating or (B) dephosphorylating membrane proximal-to-distal (123456) compared to membrane distal-to-proximal (654321). Colors represent simulations of different ranges of local stiffening/unstiffening per binding event. No stiffening (0 stiffening) shown in green. (C) Probability of the second-to-last binding event occurring at a specific site. Each of these is shown for: (i) with a membrane, assuming sites spaced like tyrosines of ζ, (ii) without a membrane, assuming sites spaced like tyrosines of ζ, and (iii) without a membrane, assuming sites spaced evenly along the length of ζ. The black dotted lines indicate the probability if all events were equally likely (A,B: 1/6!; C: 1/6). Error bars (A,B) represent standard error of the mean, treating each Gillespie run as an individual Bernoulli trial. Phosphorylation and dephosphorylation are more likely to occur membrane distal-to-proximal compared to proximal-to-distal for both the membrane bound and cytosolic ζ chain. For cytosolic ζ with evenly spaced tyrosines, both (de)phosphorylation sequences are approximately equally probable.

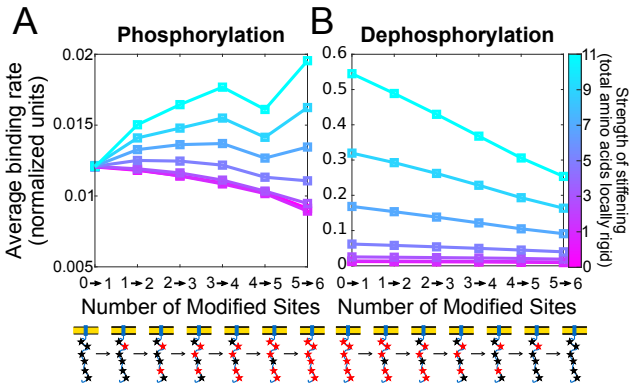

Figure 3: **Local stiffening modulates binding rates to multi-site disordered domain.** (A) Average binding rates of a kinase binding to ζ at different phosphorylation states for varying range of local stiffening (pink: no residues are stiffened; blue: 11 residues are stiffened, 5 on each side of site). The kinase average binding rate increases with each phosphorylation and also with local stiffening range. (B) Average binding rates of a phosphatase binding to ζ at different dephosphorylation states for varying ranges of local un-stiffening per dephosphorylation event. When local stiffening occurs, the phosphatase average binding rate decreases with each phosphorylation and also with local stiffening level. For both (A) and (B), schematic below axis shows example configuration for each phosphorylation state. Unphosphorylated residues represented by black stars, phosphorylated residues are red stars. Kinase and phosphatase radius is 2.1nm. Rates are normalized to the free-space binding rate.

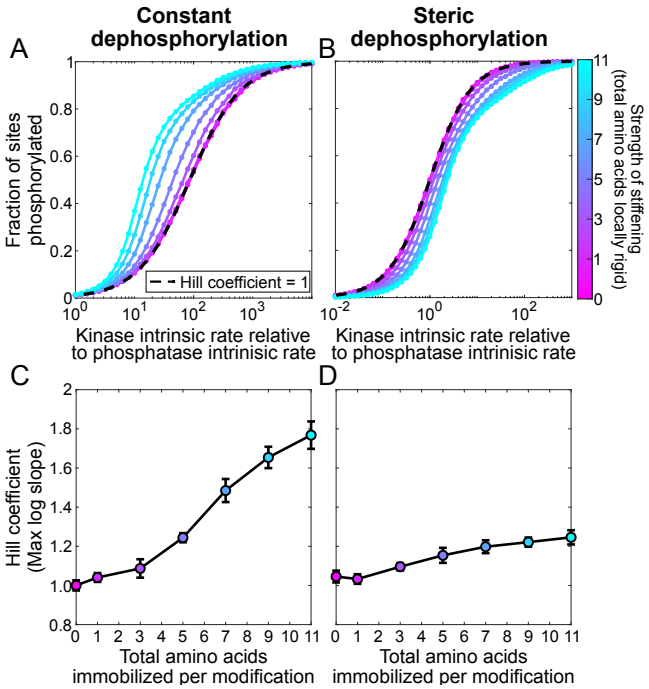

Figure 4: **Emergent cooperativity from binding rate enhancement implies switch-like dose-response, even in symmetric phosphorylation/dephosphorylation cycles.** (A,B) Fraction of sites phosphorylated as a function of the kinase-to-phosphatase activity ratio (measured by the ratio of free-space rates) for different ranges of local stiffening (colorbar), assuming a phosphatase with (A) negligible size or (B) radius of 2.1 nm, equal to the kinase. Black dashed line indicates a linear dose-response, i.e., with Hill coefficient 1. (C,D) Hill coefficients for different ranges of local stiffening per phosphorylation event, assuming a phosphatase of (C) negligible size or (D) radius of 2.1 nm. Hill coefficients calculated from maximum log-log slope of the dose response curves. Error bars indicate root-mean-square error from a cubic polynomial fit to slope. In both cases, the Hill coefficient increases with the range of local stiffening, even when dephosphorylation is assumed to be sterically hindered (D).

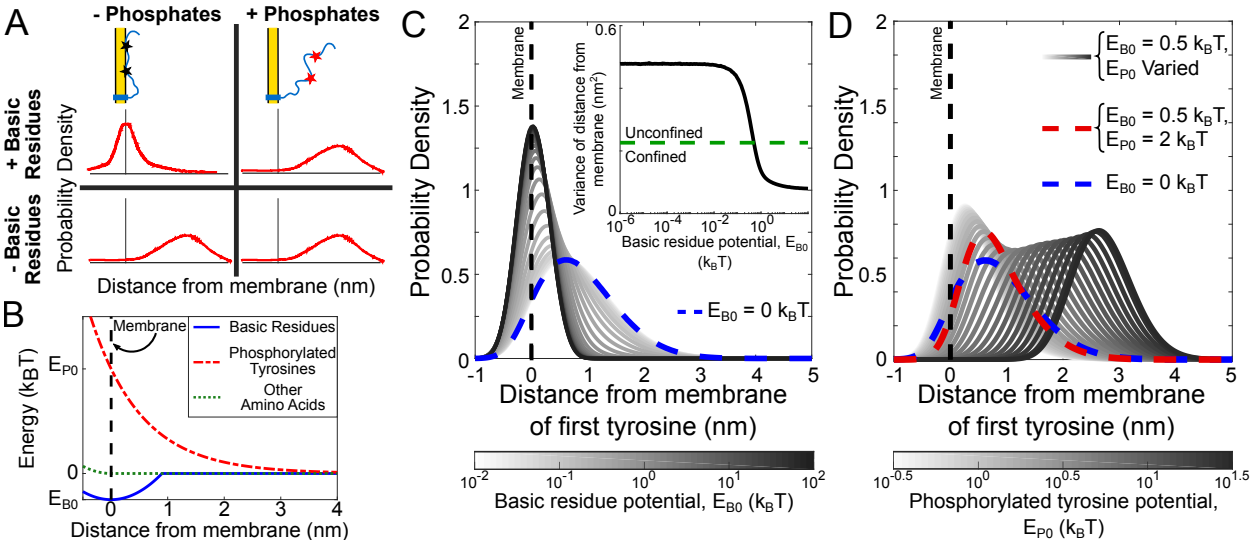

Figure 5: **Simplified membrane interaction model sets constraints on the strength of basic residue attraction and phosphorylation-driven repulsion from membrane.** (A) Schematic diagrams reflecting probability density of tyrosines under different conditions. Based on previous experimental studies, our model must be consistent with the following: Probability density of tyrosines is narrow and close to the membrane for wild type ϵ, but widens and shifts away from the membrane when it is phosphorylated or the basic residues are mutated. (B) Interaction potentials used in simplified model. Basic residues experience an attractive potential of depth E_{B0} near the membrane and experience zero potential one Debye length (~1nm) away from the membrane (blue solid line). Phosphorylated tyrosines experience a repulsive potential with strength E_{P0} (red dashed line). Unphosphorylated tyrosines and all other amino acids are hindered from entering the membrane but otherwise experience no potential (green dotted line). (C) Probability density of the distance from the membrane of the 1st tyrosine of ϵ, assuming no phosphorylated tyrosines, for varying strengths of the basic residue potential, E_{B0} (white: low; black: high). The tyrosine moves close to the membrane as E_{B0} is increased. Probability density when there is no basic residue potential ($E_{B0} = 0\ k_BT$) is shown as blue dotted line. (Inset) Variance of probability density of 1st tyrosine over range of basic residue potential strengths. Green dashed line shows the characteristic $E_{B0} = 0.5k_BT$ required to confine the tyrosine to the membrane, defined in the text. (D) Probability density of the location of 1st tyrosine assuming all tyrosines are phosphorylated, for $E_{B0} = 0.5\ k_BT$ (the value required to confine the tyrosine to the membrane assuming no tyrosines are phosphorylated), and varying phosphorylated tyrosine potential strength, E_{P0} (low - white, high - black). Probability density when $E_{P0} = 2\ k_BT$ is shown as red dashed line, reflecting the E_{P0} value needed to approximately return to the distribution when $E_{B0} = 0\ k_BT$ (blue dashed line).

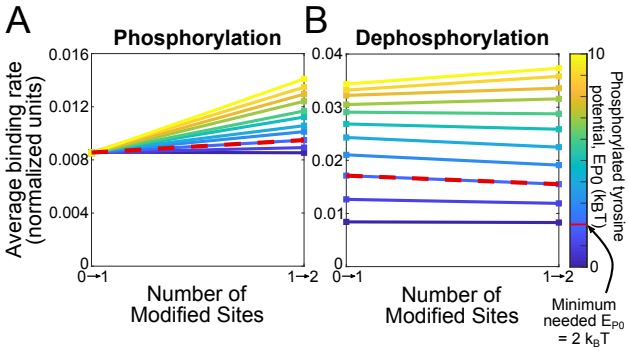

Figure 6: **Phosphorylation-driven modulation of membrane association allows tyrosines to operate as a regulatory switch yet still remain accessible in the "off" state.** Average binding rates of (A) kinase and (B) phosphatase binding to ϵ at different (de)phosphorylation states for varying strengths of phosphorylated tyrosine potential (E_{P0}) (blue: weak; yellow: strong). Both kinase and phosphatase are assumed to have radius 2.1 nm. Rates are normalized to the free-space binding rate.

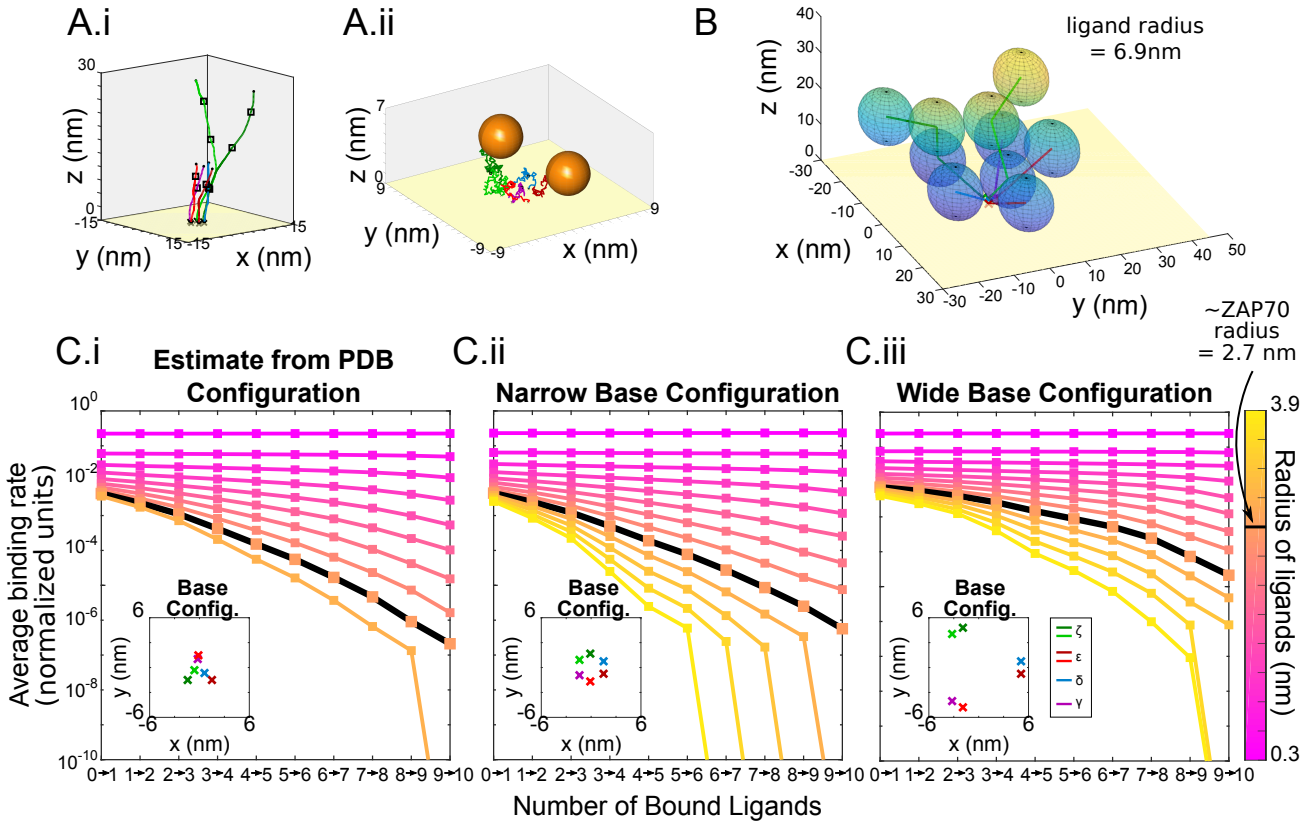

Figure 7: **Simultaneously-bound disordered regions have a reduced binding rate as they become more crowded, even before the steric limit.** (A) Snapshots of simulated TCR subunits and binding sites (black squares) (Ai) in extended conformation and (Aii) at equilibrium with two bound ligands. (B) Simultaneous binding of full TCR is not sterically prohibited at physiological ligand radii. View of single TCR configuration with ten bound ligands where each ligand has radius of 6.9 nm. In this configuration, ligands do not interact with membrane (yellow) or other ligands. Colored lines represent TCR cytoplasmic domains. (C) Average binding rates to TCR against number of ligands bound for varying ligand size (color bar; pink: small; blue: large) and TCR subunit configuration (Ci) Estimated from PDB structure of TCR, (Cii) 1.5nm, and (Ciii) 5nm.

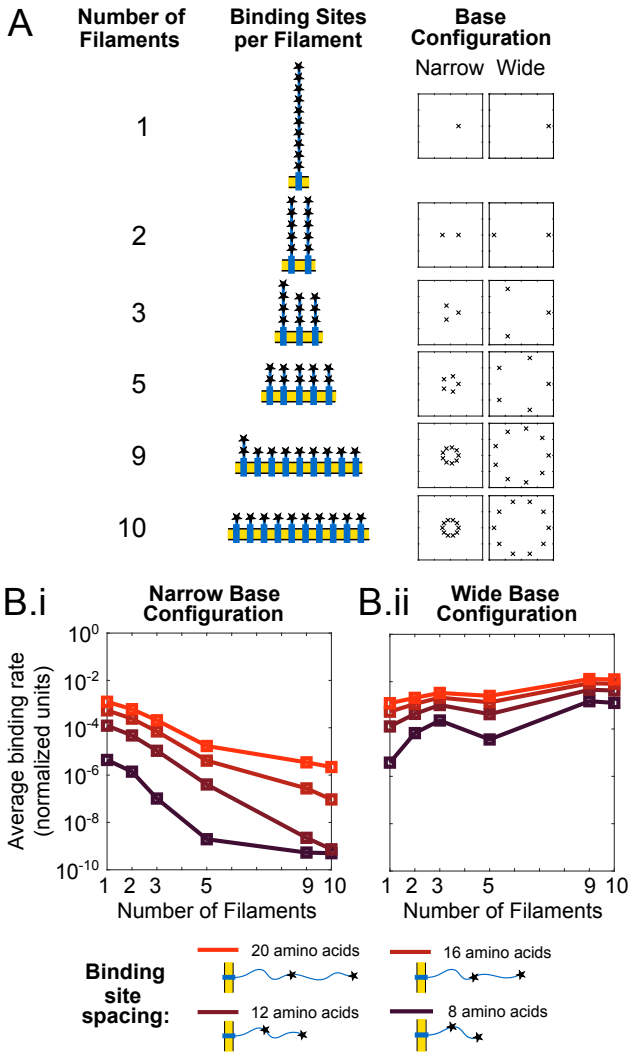

Figure 8: **Optimal distribution of ten binding sites on multiple chains is dependent on membrane spacing of subunits.** (A) Schematic for distribution of 10 binding sites on multiple chains. For each, chains are distributed evenly on a (1) narrow circle of radius 1.5 nm, and (2) wide circle of radius 5 nm. (B) Average path-weighted binding rates of sixth binding event to constructed domain against number of chains in domain (color bar; dark red: short spacing between binding sites; bright red: long spacing) and subunit configuration (Bi) 1.5nm radius, (Bii) 5nm radius. Simulations sweep over binding site spacing from 8 to 20 amino acids, indicated by color. Ligand radius is 2.7 nm.