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Point of VIEW

Signaling by Committee: Receptor Clusters Determine Pathways of Cellular Activation

Christopher W. Cairo*

Department of Chemistry, Alberta Ingenuity Centre for Carbohydrate Science, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

ABSTRACT Receptor clustering is a common signaling mechanism for cell surface receptors. Exogenous ligands such as antibodies or synthetic analogues can be used to artificially induce clustering. New studies using defined synthetic ligands suggest that the spatial organization of these clusters attenuates signaling in one pathway but has no effect in another.

*Corresponding author, ccairo@ualberta.ca.

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The components of the cell membrane serve many roles. Far from being a static barrier between cellular contents and the extracellular environment, the proteins and lipids of the membrane shuttle both molecules and information between these compartments. Though many integral membrane proteins that bind to extracellular components are well studied, key questions still remain about the physical nature of the process that converts receptor ligation to signal output. An area where chemists have begun to introduce new tools to understand these processes has been the clustering of receptors within the plasma membrane. On page XXX of this issue, Baird *et al.* explore the signaling pathways associated with the FcεRI immunoreceptor complex. Using synthetic multivalent ligands, they find that downstream signaling is composed of two major pathways that differ in their ability to respond to changes in the structure of receptor clusters.

It has long been recognized that the aggregation, or clustering, of receptors within the membrane can activate signaling pathways (*1*). Although the tools of molecular biology have provided exceptionally sensitive methods for observing signaling output, such as phosphospecific antibodies or intracellular calcium sensors, the structure and orientation of receptor clusters has remained a difficult problem. Traditional methods to induce clustering are antibodies, inherently multivalent structures that can induce receptor cross-linking. But antibody structures leave much to be desired from the point of view of a biologist seeking to understand receptor clustering at a molecular level: How close do the receptors need to be to be "clustered"? How many receptors form an active cluster? Must they be in a particular orientation? Although a bivalent antibody may be able to induce an active cluster, it provides no mechanism to ask these subtle structural questions.

Chemists have addressed these questions by using synthetic ligands with defined characteristics—by changing valency, distance between sites, and orientation—to examine the physical process involved in clustering (*2*). Baird *et al.* (*3*) present a comprehensive study of structurally defined multivalent ligands and their effect on the signaling of the FcεRI immunoreceptor. Building on a large body of pioneering work on this receptor system (*4*), the authors use rigid trivalent ligands to induce receptor clustering and probe the effects of altered clustering on downstream signaling. They test the effect of altering the distances between receptors and observe that two pathways appear to be in play: one that is sensitive to the distance between ligand sites (ligand-length dependent) and one that is not (ligand-length independent).

Immunoreceptor Signaling. The FcεRI immunoreceptor is a well-studied complex involved in the allergic response mediated primarily by mast cells (*5*). The complex consists of four subunits: an α, β, and two γ chains (see Figure 1). Many of the details of FcεRI downstream signaling remain obscure. Activation of FcεRI results in degranu-

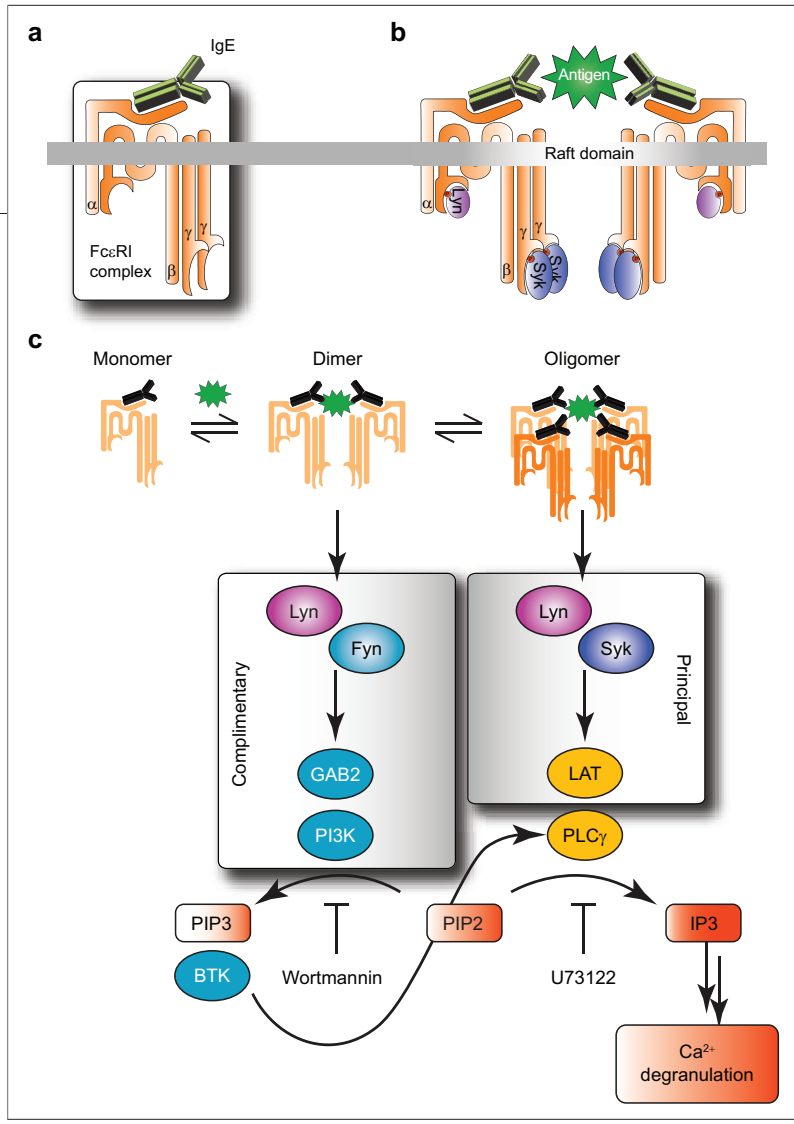

Figure 1. FcεRI signaling pathways. Schematic representation of key signaling elements downstream of the FcεRI complex. a) The receptor complex is composed of an α, β, and two γ chains. The β and γ chains contain ITAM domains, which become phosphorylated upon receptor activation. b) Multivalent antigen recognized by IgE induces clustering of the receptor. As a cluster, the complex partitions into a lipid raft domain and is phosphorylated by Src kinases, such as Syk and Fyn. c) A proposed schematic of the signaling pathway downstream of FcεRI in light of the results of Baird *et al.* (*3*). Small clusters or dimers induce activation of the complementary pathway generating phosphatidylinositol-3,4,5-triphosphate (PIP3), which may activate PLCγ through BTK inducing Ca^{2+} release and other downstream effects. This pathway is ligand-length independent and requires only minimal cross-linking of the receptors. A second ligand-length-dependent pathway, the principal pathway, also terminates in Ca^{2+} release and degranulation. Inhibitors that disrupt these pathways (U73122 and wortmannin) were used in the study. Adapted in part from Gilfillan *et al.* (*6*). Abbreviations: GAB2, GRB2-associated binding protein 2; PIP2, phosphatidylinositol-4,5-bisphosphate; and IP3, inositol-1,4,5-triphosphate.

lation of mast cells, releasing inflammatory mediators from secretory granules that lead to inflammation. The receptor binds to IgE antibody molecules; however, binding to individual IgE will not lead to activation. The α-chain of the receptor recognizes IgE molecules in complex with multivalent antigens through binding of multiple IgE molecules, which then induce clustering of the receptor complex (see Figure 1).

The signaling pathway downstream of FcεRI begins with the phosphorylation of components of the receptor complex by Src family kinases Syk (spleen tyrosine kinase), Lyn, and Fyn. The Src kinases interact with the immunoreceptor tyrosine activation motifs (ITAMs) found in the FcεRI β and γ chains, leading to phosphorylation of these subunits (*6*). The Src kinases are preferentially activated by partitioning into lipid raft domains (*7, 8*), and clustering of FcεRI leads to its recruitment into these domains (*9*). After binding and clustering of the receptor, phosphorylation of the β chain appears to be the first step of the pathway; phosphorylation of the γ chain closely follows, in a step sometimes referred to as "amplification". Phosphorylation of the γ chain results in activation of phospholipase Cγ (PLCγ) through the linker for activation of T cells (LAT). This is usually referred to as the *principal pathway* of FcεRI signaling (*6*). Alternative pathways may also be involved in FcεRI signaling. One proposal is the *complementary pathway* which involves stimulation of phosphatidylinositol 3-kinase (PI3K) by the Src kinases Lyn and Fyn (see Figure 1).

It has been long recognized that clustering of FcεRI induces signaling of the receptor, leading to degranulation (*10*). Clustering in this system is likely an advantageous way for the system to recognize its usual ligands, multivalent antigens displaying IgE antibody epitopes. A standard tool for immunologists working on this system has been to use conjugates of bovine serum albumin (BSA) displaying randomly attached groups, such as dinitrophenyl (DNP), for which IgE antibodies are known. Although useful, these conjugates suffer from structural heterogeneity that obscures the organization of the receptor cluster at the molecular level.

Cluster Organization. Clustering of the FcεRI induces downstream signaling through phosphorylation of the ITAM sites in the complex by Src kinases. Using antibodies and antibody fragments, researchers have shown that dimerization of receptors triggers signaling, and the formation of larger clusters leads to increased signaling and receptor immobilization (*11*). Antibodies are inherently limited by their structure. Synthetic ligands provide a powerful alter-

native for testing the properties of clusters with altered properties (such as distance between receptors).

Synthetic bivalent ligands for FcεRI have previously been prepared by the construction of DNA fragments that assemble into rigid double-stranded DNA complexes bearing antibody epitopes at the 5′ ends (*12*). Using these bivalent ligands, it was found that dimeric ligands are less active than multivalent BSA-DNP conjugates and that the distance between the receptors affects activity (*4*). The distance dependence of these ligands suggested that effective triggering of FcεRI signaling requires a structured cluster.

In the present study, Baird *et al.* use a similar strategy to prepare trivalent ligands to interrogate the FcεRI system. Increasing the valency of the ligand causes more complex clustering in the membrane (see Figure 2). By comparing the activities of bivalent and trivalent ligands with similar spacing, this strategy provides a way to change the organization of the receptor cluster. Varying the architecture of multivalent ligands (*13*) has been found to have dramatic effects on a variety of clustering systems (*2*); therefore, this class of ligands is an ideal tool for understanding the structural requirements of the cluster.

As expected from previous work, degranulation triggered by the trivalent ligands showed a strong dependence on distance between epitope sites (*3*). However, further examination of the signaling pathway provided some interesting results. The initial event of the signaling pathway was expected to be phosphorylation of the FcεRI β and γ chains along with LAT, and these events all showed a clear dependence on length. Kinetic resolution of these events confirmed that phosphorylation of the β chain precedes that of the γ chain and that the shorter ligands have a more pronounced effect on both events. Thus, the organization of the complex is a determining factor that controls signal amplification *via* the β- to γ-chain phosphorylation. In contrast to these results, it was also shown that phosphorylation of PLCγ was ligand-length independent. Therefore, two mechanisms of signaling appear to be in play: one that is sensitive to the distance between receptors and one that is not.

Baird *et al.* proceeded to study the details of the signaling pathway downstream of cluster formation. A common step in cellular activation is a rapid increase in intracellular calcium concentration. Calcium ions may be held in intracellular stores or can be allowed into the cell through ion channels in the plasma membrane. Experiments using calcium indicator dyes allowed quantification of the changes in intracellular Ca^{2+} from either source. Although the kinetics are slightly faster for the shortest and most potent ligand, the total Ca^{2+} released is similar for all of the ligands. Signaling components thought to be responsible for inducing intracellular calcium in mast cells are the enzymes PLCγ and PI3K (see Figure 1). Specific inhibitors of each of these enzymes are known; wortmannin is an inhibitor of PI3K and U73122 is an inhibitor of PLCγ (*14, 15*). These inhibitors were employed to test the involvement of each enzyme in FcεRI downstream signaling. It was found that in the presence the PLCγ inhibitor U73122, intracellular Ca^{2+} release is completely inhibited for both trivalent ligands tested and the BSA-DNP conju-

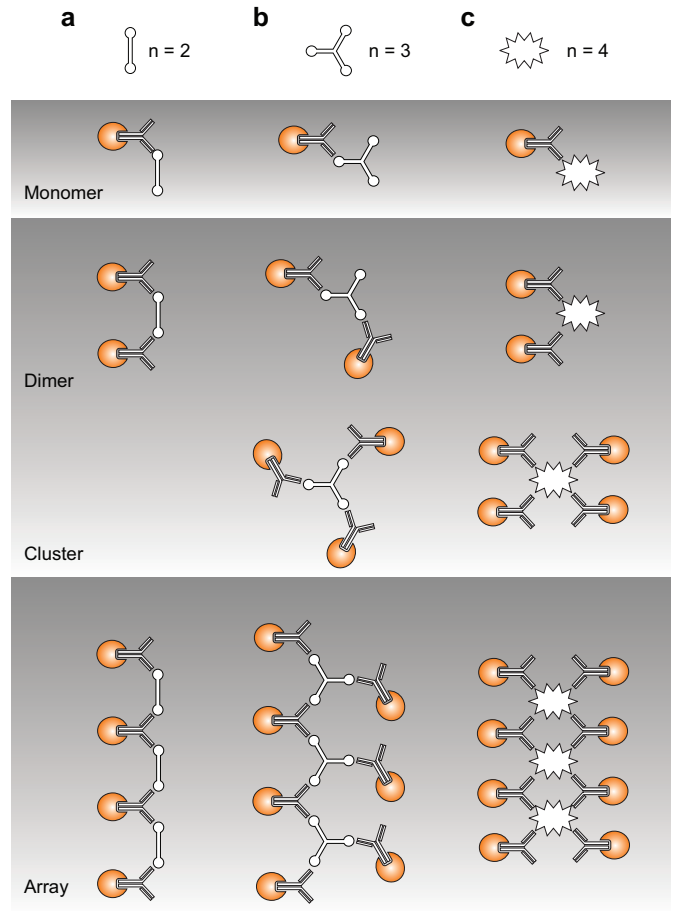

Figure 2. Cluster architecture. The valency and structure of receptor ligands can influence the formation of clusters and arrays on the cell surface. The cluster architecture is dependent on the valency, orientation, and distance between sites. A multivalent receptor and multivalent ligand can combine to form a range of possible structures. Some hypothetical structures are shown for clustering of a) divalent, b) trimeric, and c) tetrameric ligands with a divalent receptor. In addition to dimer structures, larger clusters and extended arrays are possible.

gate. In contrast, the DNP-BSA conjugate is still able to evoke a response in the presence of the PI3K inhibitor wortmannin, whereas the trivalent ligands are not. This result suggests that the DNP-BSA conjugate is better able to utilize the principal pathway of signaling. It is possible that both pathways are involved for the trivalent ligands but that the intensity of signal provided by the DNP-BSA conjugate is able to partly overcome inhibition of the complementary pathway.

How does the structure of the cluster manifest these dramatic changes in signaling? The existence of both a length-dependent pathway (principal pathway) and a length-independent pathway (complementary pathway) suggests different aspects of the cluster are recognized by each. If this is the case, we might expect that any clustering of FcεRI could activate one pathway to provide a basal "on" signal. To provide greater dynamic range, a second pathway could discriminate between small and large clusters, providing a mechanism more sensitive to the number or structure of the antigen. Models of signaling for clustered systems have suggested that one purpose of receptor clustering may be a massive improvement in dynamic range (*16*). The mechanism responsible for these effects may involve conformational change of the receptor (*17*) or a change in the proximity of active sites (*18*). In the system under investigation, it seems likely that small dimer or trimer complexes are active in both pathways; however, very large clusters induced by ligands like the DNP-BSA conjugate would be significantly more potent through a pathway that recognizes cluster size or receptor proximity.

The study by Baird *et al*. provides an exciting window into both the complexity and dynamics of receptor clustering. These results reinforce that receptor clustering is far from a nonspecific aggregation process within the membrane. Instead, it appears that the number and proximity of receptors within the cluster manifest specific molecular changes. These data suggest that the availability of defined multivalent ligands will continue to provide us with crucial data for understanding the molecular organization of membrane complexes.

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