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A Detailed Mathematical Model Predicts That Serial Engagement of IgE–FcεRI Complexes Can Enhance Syk Activation in Mast Cells

Ambarish Nag,*[,1] Michael I. Monine,*[,2] Michael L. Blinov,† and Byron Goldstein*

The term serial engagement was introduced to describe the ability of a single peptide, bound to a MHC molecule, to sequentially interact with TCRs within the contact region between a T cell and an APC. In addition to ligands on surfaces, soluble multivalent ligands can serially engage cell surface receptors with sites on the ligand, binding and dissociating from receptors many times before all ligand sites become free and the ligand leaves the surface. To evaluate the role of serial engagement in Syk activation, we use a detailed mathematical model of the initial signaling cascade that is triggered when FcεRI is aggregated on mast cells by multivalent Ags. Although serial engagement is not required for mast cell signaling, it can influence the recruitment of Syk to the receptor and subsequent Syk phosphorylation. Simulating the response of mast cells to ligands that serially engage receptors at different rates shows that increasing the rate of serial engagement by increasing the rate of dissociation of the ligand–receptor bond decreases Syk phosphorylation. Increasing serial engagement by increasing the rate at which receptors are cross-linked (for example by increasing the forward rate constant for cross-linking or increasing the valence of the ligand) increases Syk phosphorylation. When serial engagement enhances Syk phosphorylation, it does so by partially reversing the effects of kinetic proofreading. Serial engagement rapidly returns receptors that have dissociated from aggregates to new aggregates before the receptors have fully returned to their basal state. ***The Journal of Immunology*****, 2010, 185: 000–000.**

The terms serial triggering and serial engagement entered the immunological lexicon when Valitutti et al. (1, 2) reported that within the contact area between an APC and a T cell, a few antigenic peptides bound to MHC molecules (pMHCs) mediated the internalization of hundreds of TCRs. Itoh et al. (3) confirmed this result and showed that equating a pMHC engagement with an internalized TCR undercounted the number of serial engagements. They observed that TCR internalization closely followed the extent of ζ-chain phosphorylation. Thus, pMHC–TCR engagements that resulted in partial ζ-chain phosphorylation, but not TCR internalization, were not counted.

The observation that TCRs undergo serial engagement, coupled with the kinetic proofreading model for cell signaling (4, 5), led to the prediction that for T cell activation there should be an optimal range of $t_{1/2}$s for the pMHC–TCR bond (6). The basic idea of kinetic proofreading is that for a TCR to become activated, it must remain bound to a pMHC long enough for a set of biochemical modifications to occur. If the pMHC dissociates from the TCR before the necessary modifications have been completed, signaling is frustrated and activation is not achieved. For a T cell to produce a measurable response, multiple TCRs must be activated. Therefore, at low pMHC density, a single pMHC must trigger many TCRs before it diffuses out of the contact region. If the pMHC dissociates too rapidly, it will encounter many TCRs but activate few, whereas if it remains bound too long, it will activate those it encounters, but encounters will be rare. The recognition that the pMHC–TCR bond $t_{1/2}$ has opposite effects on kinetic proofreading and serial engagement led to the proposal that to achieve an optimal rate of TCR activation, there should be an optimal $t_{1/2}$, or equivalently an optimal dissociation rate constant k_{off} (6, 7). Although some studies have found an optimal $t_{1/2}$ for T cell activation (8–10), there are other results that are inconsistent with this model (reviewed in Refs. 11 and 12). For example, TCRs have been engineered with high-affinity TCR–pMHC interactions and long $t_{1/2}$s so that their cognate peptides undergo little serial engagement, yet these TCRs were efficiently stimulated by these peptides (13, 14).

Although there has been a considerable effort to unravel the role of serial engagement of TCRs in activating T cells, the role of serial engagement of other multichain immune recognition receptors in cell activation has received little attention. This is perhaps surprising, because avidity, often a manifestation of serial engagement and a property of soluble multivalent ligands, was introduced in immunology >50 y ago to distinguish between the binding properties of an Ab and its monovalent Fab fragment (reviewed in Ref. 15). It was observed that an IgG, at low concentrations, could bind to a surface containing multiple binding sites (epitopes) with an apparent affinity that was orders of magnitude greater than the equilibrium constant for binding of one of its Fab sites to an epitope (16–18). Avidity arises when the density of surface binding sites is sufficiently high that multivalent ligands are observed to dissociate from the surface more slowly than their monovalent counter

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Abbreviations used in this paper: LT, Laplace transforms; pMHC, peptide bound to MHC molecule; RBL, rat basophilic leukemia.

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parts, thus exhibiting a high apparent affinity. When a site on a doubly bound IgG dissociates, the epitope it was bound to will diffuse away. If this newly freed site on the IgG binds to another epitope before its second site dissociates, the IgG will remain bound to the surface. Thus, avidity can arise by ligands serially engaging epitopes, walking along the surface from epitope to epitope.

Avidity can arise in a second way that does not require serial engagement. For example, cross-linking IgE–FcεRI complexes on rat basophilic leukemia (RBL) cells with a highly multivalent Ag leads to the rapid formation of large stable aggregates of immobilized receptors (19, 20). This raises the possibility that a site on a bound ligand can dissociate from, and rebind to, the same immobilized receptor many times, effectively increasing its off-rate constant, before all its sites on the ligand become free and the ligand dissociates from the cell. Recent multicolor tracking experiments show that small aggregates of IgE–FcεRI complexes, made up of two to four receptors, remain mobile on the RBL surface even at doses that result in degranulation (21).

A major difficulty in assessing the role of serial engagement of receptors in cell signaling is that the phenomena cannot be studied in isolation. As we will discuss, increasing the ligand's forward cross-linking rate constant, its reverse rate constant, or its valence will increase the rate at which the ligand serial engages receptors, but each of these manipulations will effect receptor activation in different ways. To assess the role of serial engagement in mast cell signaling, we use a detailed mathematical model of the early events triggered when IgE–FcεRI complexes are exposed to a bivalent or trivalent ligand on RBL cells (22). Using this model will allow us to delineate how different alterations in the binding properties of the ligand, that result in the same rates of serial engagement of receptors, can alter receptor activation in different ways.

Mast cell responses mediated by FcεRI are initiated when a multivalent ligand, an allergen for example, aggregates the receptors. Maintenance of the response does not require that new aggregates constantly form. Kent et al. (23) exposed RBL cells briefly to covalently cross-linked oligomers of IgE and then added excess monomeric IgE to prevent further aggregation of receptors. Under these conditions, aggregated receptors continued to signal. Phosphorylation of the β and γ ITAMs, phosphorylation of the protein tyrosine kinase Syk, and the release of histamine-containing granules were sustained for substantial lengths of time after new aggregate formation was blocked. Although serial engagement of IgE–FcεRI complexes is not necessary for mast cell signaling, there remains the question: can serial engagement enhance mast cell response at low allergen concentrations? After deriving an expression for the rate at which a ligand of valence N serially engages receptors on a surface, we will refine this question and then use the model of Faeder et al. (22) to answer it.

Materials and Methods

Bivalent ligands

We use the model of Faeder et al. (22) to simulate the early response of RBL cells to the addition of a reversible bivalent ligand that binds to, and dimerizes, IgE–Fc complexes on RBL cell surfaces. The model consists of a network of 354 distinct chemical species connected by 3680 chemical reactions, 21 rate constants, and 3 concentrations, the surface concentrations of FcεRI and available Lyn and the total concentration of Syk. With the exception of the rate constants that describe the interaction of the bivalent ligand with IgE, k_{+1}, k_{-1}, k_{+2}, and k_{-2}, the parameters used in the simulations are the same as those given in Table I of Ref. (22). In the simulations, we take $k_{off} = k_{-1} = k_{-2}$. A major simplification of the model is that multiple tyrosines on receptor subunits and Syk are lumped into single units so that, for example, ITAMs are either phosphorylated or not phosphorylated. The concentrations of Lyn, Syk, and FcεRI are specific for RBL cells. The interactions among the components of the model (the reaction rules) were specified in the syntax of the second-generation version of BioNetGen, which automatically generates the set of ordinary differential equations that describe the network (24, 25). The open-source software (http://bionetgen.org) uses standard numerical methods to solve the set of ordinary differential equations and obtain the time courses for the 354 chemical species. The outputs are the weighted sums of the appropriate chemical species. For example, activated Syk is the sum of those chemical species that have a single Syk phosphorylated by a Syk and bound to a γ ITAM plus two times the sum of those chemical species that are receptor dimers and contain two such Syk molecules.

Trivalent ligands

We generalize the model of Faeder et al. (22) to simulate the early signaling events triggered by the addition of a symmetric trivalent ligand with three identical binding sites interacting with IgE bound to FcεRI on RBL cells. There is now an additional surface cross-linking reaction involving a trivalent ligand with two of its sites bound cross-linking a third IgE–FcεRI describe by rate constants k_{-3} and k_{+3}. In the simulations, we take $k_{off} = k_{-1} = k_{-2} = k_{-3}$ and $k_{+2} = k_{+3}$. It is assumed that a Lyn bound to a receptor can randomly transphosphorylate any other receptor in the aggregate with the same rates, as it was assumed for the dimeric aggregate. To go from a dimer with two identical binding sites to a trimer with three identical binding sites requires only changing one rule in the BioNetGen input file, the rule that defines the stoichiometry of the ligand and its binding from solution to a receptor on the cell surface. In the model, kinases act on all substrates in their vicinity. The kinase activity in an aggregate is summed so, for example, if three Syk molecules are in an aggregate, the rate of phosphorylation of a Syk by the two adjacent Syk is twice the rate of phosphorylation as when only two Syk molecules are in the aggregate. The BioNetGen files for the dimer and trimer are given in the Supplementary Material. For the trivalent ligand, the model expands to 2954 distinct chemical species connected by 49,948 reactions.

Results

First, we obtain an expression for the rate of serial engagement of a multivalent ligand of valence N binding to free mobile receptors on a cell surface that has a concentration of R free receptors. This expression allows us to choose parameter values that define ligands with different rates of serial engagement. We then use these parameter values to simulate the early response of mast cells to ligands that serial engage receptors at different rates. We use, as our measure of signaling response, receptors that have fully activated Syk associated with their γ ITAMs. Syk becomes partially active upon binding through its two Src homology 2 domains to the doubly phosphorylated γ ITAM (26). In our model, Syk is partially active when bound to the γ ITAM and fully active when it has been transphosphorylated on its activation loop tyrosines by a second Syk (reviewed in Ref. 22). Finally, we use the results of the simulations to evaluate the role of serial engagement in early responses of mast cell signaling.

Serial engagement of a N-valent ligand

The rate of serial engagement equals the number of receptors the ligand encounters from the time, t_a, it forms its first attachment to a cell surface receptor until the time, t_b, its last bond breaks, divided by the time this process lasted, $t_b - t_a$. Fig. 1 illustrates the reactions a pentavalent ligand can undergo once it is initially bound to a receptor on a surface.

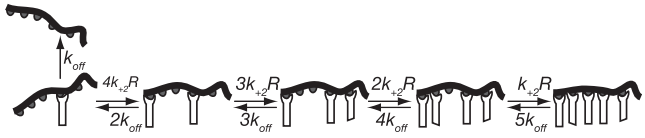

FIGURE 1. The multiple reactions that a ligand of valence five ($N = 5$) can undergo from the time it first binds a cell surface receptor until the last ligand–receptor bond breaks and the ligand leaves the surface. R is the concentration of free receptors. In these reactions, k_{+2} is the single-site rate constant for addition of a receptor to a ligand–receptor complex, and k_{off} is the corresponding reverse rate constant. By taking all of the forward and reverse rate constants to be the same, we have assumed that these rate constants are independent of the bound state of the ligand.

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The mean time an N-valent ligand, that initially has one site bound to a receptor, remains on a surface, when the concentration of free receptors is R, is (27, 28):

$$t_N = \frac{1}{k_{off}}\left[\frac{(1+K_2R)^N - 1}{NK_2R}\right], \quad (1)$$

in which $K_2 = k_{+2}/k_{off}$ is the equilibrium cross-linking constant.

In the *Appendix*, we derive the following expression for n_N, the mean number of receptors an N-valent ligand that initially has one site bound to a receptor serial engages before it dissociates from the cell surface:

$$n_N = \sum_{j=2}^{N}(K_2R)^{j-1}\frac{(N-1)!}{(j-1)!(N-j)!}. \quad (2)$$

The average rate, r_N, an N-valent ligand serially engages receptors is

$$r_N = n_N/t_N. \quad (3)$$

In Table I, we evaluate these expressions for n = 1, 2, 3, 4, and 5 and K_2R = 0.10, 1.0, and 10.0. The parameter K_2R is the product of the surface equilibrium cross-linking constant and the surface concentration of free receptors. Larger values of K_2R favor aggregate formation. Note that for large values of K_2R, as the valence is increased, the number of serial engagements and the mean time the ligand remains bound to the cell rises rapidly, but their ratio, the rate of serial engagement, increases only modestly. From Equations 1–3, it can be shown that the maximal rate of serial engagement achievable by an N-valent ligand when k_{off} is fixed and $k_{+2}R$ is increased is Nk_{off}. When $k_{+2}R$ is fixed and k_{off} is increased, the maximal rate is $k_{+2}R$.

The rates of serial engagement of receptors for bivalent and trivalent ligands

We now focus on bivalent and trivalent ligands interacting with monovalent receptors. From Equations 1–3, we have that the rate of serial engagement of a bivalent ligand is

$$r_2 = 2k_{off}\frac{K_2R}{2+K_2R}. \quad (4)$$

A second way this result can be obtained is to note that one receptor is bound and released by a bivalent ligand in one cycle time t_c so that $r_2 = 1/t_c$. The cycle time is the sum of the mean times for a site on a doubly bound ligand to open and for the singly bound ligand to bind to another receptor [i.e., $t_c = 1/(2k_{off}) + 1/(k_{+2}R)$]. [When the ligand is a pMHC rather than a bivalent ligand, the factor of two on the right hand side of Equation (4) becomes a one (29).] This simple approach for calculating r_2 cannot easily be generalized to ligands with valence >2, because for these ligands, there is >1 recycling time.

From Equations 1–3, we obtain the following expression for the rate of serial engagement of trivalent ligand:

$$r_3 = 3k_{off}\frac{2K_2R + (K_2R)^2}{3 + 3K_2R + (K_2R)^2}. \quad (5)$$

It is easy to see from these expressions that for large values of K_2R, the rate of serial engagement approaches its maximal value of Nk_{off}. We use Equations 4 and 5 to pick the parameter values that characterize the ligands for our simulations.

There are three separate ways to vary the properties of a ligand to enhance serial engagement: increase k_{off}, increase k_{+2}, or increase the valence of the ligand.

1. Increasing k_{off} increases the rate at which the ligand serial engages receptors, but this has the deleterious effect of enhancing kinetic proofreading by reducing the time a receptor remains in an aggregate. Once a receptor leaves an aggregate, unless it can rapidly enter into a new aggregate, all modifications it has undergone will be reversed, and it will return to its basal state (30, 31).

2. Increasing k_{+2} reduces the time it takes a free site on a ligand in a ligand–receptor complex to bind to a new receptor. This enhances the effects of serial engagement by making it more likely that a ligand–receptor complex will form a new dimer before the ligand dissociates and the receptor returns to its basal state.

3. Increasing the valence of the ligand has a similar effect to increasing the forward rate constant. For an N-valent ligand bound through a single site to a receptor, the forward rate of binding a second receptor and forming a dimer is proportional to $(N - 1)k_{+2}$. However, increasing the valence introduces other effects as well. A higher valent ligand can form larger aggregates and large aggregates are more effective at signaling than smaller aggregates, even when the ligands are oligomers of IgE that do not undergo serial engagement (32). In RBL cells, Lyn, the kinase that is responsible for ITAM phosphorylation, is limiting (33, 34). Therefore, larger aggregates have a higher probability than receptor dimers of containing a receptor associated with Lyn, and a Lyn in an aggregate can serial phosphorylate all its neighboring receptors in the aggregate.

Simulations of mast cell responses to bivalent and trivalent ligands for different rates of serial engagement

We use the model of Faeder et al. (22) to assess the role of serial engagement in mast cell signaling (see *Materials and Methods*). The predicted outputs of the model that we use in our study are the concentrations (number per cell) of the phosphorylated β and γ ITAMs of FcεRI, and the concentration of Syk bound to a phosphorylated γ ITAM and transphosphorylated on its activation loop

Table I. *The influence of the parameter* K_2R *(the product of the equilibrium cross-linking constant and the free receptor concentration) on the mean time an N-valent ligand remains bound to the surface* (t_N)*, the mean number of receptors the ligand engages while it remains bound to the surface* (n_N)*, and the rate the ligand serial engages reptors* (r_N)

	K_2R = 0.1			K_2R = 1.0			K_2R = 10.0		
N	$k_{off}t_N$	n_N	$r_Nk_{off}^{-1}$	$k_{off}t_N$	n_N	$r_Nk_{off}^{-1}$	$k_{off}t_N$	n_N	$r_Nk_{off}^{-1}$
2	1.05	0.10	0.095	1.50	1.00	0.67	6.00	10.0	1.67
3	1.10	0.21	0.190	2.33	3.00	1.29	44.33	120.00	2.71
4	1.16	0.33	0.285	3.75	7.00	1.87	366.00	1330.00	3.63
5	1.22	0.46	0.380	6.20	15.00	2.42	3221.00	14640.00	4.55

Increasing K_2R increases the rate at which a ligand cross-links a receptor compared with the rate at which a receptor dissociates from the ligand.

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tyrosines by a Syk bound to an adjacent receptor. We refer to Syk bound to the γ ITAM and phosphorylated on its activation loop tyrosines as fully activated Syk or phosphorylated Syk. Using Abs specific for phosphorylated tyrosines in the activation loop of Syk, it has been shown that upon FcεRI aggregation, these tyrosines phosphorylated, and this phosphorylation depended on the kinase activity of Syk (35). The presence of these activation loop tyrosines is necessary for Syk-mediated propagation of FcεRI signaling (36).

Increasing serial engagement by increasing k_{off} reduces Syk activation

In Fig. 2, we compare the predicted levels of β and γ ITAM phosphorylation and full Syk activation induced by four different hypothetical ligands, two bivalent (circles) and two trivalent (squares), that have the same single-site forward rate constants $k_{+2} = 2.0 \times 10^{-11}$ cm^2/s. (In the simulations, we take the surface area of the cell $A = 8 \times 10^{-6}$ cm^2 and the total number of receptors per cell $N_T = 4 \times 10^5$ so that the receptor density $R_T = 5 \times 10^{10}$ cm^{-2}. Therefore, at low ligand concentrations when most of the receptors are free so that $R \approx R_T$, the rate at which a bound ligand with one site free cross-links a receptor is $k_{+2}R = 1.0$ s^{-1}.) Each pair of bivalent and trivalent ligands have dissociation rate constants $k_{off} = 0.01$ s^{-1} (Fig. 2, solid lines and symbols) and 10 s^{-1} (Fig. 2, dashed lines and open symbols). For the dimers, increasing k_{off} from 0.01 s^{-1} to 10 s^{-1} corresponds to increasing the rate of serial engagement from $r_2 = 0.017$ s^{-1} to 0.10 s^{-1} (Equation 4), whereas for the trimer, the increase is from $r_3 = 0.027$ s^{-1} to 0.20 s^{-1} (Equation 5). In addition to increasing the rate of serial engagement, increasing k_{off} reduces the lifetime of the bond between a site on the ligand and a receptor site and thus increases the effects of kinetic proofreading (4). As previously discussed (22), to equalize the comparison, the levels of phosphorylation are plotted as a function of the number of receptors in aggregates. Fig. 2*A* shows the ligand concentrations needed to achieve the same level of receptor aggregation for the different ligands. As k_{off} is increased while keeping $k_{+2}RT$ fixed, for both bivalent and trivalent ligands, a higher ligand concentration is required to yield the same number of aggregated receptors, because the rate at which the receptor cross-link is broken is enhanced, whereas the rate at which the cross-links form remains unaltered. In Fig. 2*D*, we see that as the lifetime of a receptor in an aggregate is decreased (i.e., k_{off} is increased), full Syk activation is dramatically reduced for both bivalent and trivalent ligands. Kinetic proofreading dominates any positive effects serial engagement may have on Syk activation. This result is similar to that seen in experiments using fast and slowly dissociating multivalent ligands that aggregate IgE on RBL cells (37).

Fig. 2*B* and 2*C* show that the proximal signaling events β and γ ITAM phosphorylation, compared with Syk phosphorylation, are effected less by the changes in the ligand–receptor complex lifetime as expected from a kinetic proofreading model (4). The reduction in Syk phosphorylation is pronounced because it is much further down the signaling cascade than phosphorylation of the receptor γ-chain. A number of reversible steps must occur to go from γ-chain phosphorylation to Syk phosphorylation. A Syk molecule must first bind to a phosphorylated γ-chain before it is dephosphorylated. For transphosphorylation to occur, a second Syk molecule must bind, which requires a second γ-chain to be phosphorylated in the same aggregate. Finally, transphoshorylation among the Syk molecules must occur before either of the Syk molecules dissociates.

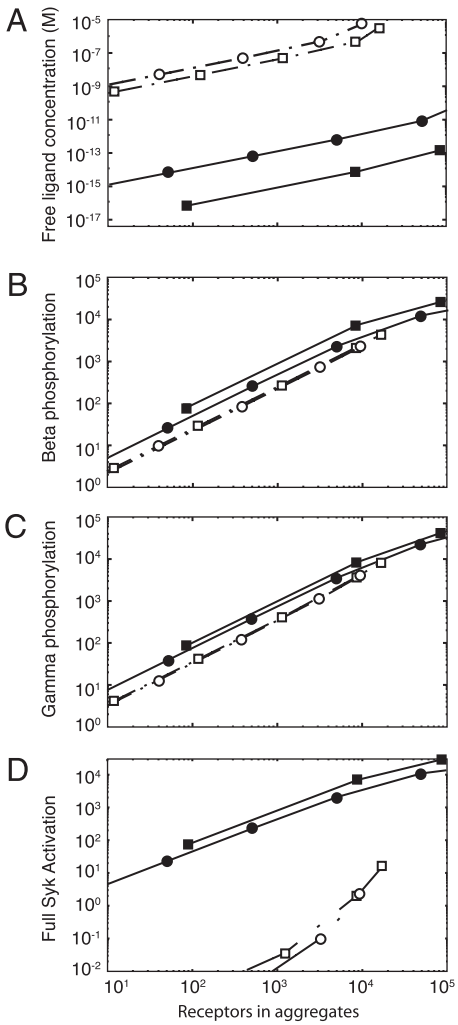

FIGURE 2. Simulation of the effects of increasing the rate of serial engagement of bivalent and trivalent ligand by increasing the ligand-receptor dissociation constant, k_{off}. The four curves correspond to $k_{+2}R_T = 1.0$ s^{-1} and bivalent (circles) and trivalent (squares) ligands with $k_{off} = 0.01$ s^{-1} (solid lines and symbols) and 10 s^{-1} (dashed lines and open symbols). The additional parameters used in the simulations are given in Table I of Ref (22). The *x*-axis indicates levels of FcεRI aggregation, given as the number of receptors per cell in aggregates. One percent of the cell's receptors in aggregates correspond to 4×10^3 receptors. *A*, Free ligand concentrations corresponding to specified levels of receptor aggregation. *B–D* are, respectively, the predicted number of receptors per cell with the β-ITAM phosphorylated, the γ-ITAM phosphorylated, and Syk bound to the phosphorylated γ-ITAM and transphosphorylated on its activation loop by another Syk.

Increasing serial engagement by increasing k_{+2} increases Syk activation

In Fig. 3, we compare the levels of β and γ ITAM phosphorylation and full Syk activation induced by four different hypothetical ligands, two bivalent (circles) and two trivalent (squares), that have the same dissociation rate constant $k_{off} = 0.1$ s^{-1} and different surface cross-linking constants. Each pair of bivalent and trivalent ligands have surface cross-linking constants such that $k_{+2}R_T = 0.1$ s^{-1} (Fig. 3, solid lines and open symbols) and 100 s^{-1} (Fig. 3, dashed lines and closed symbols). As in Fig. 2, we compare ligands at different concentrations that result in the same number of receptors being in aggregates at equilibrium. Because k_{off} is fixed, the lifetime of a receptor in an aggregate is the same for all four ligands. As $k_{+2}R_T$ is increased from 0.1 s^{-1} to 100 s^{-1}, the rate of serial engagement for the bivalent ligand increases from 0.067 s^{-1} to 0.20 s^{-1} (Equation 4) and for the trivalent ligand from 0.13 s^{-1} to 0.30 s^{-1} (Equation 5). These rates of serial engagement were calculated for low ligand concentrations, when $R \approx R_T$ and $k_{+2}R \approx k_{+2}R_T$. We see that the simulations predict that increasing the rate

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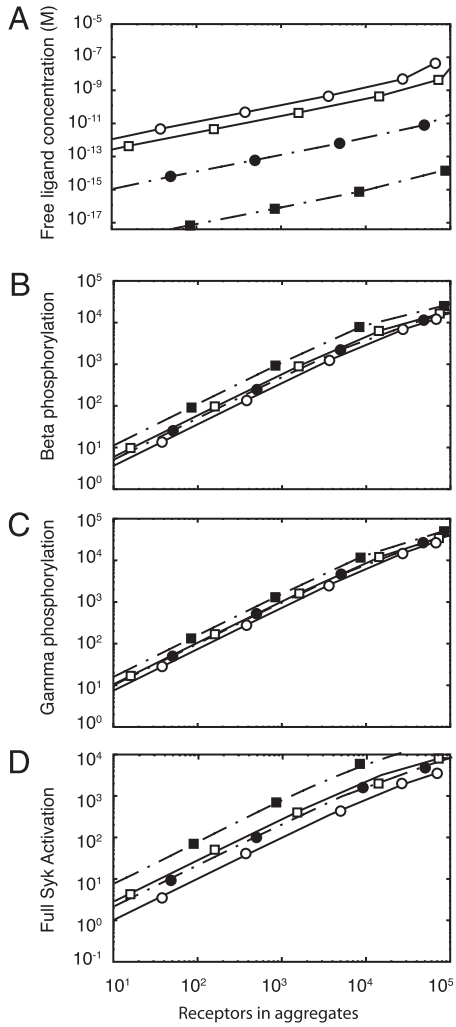

FIGURE 3. Simulation of the effects of increasing the rate of serial engagement of bivalent and trivalent ligand by increasing the rate of cross-linking, $k_{+2}R$. The four curves correspond to $k_{off} = 0.1\ s^{-1}$ and bivalent (circles) and trivalent (squares) ligands with $k_{+2}R = 0.1\ s^{-1}$ (solid lines and open symbols) and $100\ s^{-1}$ (dashed lines and closed symbols). All other parameters are the same as in Fig. 2. The *x*-axis indicates levels of FcεRI aggregation, given as the number of receptors per cell in aggregates. *A*, Free ligand concentrations corresponding to specified levels of receptor aggregation. *B–D* are, respectively, the predicted number of receptors per cell with the β-ITAM phosphorylated, the γ-ITAM phosphorylated, and Syk bound to the γ-ITAM and transphosphorylated by Syk on its activation loop.

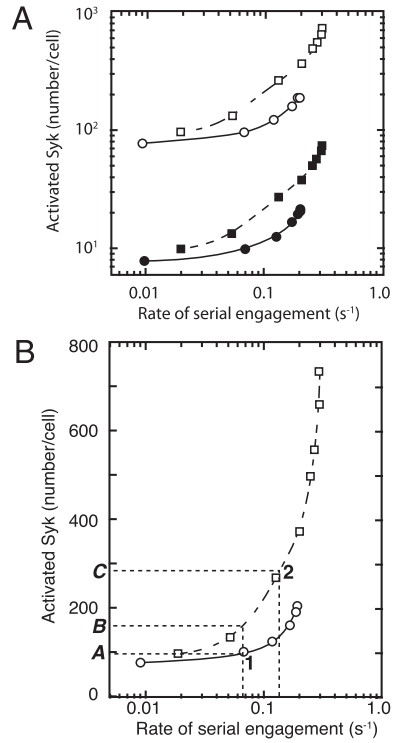

FIGURE 4. Predicted number of activated Syk (Syk transphosphorylated by Syk) as a function of the rate of serial engagement. *A*, The circles refer to bivalent ligand simulations and the squares to trivalent ligand simulations. Open and solid symbols refer to 1000 and 100 receptors in aggregates, respectively. *B*, Replot of the bivalent and trivalent curves in *A* for 1000 receptors in aggregates. The symbol 1 corresponds to a dimer with $k_{+2}R = 0.01\ s^{-1}$ and $k_{off} = 0.10\ s^{-1}$. The symbol 2 corresponds to a trimer with the same rate constants. *A* equals 100, *B* equals 161, and *C* equals 290 fully activated Syk per cell. The net increase in activated Syk ($C - A$) is 190 Syk/cell, of which $C - B = 129$ Syk/cell can be attributed to serial engagement.

of serial engagement while holding k_{off} fixed has a minor effect on receptor phosphorylation (Fig. 3*B*, 3*C*) but a more pronounced effect on the phosphorylation of Syk by Syk (i.e., on full Syk activation) (Fig. 3*D*).

Increasing the valence of a ligand increases Syk activation, partly as a result of increased serial engagement

From Equations 4 and 5, we can calculate the increase in the rate of serial engagement as a result of increasing the valence of a ligand from two to three while keeping its rate constants unchanged. The factor by which the rate of serial engagement increases, r_3/r_2, depends only on the parameter $k_{+2}R$, and monotonically decreases from 2 to 3/2 as $k_{+2}R$ increases from zero to infinity. In Fig. 4, as in Fig. 3, we compare the predicted full Syk activation from simulations where the stimulating ligand is either bivalent (circles) or trivalent (squares), the rate of serial engagement is varied by varying k_{+2}, and for both ligands, the lifetime of a receptor bound to a ligand is the same, 10 s ($k_{off} = 0.10\ s^{-1}$). The range over which the rate of serial engagement varies in Fig. 4 corresponds to $k_{+2}R = 0.01$ — $100\ s^{-1}$. In Fig. 4*A*, the two top curves and two bottom curves correspond to 1000 and 100 receptors per cell in aggregates. For the same rate of serial engagement and the same number of receptors in aggregates, the model predicts that the trivalent ligand is more effective than the bivalent ligand at activating Syk.

In Fig. 4*B*, we replot the simulations for the upper two curves in Fig. 4*A*. To illustrate how we can assess the contribution of serial engagement in enhancing Syk phosphorylation by Syk when the valence is increased, we consider the point labeled 1 on the Syk activation curve for the bivalent ligand in Fig. 4*B*. This point corresponds to the serial engagement rate for a bivalent ligand with $k_{+2}R = 0.01\ s^{-1}$ and $k_{off} = 0.10\ s^{-1}$. When the valence is increased from two to three with the rate constants unchanged, the rate of serial engagement for the trimer increases above that of the dimer to the point on the trivalent ligand curve labeled 2. We see that there would be an increase in Syk phosphorylation by Syk for a trivalent ligand even if there were no increase in the rate of serial engagement. This is indicated by the difference B − A on the *y*-axis and is a result of effects other than serial engagement. The increase in Syk activation that is attributable to the increase in serial engagement is indicated by the difference C − B on the *y*-axis, which can be substantial.

Lyn is strongly regulated by Cbp and Csk (38, 39) on RBL cells, and, as a result, the amount of Lyn available to the receptor is in short supply (33, 34). Although Lyn regulation is not in the model, the Lyn concentration is chosen so that Lyn is limiting with respect to availability to the receptor. In the model, in the absence of ligand, <5% of the receptors are associated with Lyn, which is

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consistent with the observations of Yamashita et al. (40). Upon aggregation, large receptor aggregates are more likely to contain a Lyn than small receptor aggregates, and once a Lyn is in an aggregate, it can transphosphorylate all receptors in its proximity. It is these effects that the model captures when the valence of the ligand is increased from two to three and that account for the increase in Syk phosphorylation that is not a result of serial engagement. We investigate this further by considering ligands that cannot serial engage receptors.

Serial engagement is not necessary for Syk activation

Although the aggregation of FcεRI is an absolute requirement for mast cell signaling mediated by FcεRI, serial engagement is not, as has been demonstrated using oligomers of IgE (23). As a test of our model (22), we have carried out simulations for bivalent and trivalent oligomers of IgE, taking the dissociation rate constant $k_{off} = 0$, which ensures that there is no serial engagement. [Because the $t_{1/2}$ for dissociation of an IgE–Fcε complex is close to a day (41), this is an excellent approximation in simulating experiments that last a few hours or less.] The predicted results for γ-phosphorylation and Syk activation for IgE dimers and trimers at 1 h are shown in Fig. 5*A* and 5*B*. These results are compared with histamine release dose-response curves for RBL-2H3 cells exposed to IgE oligomers for 1 h. Because of the slow forward rate constant for IgE binding to FcεRI (42), equilibrium is achieved at 1 h only for IgE concentrations >~5 μg/ml. The simulations indicate that to achieve measureable histamine release from RBL cells requires the activation of substantial numbers of Syk molecules. For example, the simulations predict that 10% histamine release with trivalent ligands, which is ~25% of maximal release, occurs when ~10,000 Syk are fully activated.

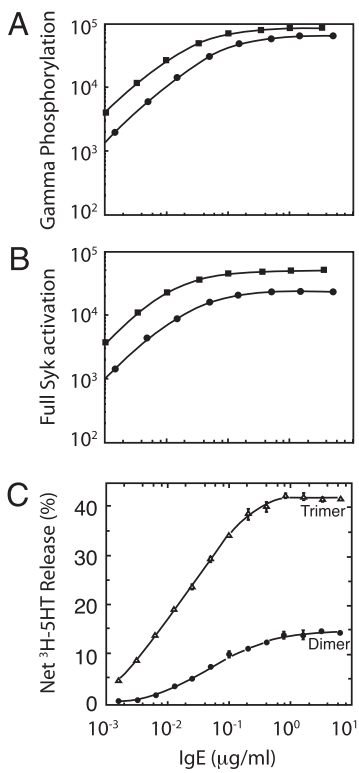

FIGURE 5. Predicted γ and Syk phosphorylation and measured RBL-2H3 cell degranulation for dimeric and trimer oligomers of IgE. In *A* and *B*, the circles refer to IgE dimer simulations and the squares to IgE trimer simulations. The rate constants characterizing the oligomers are taken to be $k_{off} = 0\ s^{-1}$, $k_{+1} = 8 \times 10^4\ M^{-1}\ s^{-1}$, and $k_{+2}\ R_T = 100\ s^{-1}$. The simulations correspond to exposure of the ligands to RBL-2H3 cells for 1 h. *C*, Experimentally determined degranulation of RBL-2H3 cells after 1 h of exposure to either dimers or trimers of IgE (32).

Discussion

When surface densities of receptors are sufficiently high, multivalent ligands can bind to cell surfaces and serial engage numerous receptors before dissociating (i.e., before all of the ligand's binding sites are simultaneously free).

Even with a valence as low as two, a ligand can engage multiple receptors from the time it first attaches to the surface until the time it dissociates (see Table I). A single ligand binding site may bind a receptor, dissociate from it, and repeat the cycle with a new receptor multiple times before the ligand leaves the surface. Using a detailed mathematical model, we have investigated the role of serial engagement of FcεRI in mast cell signaling. The model was originally developed for oligomers of IgE binding to FcεRI on the surface of RBL cells (22). For multivalent ligands binding reversibly to IgE–FcεRI complexes, the model applies when IgE acts as a monovalent receptor. We use the model for multivalent ligands at low concentration, when the binding of two ligands to one IgE is negligible, and the concentration of free receptors is high so that serial engagement is favored. The model includes the binding and cross-linking reactions between a bivalent or trivalent ligand and an IgE–FcεRI complex, the reactions among the subunits of FcεRI, Lyn, and Syk, and phosphatases that are not explicitly included in the model but are assumed to be present in the background and that rapidly dephosphorylate unprotected phosphotyrosines. Previously (22), the concentrations of all the components of the model in RBL cells were determined. All of the parameters of the model were either directly measured or estimated based on fits of model predictions to experiment. The model was shown to be consistent with a variety of experimental data in which RBL cells were exposed to covalently cross-linked dimers of IgE including the kinetics of receptor subunit phosphorylation and Syk phosphorylation. The model was also consistent with dephosphorylation experiments (30, 31) in which RBL cells, sensitized with IgE, were first exposed to a multivalent ligand and then receptor aggregates were broken up with the addition of excess monovalent hapten. It is well suited to investigate the role of serial engagement in the early cell signaling events initiated when RBL cells, sensitized with monoclonal IgE, are exposed to low concentrations of multivalent ligand that can aggregate the IgE into small aggregates of size two or three.

A basic assumption of the model is that when a ligand is bound to >1 receptor and a bond opens, the freed receptor diffuses away before the site on the ligand can rebind to it. This is a reasonable assumption as long as receptors remain mobile on the cell surface. On RBL cells, receptors in small aggregates remain mobile (21), but receptors in large aggregates do not (19, 20). When receptors are immobile, we expect serial engagement to be markedly reduced and have little effect on cell signaling. We have only considered the case when receptors remain mobile on the RBL surface and results do not apply to large aggregates where receptors are immobile. The expression we have derived for the rate of serial engagement for a ligand of valence *N*, Equation 3, holds only when receptors are mobile.

We considered three ways in which serial engagement could be increased: by increasing the rate at which a ligand binding site dissociates from a receptor; by increasing the rate at which a free site on a ligand that is bound to a receptor can cross-link a new receptor; and by increasing the valence of the ligand. We took as our measure of signaling response the predicted number of Syk per cell that were bound to phosphorylated γ-ITAMs and that were phosphorylated on their activation loop tyrosines. In the model, this phosphorylation arises through Syk transphosphorylation and requires that at least

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two Syk molecules are present, each bound to a different receptor in the aggregate, for the phosphorylation to occur. Model simulations showed that increasing the rate of serial engagement by increasing the rate of dissociation reduced the concentration of activated Syk (Fig. 2*D*). Increasing the rate of dissociation of a ligand–receptor bond decreases the lifetime of a receptor in an aggregate and increases the effect of kinetic proofreading. The further down the signaling pathway an event occurs, the more pronounced are the effects of kinetic proofreading on the event, as can be seen by comparing the predicted γ-chain phosphorylation (Fig. 2*C*) with the predicted Syk phosphorylation (Fig. 2*D*). Whatever positive effects serial engagement has on signaling are outweighed by kinetic proofreading when the lifetime of the ligand–receptor bond is decreased (4, 37).

Simulations predict that increasing the rate at which cross-linking occurs, although keeping the lifetime of the bond between a ligand site and receptor constant so as not to enhance kinetic proofreading, increases Syk activation. In Fig. 4*A*, a single curve corresponds to a series of bivalent or trivalent ligands with increasing rates of serial engagement achieved by increasing their forward rate constants for cross-linking. The ligands have the same dissociation rate constants, and their concentrations have been chosen so that they aggregate the same number of receptors on the cell surface, yet the faster the ligands are able to serial engage receptors, the more Syk they are able to activate. This raises the question, why should serial engagement matter if the lifetime of a receptor in an aggregate is the same and the number of receptors in aggregates is as well? The explanation we favor is that serial engagement can partially nullify the effects of kinetic proofreading by allowing receptors that have dissociated from an aggregate to enter new aggregates before they fully return to their basal state. Previously, we estimated that when a receptor leaves an aggregate, its unprotected phosphotyrosines are dephosphorylated in <1 s, and any Syk or Lyn that is bound to phosphorylated ITAMS dissociates with $t_{1/2}$ of the order of $10-15$ s (22). Even within an aggregate, phosphorylation and dephosphorylation is constantly occurring (23). If a receptor can enter into a new aggregate before all the modifications it has undergone have been reversed, full activation of Syk will be more efficient. As depicted in Fig. 6 for a bivalent ligand, at low ligand concentration, a receptor that remains bound to a ligand when an aggregate breaks up is much more likely to quickly enter into a new aggregate than a receptor with its binding site free, provided the rate for cross-linking a new receptor is equal to or faster than the rate of dissociation of the ligand-receptor bond (i.e., $k_{+2}R \geq k_{off}$), or equivalently $K_2R \geq 1$. Even when this condition on the rate constants is met, serial engagement can only partially reduce the effects of kinetic proofreading because free receptors that leave an aggregate will most likely return to the basal state before they form new aggregates.

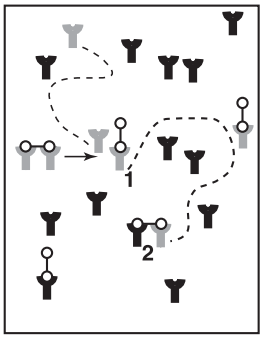

FIGURE 6. At low ligand concentration, when most receptors are unbound, a receptor–ligand complex that dissociates from an aggregate (1) can rapidly enter a new aggregate (2), provided that the rate of cross-linking is greater than the rate of dissociation of its ligand–receptor bond. If this is not the case, the ligand–receptor complex will be rapidly converted to a free receptor, reducing its chance of quickly finding a binding partner.

The model also predicts that increasing the valence of a ligand from two to three, while keeping the rate constants the same, increases Syk activation (Fig. 4). The expressions we have derived for the rates of serial engagement of dimers and trimers, Equations 4 and 5, show that increasing the valence, although keeping all other quantities fixed, increases the rate of serial engagement. However, the predicted increase in Syk activation is not solely due to the increase in serial engagement. Both the experiment (32) and the model predictions (Fig. 5) indicate that trimeric oligomers of IgE are more effective than dimers at activating RBL cells, even though these ligands cannot serial engage receptors. This is because Lyn is limiting in RBL cells with most receptors not associated with a Lyn (40, 33, 34). Large aggregates are more likely than small aggregates to have a Lyn bound to a receptor in the aggregate and initiate signaling. Further, the larger the aggregate, the higher is the number of receptors that can be phosphorylated by a single Lyn. Serial engagement can enhance this signaling. We have estimated the additional contribution to Syk activation that the increase in serial engagement makes when a trivalent ligand is substituted for a bivalent ligand and shown that it can be substantial (Fig. 4*B*).

Ligand-induced receptor aggregation initiates a chemical cascade that involves chemical reactions that build and use transient molecular scaffolds. Upon aggregation of FcεRI on mast cells, the cytoplasmic domains of the receptor become sites for the coalescence of the kinases Lyn and Syk, with Syk undergoing rapid phosphorylation. However, the structures formed around the cytoplasmic domains of the receptor, as well as other scaffolding proteins, are ephemeral, with components going on and off rapidly. How successful this construction will be depends on the lifetime of a receptor in an aggregate. If the lifetime is too short, most of the chemical cascades that are initiated will not go to completion, and signaling will be dampened or completely prevented. This is the idea behind kinetic proofreading (reviewed in Ref. 5), introduced in the context of cell signaling by McKeithan (4), and kinetic proofreading has highlighted the role of the pMHC–TCR bond dissociation rate constant in T cell signaling. We have used a mathematical model of the initial steps in the chemical cascade triggered by FcεRI aggregation on mast cells to investigate the role of serial engagement in signaling and shown that for a series of ligands with the same dissociation rate constant, increasing the forward rate constant for cross-linking, or increasing the ligand valence increases serial engagement and Syk activation. Serial engagement is able to partially reverse the effects of kinetic proofreading and enhance mast cell signaling.

Appendix

We derive an expression for n_N, the average number of receptors an N-valent ligand, which initially has one site bound to a receptor, serial engages before it dissociates from the cell surface. We consider a cell with monovalent receptors on its surface that is in equilibrium with a homogeneous concentration of N-valent ligands. At equilibrium, there is a distribution of bound ligands, some with one site bound, some with two sites bound, up to some with N sites bound. We consider an ensemble of N-valent ligands that at $t = 0$ have one and only one site bound to a receptor. We let $B_i(t)$ be the fraction of these ligands that have i sites bound at time t or equivalently, the probability that a ligand has i sites bound at time t when initially it had one site bound. We keep tract of the number of receptors a ligand serial engages by keeping track of how many receptors dissociate from a ligand until the ligand leaves the cell surface. Because the rate at which a receptor dissociates from a ligand with i sites bound is ik_{off}, the average number of receptors a ligand

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will serial engage from the time it binds to the cell until the time it leaves the cell is:

$$n_N = k_{off}\int_0^\infty (2B_2(t') + 3B_3(t')\cdots + NB_N(t'))dt'. \quad (6)$$

Before solving the general problem, we illustrate our approach by finding the number of receptors a bivalent ligand serial engages during its time on the cell surface.

The equilibrium concentration of free receptors is represented by R. For a bivalent ligand, we have the following set of ordinary differential equations:

$$\frac{dB_1}{dt} = -k_{off}B_1 - k_{+2}RB_1 + 2k_{off}B_2, \text{and} \quad (7)$$

$$\frac{dB_2}{dt} = k_{+2}RB_1 - 2k_{off}B_2. \quad (8)$$

The initial conditions are that $B_1 = 1$ and $B_2 = 0$.

The average number of receptors a bivalent ligand engages during its time on the cell surface is:

$$n_2 = 2k_{off}\int_0^\infty B_2(t')dt'. \quad (9)$$

As is standard, we use Laplace transforms (LT), to transform the problem into solving a set of algebraic equations, taking s to be the transform variable and $\bar{B}_i(s) = LT[B_i(t)]$. Note that

$$n_N = \lim_{s\to 0} k_{off}(2\bar{B}_2(s) + 3\bar{B}_3(s)\cdots + N\bar{B}_N(s)). \quad (10)$$

This follows because the LT of

$$\int_0^t f(u)du = \bar{f}(s)/s \text{ and}$$

$$\lim_{t\to\infty} g(t) = \lim_{s\to 0} s\bar{g}(s). \quad (11)$$

Taking LT of both sides of Equations 7 and 8, we have

$$\begin{aligned} s\bar{B}_1 - B_1(0) &= -k_{off}\bar{B}_1 - k_{+2}R\bar{B}_1 + 2k_{off}\bar{B}_2 \\ s\bar{B}_2 &= k_{+2}R\bar{B}_1 - 2k_{off}\bar{B}_2. \end{aligned}$$

We can take the limit before we solve the equations. Setting $s = 0$, $k = k_{+2}R/k_{off} = K_2R$ and $B_1(0)=1$,

$$\begin{aligned} 1/k_{off} &= (1+k)\bar{B}_1 - 2\bar{B}_2 \\ 0 &= k\bar{B}_1 - 2\bar{B}_2. \end{aligned}$$

Solving, we have that $\bar{B}_2 = k/(2k_{off})$

$$n_2 = \lim_{s\to 0} k_{off}2\bar{B}_2(s) = k = K_2R. \quad (12)$$

For a ligand with valence N,

$$\begin{aligned} \frac{dB_i}{dt} &= (i+1)k_{off}B_{i+1} + (N-i+1)k_{+2}RB_{i-1} - ik_{off}B_i \\ &\quad - (N-i)k_{+2}RB_i, \end{aligned} \quad (13)$$

B_i can appear through dissociation of a receptor from any of i+1 receptor-occupied sites of B_{i+1}, which correspond to a term $(i+1)k_{off}B_{i+1}$; through engaging of a receptor by any of $(N-(i-1))$ free sites of B_{i-1}, which corresponds to a term $(N-i+1)k_{+2}RB_{i-1}$. B_i can disappear by losing a receptor from any of its i receptor-bound sites and by adding a receptor to any of (N−i) free sites, which corresponds to subtraction of terms $ik_{off}B_i$ and $(N-i)k_{+2}RB_i$, respectively.

Putting $k = k_{+2}R/k_{off}$, $\tau = k_{off}t$, $B_0 = B_{N+1} = 0$, and collecting terms,

$$\begin{aligned} \frac{dB_i}{d\tau} &= (i+1)B_{i+1} - (i + k(N-i))B_i \\ &\quad + k(N-i+1)B_{i-1}, i = 1, \ldots, N. \end{aligned} \quad (14)$$

Note that $B_0 = B_{N+1} = 0$ implies that the corresponding Laplace transforms $\bar{B}_0 = \bar{B}_{N+1} = 0$.

As before, taking LT and setting the transform variable equal to zero, we obtain

$$\begin{aligned} -1 &= 2\bar{B}_2 - (1 + k(N-1))\bar{B}_1 \\ 0 &= 3\bar{B}_3 - (2 + k(N-2))\bar{B}_2 + (N-1)k\bar{B}_1 \\ 0 &= 4\bar{B}_4 - (3 + k(N-3))\bar{B}_3 + (N-2)k\bar{B}_2 \\ &\vdots \\ 0 &= -N\bar{B}_N + k\bar{B}_{N-1}. \end{aligned}$$

First, we solve this system of equations for $\bar{B}_1$by summing all the equations:

$$\begin{aligned} -1 &= \sum_{i=1}^{N}((i+1)\bar{B}_{i+1} - (i + k(N-i))\bar{B}_i \\ &\quad + k(N-i+1)\bar{B}_{i-1}) \\ &= \sum_{i=2}^{N} i\bar{B}_i - \sum_{i=1}^{N}(i + k(N-i))\bar{B}_i + \sum_{i=1}^{N-1} k(N-i)\bar{B}_i \\ &= \sum_{i=1}^{N}(i - (i + k(N-i)) + k(N-i))\bar{B}_i - \bar{B}_1. \end{aligned}$$

The sum on the right hand side equals zero, so that $\bar{B}_1 = 1$. To find $\bar{B}_2$, we sum up all equations except the first one:

$$\begin{aligned} 0 &= \sum_{i=2}^{N}((i+1)\bar{B}_{i+1} - (i + k(N-i))\bar{B}_i \\ &\quad + k(N-i+1)\bar{B}_{i-1}) \\ &= \sum_{i=3}^{N} i\bar{B}_i - \sum_{i=2}^{N}(i + k(N-i))\bar{B}_i + \sum_{i=1}^{N-1} k(N-i)\bar{B}_i \\ &= \sum_{i=2}^{N}(i - (i + k(N-i)) + k(N-i))\bar{B}_i - 2\bar{B}_2 \\ &\quad + k(N-1)\bar{B}_1. \end{aligned}$$

Again, the sum on the right hand side equals zero so that

$$\bar{B}_2 = k(N-1)\bar{B}_1/2 = k(N-1)/2.$$

Similarly,

$$\bar{B}_3 = k(N-2)\bar{B}_2/3 = k^2(N-1)(N-2)/(1\cdot 2\cdot 3).$$

Following this approach, it is straightforward to show that

$$\begin{aligned} \bar{B}_j &= k^{j-1}\frac{(N-j+1)(N-j+2)\cdots(N-1)}{j!} \\ &= k^{j-1}\frac{(N-1)!}{j!(N-j)!} \end{aligned} \quad (15)$$

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and, therefore, that

$$n_N = \sum_{j=2}^{N} j\bar{B}_j = \sum_{j=2}^{N} k^{j-1}\frac{(N-1)!}{(j-1)!(N-j)!}. \qquad (16)$$

Disclosures

The authors have no financial conflicts of interest.

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Supplementary Information

A. Sample BioNetGen input file for simulating the early response of RBL cells stimulated by a reversible bivalent ligand that binds to, and dimerizes, IgE-Fc complexes on RBL cell surfaces. In this input file, the first and second ligand dissociation rate constants k_{-1} and k_{-2} are given by $k_{-1}=k_{-2}= 10s^{-1}$. The total number of receptors per RBL cell, $N_T = 4*10^5$ molecules and the surface area of the cell is taken as $A_{cell} = 8 * 10^{-6}$ cm^2, so that the total receptor surface density is given by $R_T = 5*10^{10}$ molecules cm^{-2} . The first ligand-receptor binding rate constant $k_{+1}= 10^6$ $M^{-1}s^{-1}$. This second order rate constant is divided by the inverse RBL cell density in order to obtain the rate constant in units of $molecule^{-1}s^{-1}$ used in the BioNetGen input file. Assuming RBL cell density to be of 10^6 cells/ml [1], i.e. an inverse cell density (V_{cell}) of 10^{-6} ml/cell, k_{+1} reduces to $1.66058*10^{-9}$ $molecule^{-1}$ s^{-1}. The cross-linking rate constant k_{+2} is chosen as $2*10^{-11}$ $molecule^{-1}$ cm^2 s^{-1} so that $k_{+2}R_T = 1.0s^{-1}$. This rate constant is divided by the RBL surface area A_{cell}, to obtain the rate constant in units of $molecule^{-1}$ s^{-1} used in the BioNetGen input file. All the other rate constants and total cellular Lyn and Syk expression levels are taken from Faeder et al . JI 2003. The total number of ligands per RBL cell, L_T, is chosen in such a way that $2K_1L =1$, where L is the free ligand concentration at equilibrium and $K_1 = k_{+1}/k_{-1}$.

```
begin parameters
Lig_tot      3.0112e9
Rec_tot      4.0e5
Lyn_tot      2.8e4
Syk_tot      4.0e5

kp1  1.66058e-9
km1  10.0
kp2  2.5e-6
km2  10.0
kpL  5e-5
kmL  20
kpLs  5e-5
kmLs  0.12
kpS  6e-5
kmS  0.13
kpSs  6e-5
kmSs  0.13
pLb  30
pLbs  100
pLg   1
pLgs   3
pLS   30
pLSs  100
pSS  100
pSSs  200
dm   20
dc   20
end parameters
```

```
begin species
Lig(l,l) Lig_tot
Lyn(U,SH2) Lyn_tot
Syk(tSH2,l~Y,a~Y) Syk_tot
Rec(a,b~Y,g~Y) Rec_tot
end species

begin reaction_rules
# Ligand-receptor binding
1 Rec(a) + Lig(l,l) <-> Rec(a!1).Lig(l!1,l) kp1, km1

# Receptor-aggregation
2 Rec(a) + Lig(l,l!1) <-> Rec(a!2).Lig(l!2,l!1) kp2,km2

# Constitutive Lyn-receptor binding
3 Rec(b~Y) + Lyn(U,SH2) <-> Rec(b~Y!1).Lyn(U!1,SH2) kpL, kmL

# Transphosphorylation of beta by constitutive Lyn
4 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~Y) -> \
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~pY) pLb

# Transphosphorylation of gamma by constitutive Lyn
5 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~Y) -> \
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY) pLg

# Lyn-receptor binding through SH2 domain
6 Rec(b~pY) + Lyn(U,SH2) <-> Rec(b~pY!1).Lyn(U,SH2!1) kpLs, kmLs

# Transphosphorylation of beta by SH2-bound Lyn
7 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~Y) -> \
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~pY) pLbs

# Transphosphorylation of gamma by SH2-bound Lyn
8 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~Y) -> \
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY) pLgs

# Syk-receptor binding through tSH2 domain
9 Rec(g~pY) + Syk(tSH2) <-> Rec(g~pY!1).Syk(tSH2!1) kpS, kmS

# Transphosphorylation of Syk by constitutive Lyn
10 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \
   Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLS

# Transphosphorylation of Syk by SH2-bound Lyn
11 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \
   Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLSs

# Transphosphorylation of Syk by Syk not phosphorylated on aloop
12 Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \
   Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSS
```

```
# Transphosphorylation of Syk by Syk phosphorylated on aloop
13 Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \
   Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSSs

# Dephosphorylation of Rec beta
14 Rec(b~pY)-> Rec(b~Y) dm

# Dephosphorylation of Rec gamma
15 Rec(g~pY)-> Rec(g~Y) dm

# Dephosphorylation of Syk at membrane
16 Syk(tSH2!1,l~pY)-> Syk(tSH2!1,l~Y) dm
17 Syk(tSH2!1,a~pY)-> Syk(tSH2!1,a~Y) dm

# Dephosphorylation of Syk in cytosol
18 Syk(tSH2,l~pY)-> Syk(tSH2,l~Y) dc
19 Syk(tSH2,a~pY)-> Syk(tSH2,a~Y) dc

end reaction_rules

begin observables
Species      Ligfree     Lig(l,l)
Species      RecMon      Rec(a) Rec(a!1).Lig(l!1,l)
Species      RecDim      Rec(a!1).Lig(l!1,l!2).Rec(a!2)
Molecules    RecPbeta    Rec(b~pY!?)
Molecules    RecPgamma   Rec(g~pY!?)
Molecules    RecSykPS    Rec(g~pY!1).Syk(tSH2!1,a~pY)
end observables

generate_network({overwrite=>1});
simulate_ode({suffix=>"3.01120e+09",t_end=>1000000,n_steps=>50000, \
atol=>1e-11,rtol=>1e-11,steady_state=>1});
```

B. Sample BioNetGen input file for simulating the early response of RBL cells stimulated by a reversible trivalent ligand that binds to, and forms trimeric IgE-Fc complexes on RBL cell surfaces. For the trivalent ligand, it is assumed that $k_{-1}=k_{-2}=k_{-3}$ and the k_{-1} and k_{-2} values are the same as in the bivalent ligand case and $k_{+3}=k_{+2}$ where k_{+2} is the same as in the bivalent ligand case. All other rate constants and total cellular concentrations are taken to be the same as in the bivalent ligand case.

```
begin parameters
Lig_tot      3.0112e9
Rec_tot      4.0e5
Lyn_tot      2.8e4
Syk_tot      4.0e5

kp1  1.66058e-9
km1  10.0
kp2  2.5e-6
```

```
km2  10.0
kpL  5e-5
kmL  20
kpLs  5e-5
kmLs  0.12
kpS  6e-5
kmS  0.13
kpSs  6e-5
kmSs  0.13
pLb  30
pLbs  100
pLg    1
pLgs    3
pLS   30
pLSs  100
pSS  100
pSSs  200
dm   20
dc   20
end parameters

begin species
Lig(l,l,l) Lig_tot
Lyn(U,SH2) Lyn_tot
Syk(tSH2,l~Y,a~Y) Syk_tot
Rec(a,b~Y,g~Y) Rec_tot
end species

begin reaction_rules
# Free ligand - receptor binding
1 Rec(a) + Lig(l,l,l) <-> Rec(a!1).Lig(l!1,l,l) kp1, km1

# Receptor-aggregation (ligand is already bound at one site)
2 Rec(a) + Lig(l,l,l!+) <-> Rec(a!1).Lig(l!1,l,l!+) kp2, km2

# Receptor-aggregation (ligand is already bound at two sites)
3 Rec(a) + Lig(l,l!+,l!+) <-> Rec(a!1).Lig(l!1,l!+,l!+) kp2, km2

# Constitutive Lyn-receptor binding
4 Rec(b~Y) + Lyn(U,SH2) <-> Rec(b~Y!1).Lyn(U!1,SH2) kpL, kmL

# Transphosphorylation of beta by constitutive Lyn
5 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~Y) -> \
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~pY) pLb

# Transphosphorylation of gamma by constitutive Lyn
6 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~Y) -> \
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY) pLg

# Lyn-receptor binding through SH2 domain
7 Rec(b~pY) + Lyn(U,SH2) <-> Rec(b~pY!1).Lyn(U,SH2!1) kpLs, kmLs
```

```
# Transphosphorylation of beta by SH2-bound Lyn
8 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~Y) -> \
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~pY) pLbs

# Transphosphorylation of gamma by SH2-bound Lyn
9 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~Y) -> \
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY) pLgs

# Syk-receptor binding through tSH2 domain
10 Rec(g~pY) + Syk(tSH2) <-> Rec(g~pY!1).Syk(tSH2!1) kpS, kmS

# Transphosphorylation of Syk by constitutive Lyn
11 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \
   Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLS

# Transphosphorylation of Syk by SH2-bound Lyn
12 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \
   Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLSs

# Transphosphorylation of Syk by Syk not phosphorylated on aloop
13 Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \
   Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSS

# Transphosphorylation of Syk by Syk phosphorylated on aloop
14 Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \
   Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSSs

# Dephosphorylation of Rec beta
15 Rec(b~pY)-> Rec(b~Y) dm

# Dephosphorylation of Rec gamma
16 Rec(g~pY)-> Rec(g~Y) dm

# Dephosphorylation of Syk at membrane
17 Syk(tSH2!1,l~pY)-> Syk(tSH2!1,l~Y) dm
18 Syk(tSH2!1,a~pY)-> Syk(tSH2!1,a~Y) dm

# Dephosphorylation of Syk in cytosol
19 Syk(tSH2,l~pY)-> Syk(tSH2,l~Y) dc
20 Syk(tSH2,a~pY)-> Syk(tSH2,a~Y) dc

end reaction_rules

begin observables
Species      Ligfree     Lig(l,l,l)
Species      RecMon      Rec(a) Rec(a!1).Lig(l!1,l,l)
Species      RecDim      Rec(a!1).Lig(l!1,l!2,l).Rec(a!2)
Species      RecTri      Rec(a!1).Lig(l!1,l!2,l!3).Rec(a!2).Rec(a!3)
Molecules    RecPbeta    Rec(b~pY!?)
Molecules    RecPgamma   Rec(g~pY!?)
```

```
Molecules     RecSykPS    Rec(g~pY!1).Syk(tSH2!1,a~pY)
end observables

generate_network({overwrite=>1});
simulate_ode({suffix=>"3.01120e+09",t_end=>1000000,n_steps=>50000, \
atol=>1e-11,rtol=>1e-11,steady_state=>1});
```

C. Sample Perl script to write out a BioNetGen Input file for simulating the early response of RBL cells stimulated by a reversible bivalent ligand that binds to, and dimerizes, IgE-Fc complexes on RBL cell surfaces, and run simulations using this input file for a list of total number of ligands per RBL cell. In this particular example, the total number of ligands per RBL cell, L_T is chosen in such a way that $\log_{10}(2K_1L)$, varies from -10 to 10, where L is the free ligand concentration at equilibrium and $K_1 = k_{+1}/k_{-1}$. Running this Perl script requires the definition of an environment variable $BNGPATH in the script, which defines the path to the directory where the BioNetGen program is installed. This script requires on the command line input one argument which is the name of the directory created on running the Perl script and which contains the BioNetGen input and output files generated. For example, if the lone command line argument is "test" then a directory called "test" is created in the same directory that contains the Perl script. The directory called "test" contains the BioNetGen input file created, "test.bngl", a log file called "test.log" and a network generation output file called "test.net" which corresponds to the total ligand concentration in the parameter block contained in the Perl script. For each L_T value, the "test" directory contains a network file containing the results of network generation in the form of "test_X.XXXXXe±XX.net", and output files containing time course simulation results, "test_X.XXXXXe±XX.cdat" (containing simulated time courses of all species in the model) and "test_X.XXXXXe±XX.gdat" (containing simulated time courses of the species specified in the observable block of the Perl script) where X.XXXXXe±XX is the value of L_T in exponential format.

```
#!/usr/bin/perl

$BNGPATH="/Users/ambarish/bionetgen/BioNetGen_2.0.35" ;
@lig_conc=(0.284552,2.99445,30.1157,301.141,3011.42,30114.2,301142.0,3.01142e6,
3.01142e7,3.01138e8,3.0112e9,3.01104e10,3.011e11,3.011e12,3.011e13,3.011e14,
3.011e15,3.011e16,3.011e17,3.011e18,3.011e19);
($#ARGV==0) || die "Usage $0 runname";
$prefix=shift;
if (-d $prefix){
  die "Directory $prefix exists. Remove before running";
}
$script=<<"EOF";
begin parameters
Lig_tot        3.0112e9
Rec_tot        4.0e5
Lyn_tot        2.8e4
Syk_tot        4.0e5
```

```
kp1  1.66058e-9
km1  10.0
kp2  2.5e-6
km2  10.0
kpL  5e-5
kmL  20
kpLs  5e-5
kmLs  0.12
kpS  6e-5
kmS  0.13
kpSs  6e-5
kmSs  0.13
pLb  30
pLbs  100
pLg    1
pLgs    3
pLS   30
pLSs  100
pSS  100
pSSs  200
dm   20
dc   20
end parameters

begin species
Lig(l,l) Lig_tot
Lyn(U,SH2) Lyn_tot
Syk(tSH2,l~Y,a~Y) Syk_tot
Rec(a,b~Y,g~Y) Rec_tot
end species

begin reaction_rules
# Ligand-receptor binding
1 Rec(a) + Lig(l,l) <-> Rec(a!1).Lig(l!1,l) kp1, km1

# Receptor-aggregation
2 Rec(a) + Lig(l,l!1) <-> Rec(a!2).Lig(l!2,l!1) kp2,km2


# Constitutive Lyn-receptor binding
3 Rec(b~Y) + Lyn(U,SH2) <-> Rec(b~Y!1).Lyn(U!1,SH2) kpL, kmL

# Transphosphorylation of beta by constitutive Lyn
4 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~Y) -> \\
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~pY) pLb

# Transphosphorylation of gamma by constitutive Lyn
5 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~Y) -> \\
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY) pLg

# Lyn-receptor binding through SH2 domain
```

```
6 Rec(b~pY) + Lyn(U,SH2) <-> Rec(b~pY!1).Lyn(U,SH2!1) kpLs, kmLs

# Transphosphorylation of beta by SH2-bound Lyn
7 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~Y) -> \\
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~pY) pLbs

# Transphosphorylation of gamma by SH2-bound Lyn
8 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~Y) -> \\
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY) pLgs

# Syk-receptor binding through tSH2 domain
9 Rec(g~pY) + Syk(tSH2) <-> Rec(g~pY!1).Syk(tSH2!1) kpS, kmS

# Transphosphorylation of Syk by constitutive Lyn
10 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \\
   Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLS

# Transphosphorylation of Syk by SH2-bound Lyn
11 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \\
   Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLSs

# Transphosphorylation of Syk by Syk not phosphorylated on aloop
12 Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \\
   Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSS

# Transphosphorylation of Syk by Syk phosphorylated on aloop
13 Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \\
   Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSSs

# Dephosphorylation of Rec beta
14 Rec(b~pY)-> Rec(b~Y) dm

# Dephosphorylation of Rec gamma
15 Rec(g~pY)-> Rec(g~Y) dm

# Dephosphorylation of Syk at membrane
16 Syk(tSH2!1,l~pY)-> Syk(tSH2!1,l~Y) dm
17 Syk(tSH2!1,a~pY)-> Syk(tSH2!1,a~Y) dm

# Dephosphorylation of Syk in cytosol
18 Syk(tSH2,l~pY)-> Syk(tSH2,l~Y) dc
19 Syk(tSH2,a~pY)-> Syk(tSH2,a~Y) dc

end reaction_rules

begin observables
Species      Ligfree     Lig(l,l)
Species      RecMon      Rec(a) Rec(a!1).Lig(l!1,l)
Species      RecDim      Rec(a!1).Lig(l!1,l!2).Rec(a!2)
Molecules    RecPbeta    Rec(b~pY!?)
Molecules    RecPgamma   Rec(g~pY!?)
```

```
Molecules     RecSykPS    Rec(g~pY!1).Syk(tSH2!1,a~pY)
end observables

generate_network({overwrite=>1});
EOF

mkdir $prefix ;
chdir $prefix;

$fname = sprintf "${prefix}.bngl";
open(BNGL,">$fname") || die "Couldn't write to $fname" ;
print BNGL $script ;
$irun = 0 ;
foreach $i(@lig_conc){
  $irun += 1;
  $srun = sprintf "%6.5e",$i;
  if ($irun > 1){
    print BNGL "resetConcentrations()\n";
  }
  printf BNGL "setParameter(Lig_tot,$i);\n";
  printf BNGL "simulate_ode({suffix=>\"$srun\",t_end=>1000000,n_steps=>50000, \\
  atol=>1e-11,rtol=>1e-11,steady_state=>1});\n";
}
close(BNGL);

# Run BioNetGen on file
print "Running BioNetGen on $fname\n";
system("${BNGPATH}/Perl2/BNG2.pl $fname > $prefix.log");
```

D. Sample Perl script to write out a BioNetGen Input file for simulating the early response of RBL cells stimulated by a reversible trivalent ligand that binds to, and trimerizes, IgE-Fc complexes on RBL cell surfaces, and run simulations using this input file for a list of total number of ligands per RBL cell. For the trivalent ligand, it is assumed that k-1=k-2=k-3 and the k-1 and k-2 values are the same as in the bivalent ligand case and k+3=k+2 where k+2 is the same as in the bivalent ligand case. All other rate constants and total cellular concentrations are taken to be the same as in the bivalent ligand case.

```
#!/usr/bin/perl

$BNGPATH="/Users/ambarish/bionetgen/BioNetGen_2.0.35" ;
@lig_conc=(0.284552,2.99445,30.1157,301.141,3011.42,30114.2,301142.0,3.01142e6,
3.01142e7,3.01138e8,3.0112e9,3.01104e10,3.011e11,3.011e12,3.011e13,3.011e14,
3.011e15,3.011e16,3.011e17,3.011e18,3.011e19);
($#ARGV==0) || die "Usage $0 runname";
$prefix=shift;
if (-d $prefix){
  die "Directory $prefix exists. Remove before running";
}
$script=<<"EOF";
begin parameters
Lig_tot        3.0112e9
```

```
Rec_tot        4.0e5
Lyn_tot        2.8e4
Syk_tot        4.0e5

kp1  1.66058e-9
km1  10.0
kp2  2.5e-6
km2  10.0
kpL  5e-5
kmL  20
kpLs  5e-5
kmLs  0.12
kpS  6e-5
kmS  0.13
kpSs  6e-5
kmSs  0.13
pLb  30
pLbs  100
pLg    1
pLgs    3
pLS   30
pLSs  100
pSS  100
pSSs  200
dm   20
dc   20
end parameters

begin species
Lig(l,l,l) Lig_tot
Lyn(U,SH2) Lyn_tot
Syk(tSH2,l~Y,a~Y) Syk_tot
Rec(a,b~Y,g~Y) Rec_tot
end species

begin reaction_rules
# Free ligand - receptor binding
1 Rec(a) + Lig(l,l,l) <-> Rec(a!1).Lig(l!1,l,l) kp1, km1

# Receptor-aggregation (ligand is already bound at one site)
2 Rec(a) + Lig(l,l,l!+) <-> Rec(a!1).Lig(l!1,l,l!+) kp2, km2

# Receptor-aggregation (ligand is already bound at two sites)
3 Rec(a) + Lig(l,l!+,l!+) <-> Rec(a!1).Lig(l!1,l!+,l!+) kp2, km2

# Constitutive Lyn-receptor binding
4 Rec(b~Y) + Lyn(U,SH2) <-> Rec(b~Y!1).Lyn(U!1,SH2) kpL, kmL

# Transphosphorylation of beta by constitutive Lyn
5 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~Y) -> \\
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,b~pY) pLb
```

```
# Transphosphorylation of gamma by constitutive Lyn
6 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~Y) -> \\
Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY) pLg

# Lyn-receptor binding through SH2 domain
7 Rec(b~pY) + Lyn(U,SH2) <-> Rec(b~pY!1).Lyn(U,SH2!1) kpLs, kmLs

# Transphosphorylation of beta by SH2-bound Lyn
8 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~Y) -> \\
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,b~pY) pLbs

# Transphosphorylation of gamma by SH2-bound Lyn
9 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~Y) -> \\
Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY) pLgs

# Syk-receptor binding through tSH2 domain
10 Rec(g~pY) + Syk(tSH2) <-> Rec(g~pY!1).Syk(tSH2!1) kpS, kmS

# Transphosphorylation of Syk by constitutive Lyn
11 Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \\
   Lig(l!1,l!2).Lyn(U!3,SH2).Rec(a!2,b~Y!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLS

# Transphosphorylation of Syk by SH2-bound Lyn
12 Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~Y) -> \\
   Lig(l!1,l!2).Lyn(U,SH2!3).Rec(a!2,b~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,l~pY) pLSs

# Transphosphorylation of Syk by Syk not phosphorylated on aloop
13 Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \\
   Lig(l!1,l!2).Syk(tSH2!3,a~Y).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSS

# Transphosphorylation of Syk by Syk phosphorylated on aloop
14 Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~Y) -> \\
   Lig(l!1,l!2).Syk(tSH2!3,a~pY).Rec(a!2,g~pY!3).Rec(a!1,g~pY!4).Syk(tSH2!4,a~pY) pSSs

# Dephosphorylation of Rec beta
15 Rec(b~pY)-> Rec(b~Y) dm

# Dephosphorylation of Rec gamma
16 Rec(g~pY)-> Rec(g~Y) dm

# Dephosphorylation of Syk at membrane
17 Syk(tSH2!1,l~pY)-> Syk(tSH2!1,l~Y) dm
18 Syk(tSH2!1,a~pY)-> Syk(tSH2!1,a~Y) dm

# Dephosphorylation of Syk in cytosol
19 Syk(tSH2,l~pY)-> Syk(tSH2,l~Y) dc
20 Syk(tSH2,a~pY)-> Syk(tSH2,a~Y) dc

end reaction_rules
```

```
begin observables
Species       Ligfree     Lig(l,l,l)
Species       RecMon      Rec(a) Rec(a!1).Lig(l!1,l,l)
Species       RecDim      Rec(a!1).Lig(l!1,l!2,l).Rec(a!2)
Species       RecTri      Rec(a!1).Lig(l!1,l!2,l!3).Rec(a!2).Rec(a!3)
Molecules     RecPbeta    Rec(b~pY!?)
Molecules     RecPgamma   Rec(g~pY!?)
Molecules     RecSykPS    Rec(g~pY!1).Syk(tSH2!1,a~pY)
end observables

generate_network({overwrite=>1});
EOF

mkdir $prefix ;
chdir $prefix;

$fname = sprintf "${prefix}.bngl";
open(BNGL,">$fname") || die "Couldn't write to $fname" ;
print BNGL $script ;
$irun = 0 ;
foreach $i(@lig_conc){
  $irun += 1;
  $srun = sprintf "%6.5e",$i;
  if ($irun > 1){
    print BNGL "resetConcentrations()\n";
  }
  printf BNGL "setParameter(Lig_tot,$i);\n";
  printf BNGL "simulate_ode({suffix=>\"$srun\",t_end=>1000000,n_steps=>50000, \\
  atol=>1e-11,rtol=>1e-11,steady_state=>1});\n";
}
close(BNGL);

# Run BioNetGen on file
print "Running BioNetGen on $fname\n";
system("${BNGPATH}/Perl2/BNG2.pl $fname > $prefix.log");
```

E. How to choose L_T values for the bivalent ligand so that $\log_{10}(2K_1L)$ is a desired value?

In order to achieve this goal we need an expression relating L_T to $2K_1L$. The single site equilibrium constants for binding and cross-linking are $K_1=k_{+1}/k_{-1}$ and $K_2=k_{+2}/k_{-2}$ respectively. The concentrations at equilibrium of free receptor and receptor dimmers are denoted by R and D respectively. A 1:1 ligand receptor complex, in which one site on the bivalent ligand is bound to a receptor but the other site is free, is denoted by B. If there are RT total receptors on the cell surface and there are no processes to remove or add new receptors, the total number of receptors is conserved and

$$R_T = R + B + 2D = R + 2K_1LR + 2(2K_1L)(K_2/2)R^2$$
$$= (1+2K_1L)R + 2K_1K_2LR^2 \qquad (1)$$

Defining $r = R/R_T$ as the fraction of free receptors, the above equation reduces to

$$1 = (1+2K_1L)r + 2K_1K_2R_TLr^2 \qquad (2)$$

It is straightforward to show that w, the fraction of receptors not in dimers, is given by

$$w = (1+2K_1L)r = (-1 + \sqrt{(1+4\delta)})/2\delta, \qquad (3)$$

$$\text{where } \delta = (2K_1LK_2R_T)/(1+2K_1L)^2 \qquad (4)$$

It follows that

$$R = wR_T/(1+2K_1L);\ B = wR_T(2K_1L)/(1+2K_1L);\ D = (1-w)R_T/2 \qquad (5)$$

$$\begin{aligned} \text{and } L_T &= L*V_{cell}*f + (B+D)*A_{cell} \\ &= (2K_1L)*V_{cell}*f/2K_1 + (B+D)*A_{cell} \qquad (6) \end{aligned}$$

where f is a conversion factor from the given units of ligand concentration (L) to molecules per cell. For example, if L is in units of M (molar), then

$$f = N_{Av} *10^{-3} \qquad (7)$$

where N_{Av} = Avogadro Number.

A Mathematica notebook for the calculation of L_T values for bivalent ligands, given the desired $2K_1L$ values is provided as a separate file named "convl2ltot_km10_kp1_1p6M_kp2RT_1.nb"

References

1. Faeder, J.R., et al., *Investigation of early events in Fc epsilon RI-mediated signaling using a detailed mathematical model.* J Immunol, 2003. **170**(7): p. 3769-81.