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Insights into immunoglobulin E receptor signaling from structurally defined ligands

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Summary: The asymmetrical structure of bent immunoglobulin E (IgE) bound to its high-affinity receptor, FcεRI, suggests a possible role for this configuration in the regulation of signaling mediated by cross-linking of FcεRI on the surface of mast cells and basophils. Indeed, the presence of bound IgE strongly influences the capacity of cross-linked FcεRI dimers to trigger mast cell degranulation, implicating orientational constraints by bound IgE. Bivalent ligands that cross-link by binding to bivalent IgE can form linear and cyclic chains of IgE/FcεRI complexes, and these exhibit only limited capacity to stimulate downstream signaling and degranulation, whereas structurally analogous trivalent ligands, which can form branched networks of cross-linked IgE/FcεRI complexes, are more effective at cell activation. Long bivalent ligands with flexible spacers can form intramolecular cross-links with IgE, and these stable 1:1 complexes are very potent inhibitors of mast cell degranulation stimulated by multivalent antigen. In contrast, trivalent ligands with rigid double-stranded DNA spacers effectively stimulate degranulation responses in a length-dependent manner, providing direct evidence for receptor transphosphorylation as a key step in the mechanism of signaling by FcεRI. Thus, studies with chemically defined oligovalent ligands show important features of IgE receptor cross-linking that regulate signaling, leading to mast cell activation.

Keywords: FcεRI, cross-linking, transphosphorylation, mast cells

Introduction

For over three decades, immunoglobulin E (IgE) receptors on mast cells have served as a prototype for the characterization of immunoreceptor responses to foreign antigens. Prior to the discovery of the molecular structure of the T-cell receptor (TCR) for antigen, Metzger and colleagues (1, 2) showed tight but reversible binding of IgE to FcεRI on mast cells, and they firmly established that cross-linking of two or more IgE–receptor complexes on the surface of these cells is necessary and sufficient for stimulation of signals leading to degranulation. A key to rapid progress in these early studies of FcεRI structure and function was the rat basophilic leukemia (RBL) mast cell line (3, 4), which continues to serve as a useful model for cellular

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studies of FcεRI-mediated signaling. More recently, molecular genetic approaches to understanding FcεRI signaling have benefited from the use of bone-marrow-derived mouse mast cells, which can be differentiated in culture from mouse stem cells with altered genetic backgrounds (5, 6).

Biochemical and molecular cloning approaches established the subunit structure of FcεRI in the 1980s (7, 8), and the role of stimulated tyrosine phosphorylation in initiating its signaling cascade was also first identified during this time period (9). Fig. 1 provides a schematic representation of essential steps in IgE–receptor signaling leading to mediator release by degranulation. Cross-linking of receptor-bound, antigen-specific IgE through a multivalent antigen initiates phosphorylation of cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) sequences in the β and γ₂ subunits of FcεRI by Src family kinase Lyn, causing recruitment and activation of tyrosine kinase Syk, which phosphorylates phospholipase Cγ (PLCγ) and other substrates to activate this lipase (10). Hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLCγ produces inositol 1,4,5-trisphosphate (IP₃), which initiates the activation of Ca²⁺ mobilization, and diacylglycerol, activating protein kinase C (PKC). Sustained elevation of cytoplasmic calcium together with the activation of certain PKC isoforms is necessary and sufficient for degranulation in mast cells (11, 12). Not shown in Fig. 1 are a number of different adapter proteins that have been found to play important roles in binding and recruiting these and other enzymes during the tyrosine kinase signaling cascade which is activated by FcεRI, including the transmembrane adapter linker for activation of T cells (LAT) (13) and the cytoplasmic adapter Src homology 2 (SH2)-domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76) (14).

The mechanism by which cross-linking of FcεRI initiates its phosphorylation by Lyn kinase has been studied extensively. Phosphorylation of the β subunit ITAM of FcεRI by Lyn facilitates Lyn binding by its SH2 domain, which increases the local concentration of Lyn to amplify phosphorylation of the γ₂ subunits and consequent activation of Syk (15, 16). Lyn can also bind weakly to FcεRIβ in the absence of its tyrosine phosphorylation (17). However, β subunit is not essential for cross-link-dependent signal initiation by FcεRI (18) or chimeric forms of this receptor (19, 20), so the mechanism by which signaling is initiated does not require interactions between β and Lyn. Our laboratory has characterized a fundamental role for liquid-ordered (Lo) lipid domains in the plasma membrane, often called lipid rafts, in facilitating signal initiation by FcεRI. We showed that these Lo domains protect the active form of Lyn kinase from inactivation by dephosphorylation of its catalytic domain tyrosine residue (21), thus providing a pool of active Lyn that gains access to FcεRI when receptor association with this Lo environment is stabilized by cross-linking (22). Studies with chimeric IgE receptors showed that their transmembrane segments are critical to these cross-link-driven interactions with Lo domains and consequent signal initiation (20). Protection of Lyn kinase and cross-linked FcεRI from dephosphorylation by transmembrane phosphatases that are excluded from Lo domains was shown to be important for initiation of the stimulated tyrosine phosphorylation cascade, providing compelling evidence for the role of protein segregation caused by Lo/liquid-disordered (Ld) lipid domain separation (23).

Issues of valency and geometry

Hapten-specific IgE bound to FcεRI is efficiently cross-linked by multi-haptenated proteins to stimulate robust mast cell

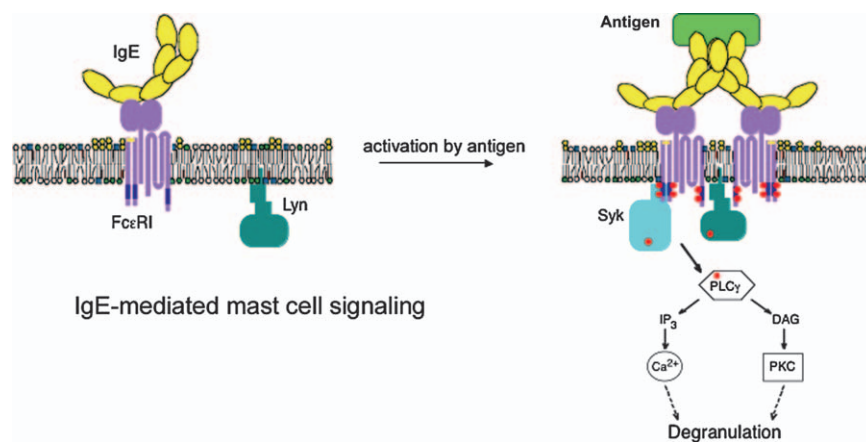


Fig. 1. Schematic diagram of essential features of antigen-stimulated IgE receptor signaling. Red dots indicate stimulation-dependent phosphorylation residues in ITAM

sequences of FcεRI, in tyrosine kinases Lyn and Syk, and in phospholipase PLCγ. Adapted from Gidwani et al. (62).

triggering and functional responses. Monoclonal IgE specific for the hapten 2,4-dinitrophenyl (DNP) and multivalent protein conjugates such as DNP₁₅-bovine serum albumen (BSA) are commonly used to trigger these robust responses. Studies of binding kinetics have shown that cross-linking of anti-DNP IgE bound to FcεRI by this multivalent antigen is complex, in part because DNP groups preferentially interact with the protein surface to which they are conjugated rather than be exposed to aqueous solution. Thus, DNP exposure becomes the rate-limiting step in binding and cross-linking anti-DNP IgE (24). This binding process results in rapid immobilization of the cross-linked IgE receptor complexes in a heterogeneous distribution of sizes and orientations (25, 26). Thus, multivalent antigens typically cause robust stimulation of FcεRI signaling, but they are not useful for examining features of the receptor cluster such as size or orientation that are critical for signal initiation.

Early studies on the sensitivity of triggering to receptor aggregate size used covalent oligomers of IgE to evaluate minimal requirements for FcεRI signaling. These showed that covalent IgE dimers are sufficient to trigger mast cell degranulation (27). Fewtrell and Metzger (28) found that highly purified IgE dimers (but not monomers) can trigger RBL mast cells, but trimers and larger oligomers trigger substantially better responses. They determined that this graded response to oligomer size represents a fundamental difference in signaling potency (per receptor) of dimers versus trimers and higher oligomers. A subsequent study showed strong correlations between aggregate size, signaling potency, and a loss of receptor lateral mobility at the cell surface (29). The conclusion from these studies, that monomeric IgE does not stimulate and dimeric IgE only poorly stimulates mast cell signaling, has been challenged in recent years in several studies. Claims that highly purified monomeric IgE can stimulate mast cell activation without aggregating FcεRI (30, 31) are inconsistent with the results of Fewtrell and Metzger (28).

The sensitivity of apparently monomeric IgE-mediated responses to inhibition by monovalent hapten (32, 33) suggests the possibility that they are caused by co-isolation of small fragments of the haptenated affinity matrix that is used to purify these IgEs. In our experience, this is a major problem when N-hydroxysuccinimide-derivatized materials are used to prepare the affinity matrix, suggesting that similar problems could occur when cyanogen bromide-conjugated matrices are used. In addition, the highly cytokinergic anti-DNP IgE monoclonal antibody clone specification SPE-7, which most strongly triggers mast cell activation in these studies, exhibits an unusual conformational isomerization in its antibody-combining site (34) that might give rise to cross-reactivity with some cell surface antigen.

In contrast to claims that monomeric IgE can trigger mast cell signaling, binding of IgE appears to limit the potency of signaling by FcεRI dimers. Several different monoclonal anti-receptor antibodies are as potent as multivalent antigen in stimulating strong mast cell responses, although most of these antibodies can form only FcεRI dimers (35, 36). These monoclonal antibodies compete for IgE binding, and so only trigger cells in the absence of bound IgE, suggesting a fundamental difference in the capacity of FcεRI with IgE bound to stimulate responses. Thus, it appears that bound IgE substantially limits the response to FcεRI dimerization. Studies with an anti-IgE monoclonal antibody that form dimers but not longer chains (37) are consistent with this conclusion. IgE-induced dissociation of interactions between a 78-kDa protein and FcεRI (38) may be relevant to this regulation. It is also possible that bound IgE simply imposes steric constraints on the interactions between cross-linked FcεRI and Lyn kinase.

Studies with synthetic bivalent haptens provide further insights into this issue. These studies show that formation of small cyclic complexes of IgE, first demonstrated by Schweitzer-Stenner et al. (39), severely restricts stimulated responses. Minimal length bivalent haptens such as N,N'-di-DNP-lysine were found to be ineffective for stimulating anti-DNP IgE-sensitized RBL and peritoneal mast cells, whereas a somewhat longer bivalent ligand, (DCT)₂-cys, was found to be more effective, especially in the presence of an enhancing reagent such as D₂O (40) or cytochalasin D (41). Bivalent ligands can form linear and/or cyclic chains of various sizes with bivalent IgE. Binding and functional studies showed that stimulation by (DCT)₂-cys is limited because of its propensity to form cyclic dimeric IgE-receptor complexes, which are ineffective stimulators even when cross-linked into larger complexes with an anti-IgE monoclonal antibody (37). This latter observation suggests that the geometry of these cyclic complexes limits their capacity for signal initiation. As depicted in Fig. 2, this restriction could limit access of kinases to ITAM sequences in the complex. Consistent with this explanation, we found that these cyclic dimers exhibit reduced phosphorylation of FcεRIβ relative to functionally active, larger complexes formed at higher concentrations of this ligand (41). Evidence for interactions between the Fc segments of IgE in these cyclic complexes comes from kinetic studies of (DCT)₂-cys dissociation from IgE versus its F(ab)₂ fragment (42), which is consistent with the structure for cyclic complexes shown in Fig. 2.

These studies indicate that dimeric IgE-receptor complexes are poor stimulators of signaling leading to degranulation, and the formation of geometrically constrained cyclic complexes contributes to this restriction. In this regard, it is possible the

covalent IgE dimers that can bind to two Fc ϵ RI on the cell surface are those in which covalent coupling occurs between the C-termini of IgE heavy chains. Such dimers would cause the same orientation of Fc ϵ RI pairs as that for cyclic complexes depicted in Fig. 2.

Inhibitory bivalent ligands

It is well established that monovalent haptens compete with multivalent antigen to block mast cell stimulation (43, 44). However, effective inhibition requires concentrations of monovalent haptens that are orders of magnitude higher than those of multivalent antigen because of the large avidity difference, especially for IgE bound to a cell surface. Consequently, competitive monovalent antagonists are not practical for therapeutic inhibition of specific allergic disease such as anti-drug allergies. We reasoned that bivalent haptens that are sufficiently long and flexible should effectively cross-link bivalent IgE intramolecularly with high avidity, and these stable 1:1 complexes should be much more potent inhibitors of stimulated degranulation than monovalent haptens. We tested this hypothesis with a series of bivalent DNP ligands with polyethylene glycol (PEG) spacers of various lengths in a range estimated to bind to both antibody-combining sites simultaneously (45). The efficacy of these ligands as inhibitors was predicated on the expectation that they would be ineffective stimulators of mast cell activation, even if they caused intermolecular IgE/Fc ϵ RI

cross-linking because of the strong tendency for such ligands to form ineffective cyclic dimers as described above. Indeed, a series of DNP-PEG_x-DNP ligands with extended lengths between 5 and 85 nm failed to stimulate degranulation at any concentration between 0.1 and 1000 nM, despite efficient cross-linking of IgE/Fc ϵ RI. The most effective of these bivalent ligands, having an extended length of 30 nm, inhibited maximal degranulation by DNP₁₅-BSA with an inhibitor concentration for 50% reduction (IC₅₀) of <10 nM, which is better than 10-fold more effective than monovalent DNP-caproate (45). Furthermore, a tetravalent ligand of the same length, in which two DNP groups are attached at each end of the PEG polymer, also fails to stimulate degranulation, and it inhibits DNP-BSA-stimulated degranulation more than 30-fold better than DNP-caproate. This enhanced competitive advantage is because of increased probability that one of the two DNP on each end will bind. For this tetravalent ligand, complete inhibition of the degranulation response occurs at a concentration of inhibitor (10 nM) that is more than 100-fold lower than the corresponding concentration of DNP-caproate. These results are summarized in Fig. 3.

We found unexpectedly that monovalent DNP-PEG derivatives of lengths similar to the effective bivalent ligands are more potent than small haptens at inhibiting degranulation, although they are less potent than their bivalent or tetravalent counterparts. Fig. 3 summarizes these inhibition data for several of these ligands as well as a small dendritic PEG ligand with a valency of four that also fails to stimulate degranulation. In more recent studies, we investigated the mechanism by which these monovalent DNP-PEG derivatives inhibit antigen-stimulated degranulation. Our binding data are consistent with a model in which long PEG tails can self-associate, effectively forming a bivalent ligand in situ when two monovalent ligands simultaneously occupy the two combining sites of a single IgE (R. Das, unpublished results) (Fig. 3). For the longest PEG derivatives tested, additional effects because of steric blockage by these highly hydrated PEG structures are observed. These results are relevant for therapeutic applications because PEG has been shown to be pharmaceutically safe in many uses (46), and the monovalent derivatives are much less likely to have stimulatory side effects that could limit the utility of these compounds as specific inhibitors of allergic responses to drugs such as penicillin and related antibiotics. Furthermore, PEG conjugates with other ligands, such as agonists or antagonists of G-protein-coupled receptors, may also provide significant advantages to these drugs in prolonging their clearance times (47) and/or in enhancing their binding affinities. Of potential importance for such applications is the introduction of an

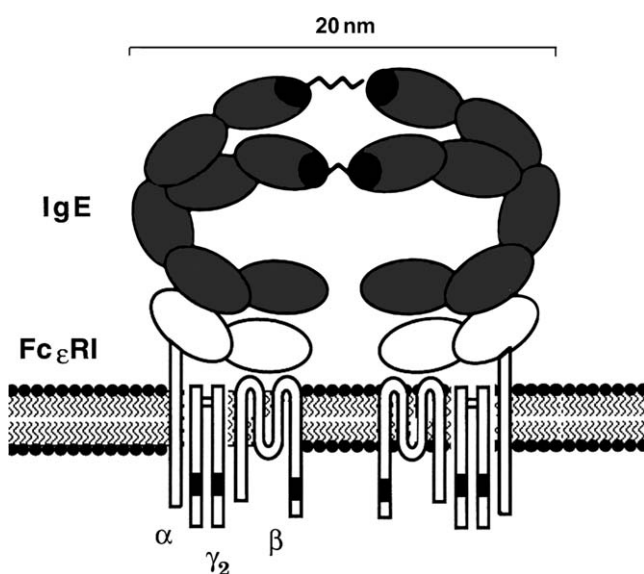


Fig. 2. Proposed structure for IgE-Fc ϵ RI complexes cross-linked into cyclic dimers by bivalent ligands. Jagged lines between IgE-antibody-combining sites represent bivalent ligand spacers, and the bent conformation for IgE and Fc ϵ RI structures is based on previous studies (61, 63). Adapted from Harris et al. (41).

appropriate spacer between the structure that fits in the receptor-binding site and the PEG chain. This proved to be a significant feature of the DNP-PEG ligands that we designed and characterized for binding IgE (45).

Spacing between cross-linked complexes

A long-standing question is whether cross-linked FcεRI must be tethered within some critical distance for signal initiation to occur. Models in which Lyn kinase bound to one receptor in a cross-linked complex can transphosphorylate a neighboring receptor (48) predict that this process will be limited if cross-linked receptors are held too far apart. In contrast, models that do not invoke the need for specific binding of Lyn to receptors for signal initiation, such as the simplest models based on Lo/Ld segregation by lipid rafts (22), do not predict strong distance dependence between cross-linked receptors for signal initiation. As discussed above, receptors that are cross-linked to form cyclic dimers by small, flexible bivalent ligands are ineffective mediators of mast cell signaling, at least in part because of steric restrictions to the access of ITAMs by the relevant kinases. For cross-linking by multivalent antigens, the heterogeneous nature of these ligands makes it likely that at least some orientations and spacings between cross-linked receptors will be favorable,

regardless of the molecular mechanism of signal initiation. Thus, well-defined ligands with known valencies and rigid spacings between haptenic groups are valuable for investigating this question.

Our early efforts to investigate the role of receptor spacing used linear polymers of avidin that were constructed with bivalent biotin ligands, as originally described by Green et al. (49). These polymers were stabilized by chemical cross-linking, and the ends were capped with DNP-biotin derivatives, providing a series of DNP antigens with one or two DNP groups at each end of a linear chain of avidin monomers that varied in length between 4 nm for monomeric avidin and 24 nm for the hexameric polymer (50). These antigens were characterized by electron microscopy, binding studies, and measured effects on IgE/FcεRI lateral diffusion, and their capacities to stimulate degranulation by cross-linking anti-DNP IgE bound to FcεRI were compared. We found the smallest monomeric avidin antigen to be substantially less effective at stimulating degranulation than longer antigens, which were similarly potent. These results suggested that cross-linked receptors do not need to be held in close proximity to effectively stimulate downstream signaling. However, limitations in obtaining sufficient quantities of purified polymers to fully characterize the homogeneity and valency of these antigens made it difficult to extend the initial observations.

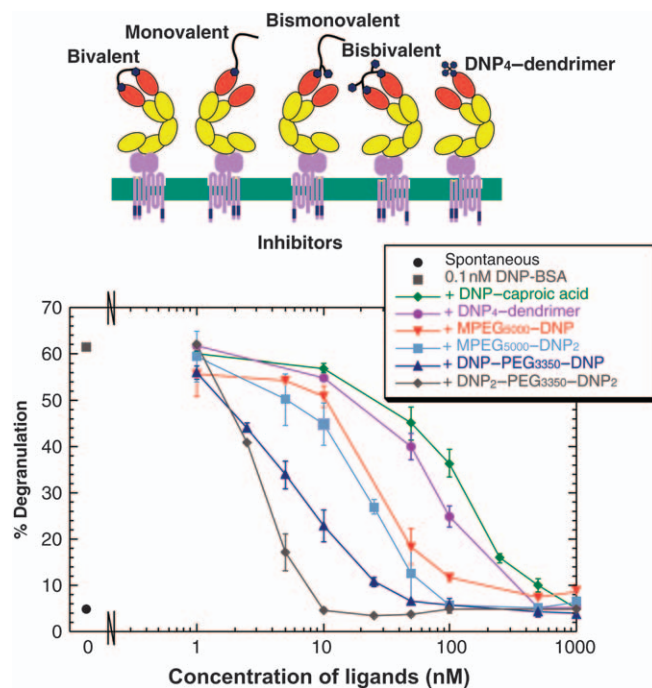


Fig. 3. Inhibition of degranulation by PEG-based ligands. Summary of various PEG-based ligands bound to IgE-FcεRI (top) and data for inhibition of DNP₁₅-BSA-mediated degranulation by these ligands (bottom). Adapted from Baird et al. (45).

Bivalent double-stranded DNA ligands

In a subsequent study, we used double-stranded DNA (dsDNA) as a rigid spacer to prepare a series of bivalent DNP ligands with increasing lengths in the range of 4–10 nm (51). These ligands all bound to anti-DNP IgE with high-affinity and efficiently cross-linked IgE on cells and in solution. When stimulated degranulation was compared, we found that the shortest ligands (4–5 nm rigid length) stimulated substantially more degranulation than ligands with longer spacers in the range of 7–10 nm rigid lengths (51). Introduction of a flexible joint in the longest ligand converted it from a non-stimulatory to a stimulatory ligand, with potency similar to the shortest ligands. As we had observed previously with (DCT)₂-cys, the dose dependence for degranulation with these rigid bivalent ligands was shifted to higher concentrations than that predicted from equilibrium binding analysis, and efficient formation of cyclic dimers at optimal concentrations for cross-linking is the most likely explanation for this limited stimulation. Sensitization with a bispecific IgE containing only a single anti-DNP-combining site limits cross-linking to linear dimers of IgE-receptor complexes by these bivalent ligands, and it resulted in

a large shift in the dose–response curve for degranulation to lower concentrations of these ligands. This finding confirms that the formation of cyclic complexes with wildtype bivalent anti-DNP IgE is indeed restrictive, although the linear dimers formed with the bispecific IgE were also only poorly stimulatory, thus limiting their utility in these studies (51).

None of these rigid bivalent ligands stimulated a detectable calcium response in the absence of cytochalasin D, and these responses were small and transient in the presence of this potentiator of FcεRI signaling. Degranulation stimulated by these ligands was also strongly dependent on potentiation by cytochalasin D, further emphasizing that these ligands trigger only limited signaling. Nonetheless, these rigid ligands are among the most potent bivalent ligands we have characterized, and the results obtained point to a clear dependence of stimulated signaling on the length of the rigid spacer between haptenic groups. Efforts to investigate the early tyrosine phosphorylation signals stimulated by these ligands were limited by the small amounts of stimulated phosphorylation observed and the difficulties in quantifying these limited responses of non-linear, near-threshold western blotting signals. These limited responses are generally consistent with those of our previous observations using synthetic bivalent ligands of various structures described above, and they underscored the need for ligands that trigger more substantial signaling to more fully characterize length-dependent responses of well-defined ligands with rigid spacers.

Trivalent DNA ligands

For enhanced cell stimulation while comparing ligands with rigid spacings of different lengths, we took advantage of new capabilities for constructing branched dsDNA (52). In particular, we prepared a series of trivalent ligands by hybridizing three single strands of DNA, each 5′-modified with a DNP derivative of N-hydroxysuccinimide (Fig. 4). DNA sequences were chosen (52) to maximize stable hybridization at physiological temperatures. The properties of these ligands are summarized in Table 1, including calculated distances between DNP haptens, binding constants for cell-bound anti-DNP IgE, and peak molecular sizes of the complexes formed with IgE in solution as determined by gel permeation chromatography. The capacity of these ligands to trigger degranulation was measured as a function of concentration in the presence and absence of cytochalasin D to enhance signaling. Similar trends were obtained under these two different conditions, although the magnitudes of the degranulation responses were approx-

imately three- to four-fold greater in the presence of cytochalasin D, consistent with previous results with paucivalent ligands described above. At the ligand concentration optimal for stimulated degranulation by the shorter ligands (30 nM), the longest ligand, Y46-DNP3, triggered only approximately 20% as much degranulation as the shorter ligands in the presence of cytochalasin D, and no significant degranulation in its absence. As shown in Table 1, degranulation stimulated by this optimal concentration of the shorter ligands ranged from 13 to 19% in absence of cytochalasin D and from 54 to 67% in its presence.

Similarly, we found that integrated calcium responses stimulated by these ligands were approximately three- to fourfold greater for the shorter trivalent ligands than for Y46-DNP3 (D. Sil, unpublished results). These trends are qualitatively similar to the results summarized above for the bivalent dsDNA ligands, in which the longer rigid spacers resulted in smaller degranulation responses. However, these trivalent ligands stimulate substantially larger calcium and degranulation responses than bivalent dsDNA ligands of similar lengths. A principal difference between the cross-linked receptor complexes formed by bivalent and trivalent ligands is the capacity to form branched networks by trivalent ligands. Bivalent ligands can only form linear or circular complexes with bivalent IgEs, and this may be a fundamental difference that accounts for their limited capacity to trigger mast cell degranulation.

We compared the capacity of dsDNA trivalent ligands of different lengths to trigger early tyrosine phosphorylation responses, including stimulated phosphorylation of FcεRIβ and γ₂ by Lyn kinase and phosphorylation of LAT by Syk kinase. Both these responses show a length dependence that is qualitatively similar to degranulation and calcium responses, such that stimulated tyrosine phosphorylation of FcεRI and LAT are strongest for the shortest ligand, Y16-DNP3, and weakest for the longest ligand, Y46-DNP3 (Fig. 5A). Quantified blotting data from multiple experiments showed a 4- to 10-fold difference between these responses for the shortest and longest ligands, indicating that length-dependent differences in early tyrosine phosphorylation by Lyn and Syk can account for the length-dependent differences in stimulated calcium and degranulation responses (D. Sil, unpublished results). Because cross-linking of IgE/FcεRI by Y46-DNP3 is similarly efficient as for cross-linking by the shorter dsDNA trivalent ligands (unpublished results) (Table 1), these results show clearly that the spacing between cross-linked complexes at the cell surface is important for initiation of FcεRI phosphorylation by Lyn kinase and subsequent signaling events.

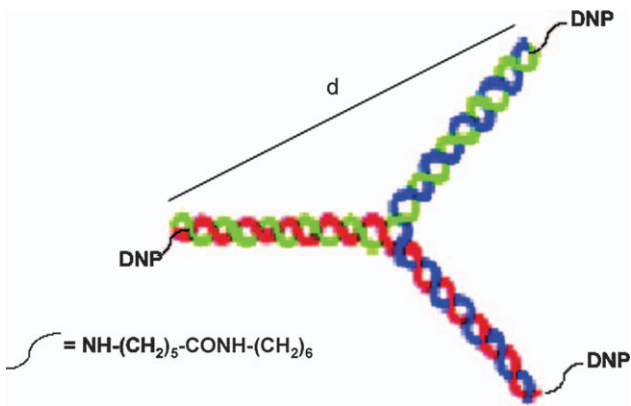


Fig. 4. Schematic diagram of a rigid trivalent DNA ligand showing three hybridized strands of DNA in different colors. The distances *d* between the tips are listed in Table 1 for the four ligands tested. Chemical structure for the spacer (1.4-nm extended length) separating DNP and the 5'-end of each polynucleotide strand is also indicated. Adapted from Li et al. (52).

These results are consistent with transphosphorylation as part of the mechanism for FcεRI activation: the spacing between cross-linked IgE–receptor pairs determines the efficiency of stimulated signal initiation. Although rigid spacings between 4.5 and 13.5 nm are all sufficient for detectable activation to occur, signaling triggered by the longest ligand, Y46-DNP3, can stimulate only a very small calcium response, and it is insufficient to trigger significant degranulation in the absence of cytochalasin D. As shown by Kinet and colleagues (15), antigen-stimulated tyrosine phosphorylation mediated by FcεRI is substantially reduced in the absence of FcεRIβ, implicating a role for Lyn binding to β subunit in this signaling process. Our results with rigid cross-linking ligands support a model in which Lyn binding to FcεRIβ facilitates the phosphorylation of an adjacent receptor(s) in the cross-linked

complex. Recent studies show that this role for FcεRIβ depends on its ITAM phosphotyrosines (16, 53), consistent with the need to initiate this phosphorylation cascade by Lyn not bound to FcεRI. As discussed above, enhanced interactions between cross-linked FcεRI and active Lyn within the protective environment of Lo lipid domains can account for this initiation. Consequent binding of Lyn to phosphorylated β-ITAMs then accelerates the process, yielding the substantially larger tyrosine phosphorylation response measured in the presence of FcεRIβ.

The apparent importance of branched cross-linked complexes described above provides further evidence that some large-scale structural organization of cross-linked IgE–receptor complexes is fundamental to their capacity to function optimally in mast cell stimulation. Cross-link-dependent nucleation of Lo membrane domains is one possible explanation for this requirement. By comparison, the length dependence for triggering by the rigid bivalent and trivalent ligands we have characterized provides the most direct evidence to date that spacing between cross-linked IgE–receptor complexes is also a critical parameter in this process. Our findings with both bivalent and trivalent ligands indicate that those with rigid spacers of 4–5 nm are more effective than ligands with shorter, flexible spacers, possibly because of the steric restrictions of these shorter cross-links described above. Ligands with rigid spacers that are two- to threefold longer than this optimal spacing stimulate progressively reduced degranulation responses, with a sharper reduction in response for rigid spacers of greater than approximately 10 nm. As indicated by the scale bar in Fig. 2, this longer spacing is likely to prevent direct receptor–receptor interactions in cross-linked complexes, despite some limited flexibility of the IgE Fab segments (54), such that Lyn bound to one receptor in a complex would need

Table 1. Summary of properties of trivalent DNA–DNP ligands

Name*	Length† (nm)	K _d (nM)‡	Ligand/IgE	Average degranulation¶	
			Complex MW§	– cyto D	+ cyto D
Y16-DNP3	4.5	1.5 ± 0.5	615 k	19	67
Y26-DNP3	7.5	1.2 ± 1.0	600 k	16	54
Y36-DNP3	10.5	1.5 ± 1.0	620 k	13	54
Y46-DNP3	13.5	1.6 ± 0.5	710 k	1	15

cyto D, cytochalasin D; MW, molecular weight.
*For Yn-DNP3, Y refers to the tribranched structure shown in Fig. 4; each of the three ends is conjugated with DNP by a flexible linker; n is the number of nucleotide bases in each of the three single-stranded DNA comprising the branched dsDNA structure.
†Calculated distance between adjacent ends of dsDNA rigid trimers (*d* in Fig. 4).
‡Equilibrium dissociation constant for ligand binding to cell-bound IgE, assuming an equivalent site model. Average values for *n* > 3 ± SD.
§Peak MW from gel permeation chromatography profile for 1:1 molar ligand: IgE mixture, monomeric IgE = 200 k.
¶Average percent stimulated release of granule marker β-hexosaminidase for *n* = 3 experiments, each in the absence or presence of cyto D; spontaneous release ≤5%.

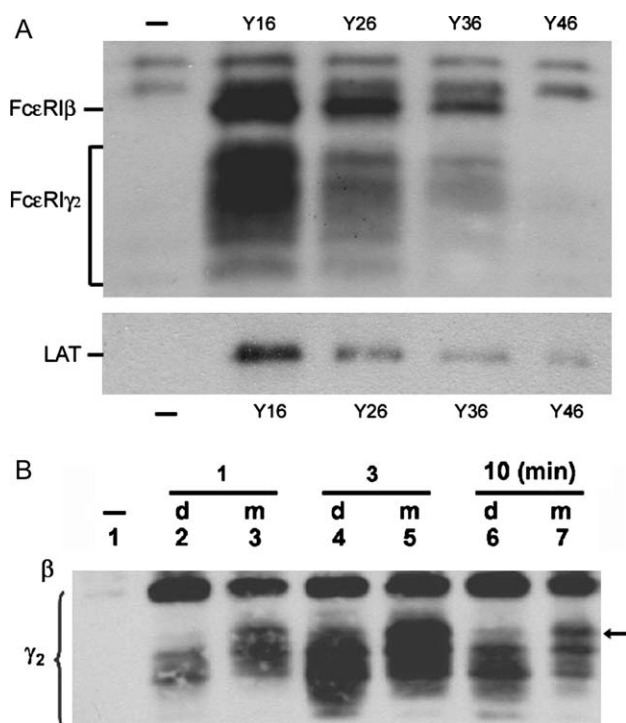


Fig. 5. Anti-phosphotyrosine western blots of immunoprecipitated FcεRI and LAT. (A) The spacer length dependence of trivalent DNA ligands (30 nM) for stimulated phosphorylation of FcεRI (upper panel) and LAT (lower panel). (B) Stimulated FcεRI phosphorylation by 0.2 nM DNP-multivalent antigen (m: lanes 3, 5, and 7) compared with 100 nM DNP-divalent DNA (d: lanes 2, 4, and 6). Note the slower mobility γ_2 band (arrow) that is strong for multivalent and weak for divalent stimulation at all time points.

to dissociate, at least transiently, to phosphorylate an adjacent receptor.

In another study, we found that bivalent ligands prepared from Y-shaped dsDNA by modifying two of the three strands with the DNP hapten stimulate a phosphorylation pattern different from that for multivalent-antigen-stimulated phosphorylation. The multiple species of FcεRI γ_2 bands phosphorylated by these bivalent DNA ligands consistently migrate at faster mobilities than those phosphorylated by multivalent ligand, independent of ligand spacer length or time of stimulation (Fig. 5B). This finding indicates a qualitative difference in the capacity of these bivalent ligands to stimulate FcεRI γ_2 phosphorylation compared with ligands of higher valency (C. Torigoe, unpublished data). These results are reminiscent of the differences in stimulation of TCR ζ_2 phosphorylation by full and partial agonist peptides (55). They suggested that the limited downstream signaling observed for stimulation by these bivalent ligands may be related to their failure to fully phosphorylate FcεRI γ ITAM tyrosines, as shown for partial agonist and antagonist peptides in TCR-mediated responses (56).

An interesting finding in our studies with trivalent dsDNA ligands is that although FcεRI and LAT exhibit length-dependent tyrosine phosphorylation, another substrate of Syk kinase, PLC γ 1, is phosphorylated to similar extents by these ligands regardless of their length (D. Sil, unpublished data). Consistent with these observations, we find that stimulated calcium release from stores for the longest trivalent DNA ligand, Y46-DNP3, is similar to that for shorter ligands, as expected if phosphorylation of PLC γ by these ligands activates IP $_3$ production by this enzyme to similar extents (10). Because both PLC γ and LAT are substrates of Syk kinase (57), it is not expected that phosphorylation of one is length dependent, whereas phosphorylation of the other is not. One possible explanation for this difference is that PLC γ is tyrosine phosphorylated by both Syk and the Tec family kinase Bruton's tyrosine kinase (Btk) (10), and Btk activation may be independent of ligand length. Alternatively, structural interactions involved in LAT phosphorylation and PLC γ phosphorylation by Syk and/or Btk may be different, such that LAT phosphorylation depends more strongly on interactions with cross-linked IgE-receptor complexes, whereas PLC γ phosphorylation does not. Because PLC γ phosphorylation depends on LAT (13), it might be expected to depend on the extent of LAT phosphorylation. Btk, in contrast, is activated by Lyn phosphorylation (58), so its activation may only depend on recruitment of Btk to the vicinity of active Lyn.

Additional experiments are needed to distinguish among these explanations. However, regardless of the mechanism by which PLC γ is activated, our results indicate that the ligand length dependence of calcium mobilization that we observe is likely to result from differences in the activation of calcium influx that are not wholly accounted for by differences in PLC γ -mediated calcium release from stores. Recent studies showed a role for translocation of the calcium sensor protein STIM1 from internal membranes to the plasma membrane, where it appears to activate the store-operated calcium influx channel, CRACM1 (59, 60). This process depends on the emptying of calcium from endoplasmic reticulum stores, but other steps, such as vesicle trafficking to the plasma membrane, may also be necessary for STIM1-CRACM1 interactions. These may have additional signaling requirements that are sensitive to ligand-length-dependent tyrosine phosphorylation of FcεRI and/or LAT.

Summary and conclusions

The use of chemically defined paucivalent ligands has enabled a better understanding of the structural constraints that IgE

binding to FcεRI imposes on cross-link-dependent signaling. As described in this review, dimers of IgE–FcεRI complexes are severely limited in their signaling capacity in RBL mast cells, whereas dimers of FcεRI produced by anti-receptor monoclonal antibodies stimulate strong activation responses similar to those stimulated by multivalent antigens. Small, flexible bivalent ligands have a high propensity to form small, cyclic IgE–receptor complexes, especially cyclic dimers, and these are generally poor stimulants, implicating geometrical constraints that limit the capacity of these complexes to initiate ITAM phosphorylation. In addition, longer flexible bivalent ligands that can form intramolecular cross-links between two antibody-combining sites on the same IgE are efficient inhibitors of intermolecular cross-linking by multivalent antigens, and they more effectively block antigen-stimulated degranulation than monovalent ligands, pointing to a potential therapeutic advantage. Monovalent haptens with long PEG tails are intermediate in effectiveness as inhibitors between bivalent PEG-based ligands and smaller monovalent haptens, suggesting that, by weak self-interaction, PEG derivatization may confer more general efficacy for pharmacologic antagonists.

A series of studies with rigid cross-linking ligands leads to the conclusion that signal initiation strongly depends on the length of the spacing between cross-linked IgE–receptor complexes. These results provide strong evidence for structural constraints within the signaling mechanism. Detailed analyses of early signaling stimulated by trivalent DNP ligands with dsDNA spacers showed a strong dependence of FcεRI ITAM phosphor-

ylation on rigid spacer lengths in the range of approximately 5–14 nm, which results in a corresponding length dependence for stimulated calcium and degranulation responses. These studies support a critical transphosphorylation step after receptor phosphorylation is initiated within *Lo* domains: Lyn kinase binding to one receptor in a cross-linked complex then amplifies the signaling by phosphorylating an adjacent receptor(s) in that complex, provided that the distance between them is less than approximately 10 nm. In contrast, at least one stimulated phosphorylation event, the phosphorylation of PLCγ, has no detectable length dependence, suggesting a different structural requirement for its activation during the signaling process.

These collective studies show a functional role for the highly asymmetric structure of IgE bound to FcεRI (61): the steric regulation of productive IgE–FcεRI cross-linking leading to mast cell activation. In addition, studies with bivalent and monovalent PEG-based ligands show new strategies for more effective inhibition of IgE receptor signaling that may have practical applications for more effective control of specific allergic responses. Last, recent results summarized provide strong evidence for the importance of receptor proximity in cross-link-dependent signal initiation, and they suggest practical strategies for further exploration of the structural details of transmembrane signaling by this immune receptor prototype. Indeed, studies of IgE–FcεRI signaling with chemically defined ligands show structural facets of signal initiation not evident from more conventional stimulation with chemically heterogeneous multivalent antigens.

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