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A conformation-induced oligomerization model for B cell receptor microclustering and signaling

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Summary

The B cell receptor (BCR) generates both antigen-independent and -dependent intracellular signals that are essential for B-cell development and antibody responses against pathogens. However, the molecular mechanisms underlying the initiation of BCR signaling remain incompletely understood. The advent of new imaging technologies is allowing the earliest events in B cell signaling to be viewed both *in vivo* in lymphoid tissues and *in vitro* in living cells, in real time, down to the single molecule level. Here we review recent progress in the use of these technologies to decipher the earliest events that follow B cell antigen recognition. Based on recent data using these techniques, we propose a model for the initiation of BCR signaling in which the binding of antigen induces a conformational change in the BCR's extracellular domains leading to BCR oligomerization and signaling. We conclude that testing this model will require an in depth understanding of the unique structural and organizational features of the BCR in the plasma membrane of living B cells in the presence and absence of antigen.

Introduction

A hallmark of adaptive immunity is the production of highly specific, high affinity antibodies that serve to eliminate pathogens from the host. The production of antibodies is triggered by direct recognition of antigens by the clonally distributed B-cell antigen receptors (BCRs) expressed on B-cell surfaces. Once bound to antigens, the BCR triggers a sequence of intracellular signaling events and the internalization of antigens that ultimately result in B-cell proliferation and differentiation into plasma cells secreting antibodies [1]. In addition to the antigen-induced initiation of antibody responses, the BCR also generates what are believed to be antigen-independent signals that are important for the development and homeostasis of B cells. In pre-B cells, the expression of the pre-BCR, containing a surrogate light chain, leads to clustering of the pre-BCR and the commencement of the development of the pre-B cells into mature B cells [2,3]. In resting mature B cells, the BCR produces continuous low level, 'tonic signals' that are critical for B cell survival [4,5]. With such a wide range of functions of the BCR, the molecular mechanism of initiation of BCR signaling is likely to be both intricate and interesting.

The BCR is a multichain receptor composed of a membrane form of immunoglobulin (mIg) and a heterodimer of Igα and Igβ accessory chains [1]. Although the mIg binds antigens, its short cytoplasmic tails do not directly connect to the B cells signaling machinery. The all-important intracellular signaling and internalization of the antigen- BCR complex are the function of the cytoplasmic domains of the Igα and Igβ chains. Over the last several years many of the components of the B cell's intracellular signaling cascades have been characterized in considerable detail [6]. The first proteins that are activated and recruited to

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the BCR following antigen binding are members of the Src-family kinases, namely Lyn, Blk and Fyn [7]. Src kinases phosphorylate essential tyrosines in the intracellular domains of Igα and Igβ. These tyrosines are part of theimmunoreceptor tyrosine-based activation motives (ITAMs) and once phosphorylated they bind the SH2 domains of the kinase Syk. The activation of the Src-kinases and Syk triggers signaling cascades that involve the activation of at least four major signaling pathways including phospholipase C, the Rho family of GTPases, Ras and phosphatidylinositol-3-kinase [4,6]. In addition, the initial signaling also triggers internalization of the BCR-antigen complex into intracellular compartments where the antigen is processed and presented on MHC class II molecules.

Although the downstream signaling pathways that connect the phosphorylated BCR Igα and Igβ chains to B-cell activation are becoming well characterized, the initial molecular events that follow antigen binding to the BCR and lead to ITAM phosphorylation still remain largely obscured. Understanding the molecular mechanisms by which antigen binding to the BCR ectodomains is transduced to the intracellular domains of the BCR's Igα and β chains to initiate ITAM phosphorylation, is essential to fully comprehend the function and regulation of the BCR both in antibody responses and in development. The key aspects of B-cell biology that are inherently dependent on the function of the BCR include: the ability of B cells to recognize and respond to the universe of foreign antigen structures that confront the immune system; the ability of B cells to discriminate the affinity of antigen binding to promote the development of high affinity B cells; the modulation of BCR signaling by coreceptors and the BCR's generation of antigen-independent tonic signals.

The first unique aspect of the BCR is that it is a clonally distributed receptor with an extraordinary diverse repertoire generated by random recombination of V region genes encoding the antigen-binding domains of the mIg. Through this diverse repertoire, B cells are able to respond to an enormous array of antigen structures ranging from components of the bacterial cell wall to small chemical compounds. The ability to respond to such a variety of ligands differing in their structure, size and valency is a unique property of B-cell immunity and is critical for antibody function. However, this property of the BCR raises a fundamental question concerning the mechanism by which signaling is initiated, namely, how does the binding of the universe of foreign antigens by BCRs ultimately engage the common mechanism of ITAM phosphorylation? In this context, understanding the mechanism of BCR activation may provide a molecular basis for the broad recognition of antigens by the BCRs.

A second unique aspect of the B cell response to antigen is the B cells' ability to discriminate the affinity of the interaction of the antigen with the BCR [8]. Affinity discrimination is essential for the affinity maturation of antibodies through iterative cycles of somatic hypermutation and antigen-driven selection, ensuring that antibodies have sufficient affinity for pathogens or their products to prevent disease. The affinities that BCRs can discriminate are in the range of 10^{-6}–10^{-10} M [8]. Presumably, BCR signaling is sensitive to the affinity of the BCR-antigen interaction because the longer the half life of the BCR-antigen complex, the longer the time the cytoplasmic domains have to initiate intracellular signaling. However, the mechanism by which the BCR discriminates such a wide range of affinities is not clear. It is particularly puzzling how affinity maturation occurs in response to multivalent antigens. The avidity of the binding of the bivalent BCR to multivalent antigens that contain many epitopes will provide a large advantage during affinity maturation over BCR binding to monovalent antigens containing only a single epitope. However, the high avidity interaction may quickly reach the ceiling of the affinity discrimination range, leading to lower then desired affinities of the IgG secreted antibodies that cannot benefit from the avidity effect. Thus, understanding the mechanism of BCR

activation will likely have important consequences for our understanding of the generation of high affinity antibodies and ultimately aid in vaccine design.

Third, B-cell responses appear to be both positively and negatively regulated at multiple levels. B-cell coreceptors that interact with the BCRs on the cell surface and modulate BCR signaling, depending on the context of the antigen or the state of the B cell, play an important role for this regulation. Recent studies focusing on CD19 and the FcγRIIB [9,10] illustrated that to understand how coreceptors interact with the BCR, we will need to understand the localization and structure of the activated BCR and as well as that of the coreceptors on the B cell surface. A clearer understanding of this process may reveal new strategies to modulate BCR signaling.

Fourth, An essential feature of the BCR is its ability to propagate tonic signals required for B-cell survival in the apparent absence of antigen binding. In this pro-survival signaling the BCR cooperates with the BAFF receptor [11]. Abrogation of either the BCR or the BAFF receptor leads to B cell death [12–14]. Conversely, excessive signaling from the BAFF receptor leads to B cell hyperplasia and autoimmunity [15]. It is possible that a similar dysregulation of the BCR's pro-survival signaling may lead to diseases. For example, there are indications that BCR signaling is required for the survival of certain types of B cell lymphomas [16]. Presumably, a better understanding of the mechanisms by which the BCR initiates tonic signals could provide opportunities to regulate B cell fate under pathological conditions.

Collectively, these examples illustrate that knowledge of the molecular mechanisms that underlie the activation of the BCR will ultimately be required to gain an in-depth understanding of how B cells develop and how antibody responses are generated. An important step in our effort to understand BCR signaling is to learn more about how B cells recognize antigens *in vivo* and how the binding of the antigens to the BCR in living B cells leads to intracellular signaling. Here we describe a new picture of BCR activation that is emerging from the use of recently developed imaging technologies. By looking at living B cells both *in vivo* in lymphoid tissue and as single cells *in vitro*, these new approaches offer a view of the activation of B cells that was not possible before. Hopefully, learning about the BCR activation in live B cells in real time will lend insights into how the BCR functions in development and how antigen binding activates the BCR and triggers antibody responses.

How B cells see antigens *in vivo*

Although a considerable amount has been learned about the mechanisms of BCR activation from studies of B cells stimulated with soluble antigens *in vitro*, studying B cell interacting with antigens *in vivo* in specialized microenvironments of the lymphoid tissues will be essential to gain a full understanding of how B cells recognize and are activated by antigens. B cells enter lymph nodes through the high endothelial venules in the paracortex and then rapidly move through cortex and B cells follicles localized underneath the lymph node capsule [17]. Recently, using two photon intravital imaging techniques, several groups were able to look inside lymph nodes and directly observe B cells engaging their antigens. Within minutes of injection of fluorescently labeled small soluble antigens in the periphery, the antigens were detected in B cell follicles, suggesting that small soluble antigens have the ability to specifically enter the follicules and activate follicular B cells [18]. In contrast, particulate antigens, such as virions and immune complexes, trafficking through the lymph were efficiently captured by a subset of macrophages lining the floor of the subcapsular sinus. Translocating the antigens from the subcapsular sinus into the lymph node cortex, the macrophages presented the antigens to B cells migrating through the cortex. This resulted in rapid accumulation and activation of B cells at the subcapsular sinus [19–21].

In addition to the contacts with the subcapsular macrophages, B cell were also seen to engage antigens that had been carried into the lymph node by dendritic cells [22]. It is well established that dendritic cells arriving from the periphery present processed antigens to T cells in the T-cell zone of the lymph node. However, unprocessed native antigen was also detected on the DC surfaces and these DC were able to stimulate B cells that migrated through the T-cell zone after they entered the lymph node through the high endothelial venules. These remarkable findings collectively indicate that antigen presenting cells (APCs) actively assist B cells in antigen recognition *in vivo*. Although at present we do not know how the antigens are captured and presented to B cells by APCs, it is likely that the B cell - APC contact represents a critical step in B cell activation *in vivo*, at least for some forms of antigens. These observations point to the importance of understanding how B cells respond to antigens presented in cellular contacts with APCs.

Imaging B cell interactions with antigen *in vitro*: defining the B cell immune synapse

Earlier work from Batista and colleagues showed that B cells avidly respond to antigens presented on the surface of APCs [23]. When binding membrane antigens, B cells form a highly organized contact area, called the immunological synapse that resembles synapses observed in T cells and NK cells engaging their APC or target cells. The B-cell immunological synapse is composed of a central aggregate of the antigen-engaged BCRs, called the cSMAC. Surrounding the cSMAC is a ring of adhesion molecules called the pSMAC, that includes the LFA-1 ICAM-1 pair. Evidence was also provided that during formation of the immunological synapse B cells are not only activated to signal, but also extracted and internalized antigen from the presenting cells. These seminal findings suggested that the organization of the BCR in the immunological synapse is important for BCR activation and antigen internalization.

In more recent studies, Batista *et al.* showed that B cell activation and immune synapse formation can also be observed in B cells interacting with antigens anchored to planar lipid bilayers providing an experimental system that offered better resolution of the initial steps of the contact of the B cell with the antigen [24–26]. These studies showed that after B cells touch antigen-containing bilayers in a few contact points, they initiate a BCR-signaling and actin-dependent spreading that allows the B cells to reach over the antigen-containing bilayer and collect a large number of antigens. The first contact and spreading of the B cells results in the formation of microclusters containing the antigen-engaged BCR. The BCR microclusters stream along actin fibers to the center of the synapse, where they accumulate to form the cSMAC. The spreading of the B cells is short-lived, however, and is quickly followed by contraction that collects all the BCR-bound antigen to the cSMAC. These remarkable observations indicated that the recognition of antigens presented by APCs is a much more active process then previously thought. Because the amount of antigen that the B cells engages depends on the spreading, which in turn is fueled by BCR signaling, B-cell spreading provides a positive feedback on the BCR-mediated collection of antigens. This feedback amplifies the differences in the collection of antigens of variable affinity for the BCR and improves the B cell's ability to discriminate between low and high affinity antigens [25].

Detailed observations of BCR microclusters as they first formed showed that they assembled almost exclusively at the sites of initial contact of the B cell with the antigen-containing membrane and in the peripheral lamellopodia of the spreading B cells [25,27]. This is despite the fact that there are BCRs available on the B cell body and antigen available on the corresponding areas of the presenting membrane. In the case of lamellopodia, the new contacts occurred through the cycles of lamellopodia lifting, protruding and adhering with

the antigen-presenting membrane. It is possible that the curvature of the membrane in the contact sites leads to confinement of the BCRs bound to antigens at a certain distance from the presenting membrane. Diffusion of new BCRs into this contact point and their binding would thus create a high concentration of the engaged BCRs promoting BCR clustering.

Although the resolution to observe microcluster formation has only been achieved imaging B cells *in vitro*, it is reasonable to think that similar mechanisms promote BCR microclustering in B cells engaging antigen on APC *in vivo* as they migrate through lymphoid tissues. Likely, the spreading of B cells is similar to the common mechanism by which cells form adhesion contacts. The mechanical activity of the lamellopodia is a result of a coordination of actin polymerization and actin-myosin contraction [28].

Eventually, pulling on the adhesion sites results in strengthening of the adhesion sites and a similar effect may result in compacting the BCRs in microclusters [29]. Consitently with this idea, disruption of the actin cytoskeleton in lymphocytes reduces the ability of the immunoreceptors to form microclusters [30].

Models for the mechanisms by which BCRs cluster

The observation that BCRs form microclusters in the first steps of the immune synapse formation suggests that BCR microclusters may be the B cell's elementary signaling units. Indeed, imaging of intracellular signaling molecules in living B cells showed that the formation of the BCR microclusters is followed within seconds by recruitment of Lyn and Syk to the clusters and the initiation of calcium signaling through PLCγ_2 [25,26,31]. The proposal that the BCR microclusters are the structures in which BCR signaling occurs, begs the questions how are these structures formed and what can we learn about the initiation of BCR signaling from the mechanism of their formation.

The current prevailing model for BCR clustering and activation is one we will refer to as the 'crosslinking model'. A shared feature of soluble antigens that are able to stimulate B cells is that they are multivalent, containing multiple BCR epitopes [32]. Although there is some controversy [33], most data confirm that for responses to soluble antigens, the BCRs must be crosslinked by the engagement of multiple binding sites on the antigen molecules [34]. These data suggest that binding of multivalent antigens crosslinks the BCR inducing clustering of the cytoplasmic domains of the BCR. Proximity of the cytoplasmic domains of two or more clustered BCRs would allow recruitment of Src-kinases and phosphorylation of the ITAMs by mechanisms that have yet to be delineated. The notion that BCR crosslinking by multivalent antigen initiates signaling was reinforced by the crystal structures of antibodies showing that binding of soluble antigens does not propagate any conformational changes from the antigen binding site to the constant domains that could initiate signaling of the BCR. In addition, the requirement for crosslinking of the BCR to initiate signaling was compatible with the ability of related ITAM containing receptors to signal only after crosslinking by multivalent ligands [34]. However, in the case of the BCR, the requirement for crosslinking does not explain B-cell responses to small, relatively soluble antigens, such as toxins. Also, B cells produce antibodies to rapidly diffusing cell membrane components, such as phospholipids, that cannot directly crosslink the BCR for any significant period of time. In addition, not all oligomeric antigens may be able to crosslink the BCR into a configuration that would bring the cytoplasmic domains of the clustered BCRs into physical proximity [35].

In the context of these limitations of the crosslinking model, an alternative explanation of the requirement for multivalency of soluble antigens warrants consideration. Reth and colleagues proposed that multivalent antigens disrupt an auto-inhibited configuration of the BCR present in preformed BCR clusters [35]. According to this 'permissive geometry'

model [36], the binding of the antigens reorganizes the BCRs in the clusters into an active geometry. In this model the individual BCRs do not change conformation but rather reorient one to another to trigger signaling. Alternatively, it is possible that the binding of antigen leads to a conformational change in the BCR ectodomains on the cell surface that promotes oligomerization, clustering and signaling. We refer to this model as the 'conformation-induced oligomerization' model (Fig. 1). In the following sections we discuss this model in greater detail and describe new single molecule imaging techniques that are providing evidence in support of the model. We then discuss how this model accommodates key aspects of B cell biology.

Insights into the mechanism of BCR microcluster formation from single molecule imaging

To analyze the molecular mechanism by which the BCRs assemble into microclusters in more detail, we recently developed imaging techniques to observe individual BCRs during microcluster formation [27]. To observe single BCR molecules, we labeled a small proportion of the BCR on the surface of B cells with fluorescent Fab fragments of Ig-specific antibodies. Under these conditions, individual labeled BCR could be observed in B cells spreading on bilayers containing antigens by total internal reflection microscopy (TIRF). The BCRs could be tracked for up to few seconds, which is long enough to observe their behavior as they form microclusters. Using this imaging technique we found that BCRs in resting cells were mostly mobile on the cell surface. However, during spreading of the B cells on the antigen-coated bilayers, BCRs immobilized as they formed microclusters. Surprisingly, the immobilization of the clustered BCRs was observed even after the BCR bound to monomeric antigen on the fluid lipid bilayers. This finding indicates that the microclusters form without the need for physical crosslinking of the BCRs. Combining fluorescence resonance energy transfer (FRET) between BCRs tagged in their cytoplasmic domains with FRET donor and acceptor fluorescent proteins with TIRF microscopy we showed that within the first seconds of microcluster formation the BCRs come into close molecular proximity even though the BCRs were not physically crosslinked by antigen (PT unpublished observation).

It is possible that the immobilization of the BCR as it forms microclusters reflects attachment of the BCR to the membrane cytoskeleton or to large complexes of intracellular signaling molecules. However, we found that the immobilization of the BCR in the microclusters was completely independent of the cytoplasmic domains of the BCR or of the presence of the Igαβ subunit [27]. Thus, the microclusters are composed of immobile oligomeric arrays of the BCR formed solely through intrinsic properties of the extracellular and transmembrane of domains the mIg. To search for the minimal requirements for the microclustering of the mIg, we carried out mutational studies and showed that the immobilization of the mIg in microclusters induced by antigen binding depends on the presence of the Cμ4 domain as well as on a WTxxST motif in the transmembrane region. Cγ4 is the membrane proximal domain that forms a homodimer at the bottom of the canonical Fc structure shared in all Ig molecules [37–39]. The WTxxST motif in the N-terminal part of the transmembrane domains is predicted to line the side of the transmembrane helix that is opposite of the putative Igα β interaction site. Notably, the WTxxST motif-containing side of the transmembrane domain has been previously implicated in the formation of BCR oligomers observed after lysis of B cells with limiting amounts of detergents [40].

Single molecule imaging showed that the mIgM molecules that lacked the Cμ4 domain and had the mutation of the WTxxST motif still accumulated and were confined in their movement inside of structures similar to microclusters [27]. However, they could not

immobilize in the microclusters, suggesting that they could not form the oligomeric structures. Measurement of signaling activity of a chimeric receptor consisting of the mutated IgM and intracellular domains of Igα or Igβ showed that the constructs were significantly compromised in the tyrosine phosphorylation in the synapses as well as in the upregulation of CD69. Conversely, the expression of the Cμ4 domain alone, but not larger parts of the Fc region of the mIg, lead to spontaneous clustering of the construct. Similar clustering was observed after expression of Cγ3, the membrane proximal domain of IgG. In addition, when expressed with the Igα β heterodimer, clustering of the Cμ4 domain lead to spontaneous recruitment of Syk into these clusters and upregulation of CD69.

These findings are consistent with a model of microcluster formation, in which the Cμ4 and the transmembrane region contain a homotypic clustering interface that is not accessible in the mIg in resting B cells. Binding of membrane antigen confines the BCR in the contact areas with the presenting membrane and unmasks the clustering interface in the Cμ4 domain, that together leads to the formation of an BCR oligomer that promotes signaling from the cytoplasmic domains. As mentioned above, we refer to this model as to the "conformation-induced oligomerization model" (Fig. 1).

Is there evidence for conformation-induced oligomerization predicted by the model?

How can monovalent membrane antigens binding to the BCRs unmask a clustering interface? As mentioned above, structural studies suggest that it is unlikely that the binding of the antigen propagates conformational changes to the Fc through a direct allosteric mechanism [41]. Structural studies as well as electron microscopy also provided no evidence for the clustering of soluble antibodies engaged by soluble antigens [42], although the Fc region of antibodies has some role in the formation of immunoprecipitates [43,44]. Nevertheless, it is possible that the binding of a membrane antigen to the BCR induces a change in the Fc region of the Ig indirectly. Stretched by the antigen binding between the B-cell membrane and the APC, the BCR could be subjected to a pulling or twisting force. The force could induce conformational changes within the Cμ4 and transmembrane domains, leading to formation of a clustering interface as depicted in Fig. 1. Alternatively, the force could induce a reorientation of the Cμ3 to allow access to a preformed clustering interface in Cμ4 and the transmembrane domains. It is also possible that similar activating changes in the BCR could be induced by soluble multivalent antigens, in which case the force would come from the binding of several BCRs to a single antigen object.

Although the structure of the Fc region of the IgM is not available, numerous structures have been solved of the Fc regions of IgG, IgE and IgA [37–39]. The canonical Fc region is composed of two angled Ig domains that pair to form a rhombus. In principle, the Fc region can undergo changes of the interdomain angle, leading to opening and closing of the top of the structure. In the available structures, the opening of the interdomain angle has been observed after binding to Fc receptors [45,46]. Although these conformational changes are relatively subtle in IgG binding to FcγRs, they are substantial in IgE binding to FcεRI, where the opening is potentially associated with a reorientation of the Cε2 domains [38]. Interestingly, the changes of the angle between Cε3 and Cε4 domains are propagated to the AB and EF loops of the Cε4 at the bottom and side of the Fcε. However, whether similar changes may be induced in the IgM and IgG BCR remains unknown. Likely, studying the Ig structure in the context of the full BCR complex will be necessary to better understand these issues.

Implications of the ‘conformation-induced oligomerization’ model for B cell biology

Although we do not currently understand the structural changes in the BCR that could initiate BCR oligomerization in the microclusters, there are interesting implications of the conformation-induced clustering model that are relevant for B-cell biology. Importantly, the presence of a clustering interface in the BCR suggests that microcluster formation is independent of the antigen valency. The homotypic interaction of the membrane proximal and transmembrane domains may thus potentially be important for B-cell responses to antigens that do not directly crosslink the BCR, or to antigens that crosslink the BCR to a configuration that does not directly bring the cytoplasmic domains of the BCR into an active configuration. Separately engaged BCRs would in this case associate laterally on the cell surface and bring the BCR into an active conformation by the interaction of their Cμ4 domains leading to efficient signaling. Thus, the clustering interface in the BCR could broaden B-cell responses to a wider range of antigens. The ability of BCRs to oligomerize and signal following monovalent binding to antigen would also alleviate the problem of avidity in the B cell’s discrimination of antigen affinity.

The specific structure of the oligomeric BCR may also contribute to BCR’s interaction with membrane signaling adaptors and BCR coreceptors that modulate B-cell activation. For example, recent studies showed that the coengagement of the BCR and FcγRIIb during recognition of membrane-bound immune complexes blocks B-cell spreading and the interactions of BCR microclusters with signaling components, suggesting that the FcγRIIb blocks early steps of BCR activation in the microclusters [10]. In addition, B-cell spreading and intracellular signaling in response to membrane antigens requires the recruitment of the positively signaling transmembrane adaptor CD19 [9]. CD19 amplifies BCR signaling by recruiting intracellular signaling proteins such as Vav and PI3 kinase to the plasma membrane. While in response to soluble antigens CD19 interacts with the BCR as a part of the CD19-CD21-CD81-TAPA-1 complex that recognizes complement-tagged antigens, the involvement of CD19 in B-cell responses to membrane antigens is independent of complement binding, and occurs through dynamic interactions of the CD19 directly with BCR microclusters. These examples raise the possibility that the oligomeric BCR is the structure that interacts with positive and negative coreceptors to provide regulation of the earliest steps in B cell activation. Understanding how coreceptors interact with BCR microclusters may lead to new ways to modulate dysregulated B-cell responses, particularly in autoimmune diseases.

The intrinsic ability of the BCR to cluster suggests that there may be a low level of spontaneous BCR clustering in resting B cells that may underlie antigen-independent tonic signaling. It is the current prevailing view that tonic BCR signaling is a result of a “leaky” regulation of the BCR’s signaling pathways that are inherently at a fine balance between receptor phosphorylation and dephosphorylation [5]. However, it is also possible that the tonic signaling is generated from the spontaneous clustering of a small fraction of BCR due to conformational flexibility of the extracellular domains. If so, the tonic signaling would arise from transient, albeit structurally defined BCR clusters. Such spontaneously forming clusters would be of interest as they may be potentially the basis of exaggerated constitutive BCR signaling under pathological conditions. For example, in the rare heavy chain disease, somatic deletions in the V_H-Cμ2 region lead to constitutive signaling from the truncated BCR resulting in a B cell proliferative disorder [47,48]. It is possible that the truncation of the mIg domains unmasks the clustering interface of the BCR, as we observed in the expression of the isolated Cμ4 domain. It will be interesting to investigate whether the mechanism of BCR clustering contributes to more common diseases such as B-cell

lymphomas. In this case, understanding the mechanism of formation of BCR clusters may provide a new target for the intervention of the pathological BCR signaling.

The unsolved problem of transducing conformational changes in the BCR ectodomains to the cytoplasmic domains

Collectively, the data reviewed here suggest that the transition of the resting BCR into its active state involves specific participation of the extracellular and transmembrane regions of the BCR. But how are changes induced by antigen binding in the extracellular domains transduced through the transmembrane domains to the intracellular domains? In vitro, peptides representing the intracellular domains of Igα β are unstructured, providing little information as to what specific changes may lead to the recruitment of Src-kinases and the phosphorylation of the ITAMs [49]. However, it is not known if the cytoplasmic domains of the native BCR complex in living cells take on a more defined structure. Using FRET to measure the distance between the BCR's Ig, Igα and Igβ cytoplasmic domains, we observed that the cytoplasmic domains of the BCRs come into close proximity in the first ~5 seconds of microcluster formation [50]. After that, the FRET between the intracellular domains rapidly drops to a level of FRET that is still higher than the FRET in resting cells. This FRET pattern was observed in cells expressing any combination of BCR chains containing donor and acceptor fluorescent proteins, reporting either inter- or intra-molecular BCR chain interactions. The FRET pattern suggests that while the BCRs remained clustered, the cytoplasmic domains opened up. The opening required phosphorylation of the ITAMs, but was independent of the recruitment of Syk or other downstream molecules. Thus, it is possible that in resting BCRs the cytoplasmic domains of Igα and Igβ are in a closed, folded conformation in which the tyrosines of the ITAMs are not accessible as depicted in Fig. 1. Binding of Src-kinases and/or phosphorylation of the ITAMs stabilizes a new, open or unfolded conformation and allows the initiation of downstream signaling.

One mechanism by which the BCR may recruit Src-kinase in the first seconds of microcluster formation is by inducing changes in local lipid composition in the microclusters. Such lipid changes could be induced by perturbation of the membrane by the local concentration of the BCR transmembrane domains, leading to transient trapping of the myristoyl and palmitoyl fatty acid membrane anchor of Src-family kinases. We recently showed by FRET in living cells that the interaction of a probe containing this lipid anchor with the BCR occurs rapidly after the onset of microcluster formation and overlaps with the very initial recruitment of Lyn to the BCR [31,51]. The interaction of the lipid probe was transient and limited to nascent microclusters in the periphery of the immune synapse, whereas Lyn interacted with the microclusters during their trafficking to the cSMAC, suggesting that protein-protein interactions, presumably mediated by the SH2 domains binding to phosphorylated ITAMs, stabilize Src-family interaction with the BCR to sustain signaling.

Conclusions

Although experimental data are far from providing a complete picture of the mechanisms by which antigen binding activates the BCR, they collectively suggest that our currently incomplete understanding of these mechanisms is due gaps in our knowledge of the structure and organization of the full BCR complex in living B cells. It will be exciting to watch these gaps be filled in the near future as new technologies allows closer and closer views of the BCR on the B cell surface. Hopefully, the knowledge of the structure of the BCR in the B-cell plasma membrane, together with a better understanding of B-cell recognition of antigens during an immune response *in vivo* will render a clearer picture of BCR activation and the

early signaling steps. With much remaining to be learned, the near future may still bring many surprises.

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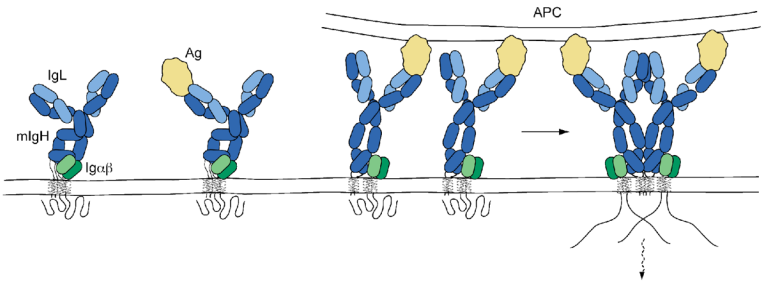

Fig 1. Schematic illustration of the 'conformation-induced oligomerization' model for BCR signaling

The BCR is preferentially in a closed, inactive conformation in resting cells. Binding of monovalent soluble antigen does not change the conformation of the BCR and does not induce signaling. Binding of membrane antigens pulls the BCR's ectodomains into an active conformation exposing an oligomerization interface in the membrane proximal region of the membrane immunoglobulin. Assembly of the BCR oligomer leads to perturbations of the local lipid environment, opening of the cytoplasmic domains and the initiation of signaling.