Membrane heterogeneities in the formation of B cell receptor–Lyn kinase microclusters and the immune synapse

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Antigen binding to the B cell receptors (BCRs) induces BCR clustering, phosphorylation of BCRs by the Src family kinase Lyn, initiation of signaling, and formation of an immune synapse. We investigated B cells as they first encountered antigen on a membrane using live cell high resolution total internal reflection fluorescence microscopy in conjunction with fluorescence resonance energy transfer. Newly formed BCR microclusters perturb the local membrane microenvironment, leading to association with a lipid raft probe. This early event is BCR intrinsic and independent of BCR signaling. Association of BCR microclusters with membrane-tethered Lyn depends on Lyn activity and persists as microclusters accumulate and form an immune synapse. Membrane perturbation and BCR–Lyn association correlate both temporally and spatially with the transition of microclustered BCRs from a “closed” to an “open” active signaling conformation. Visualization and analysis of the earliest events in BCR signaling highlight the importance of the membrane microenvironment for formation of BCR–Lyn complexes and the B cell immune synapse.

Introduction

Binding of antigens to the B cell receptors (BCRs) leads to clustering of the BCRs and triggering of a signaling cascade resulting in the activation of a variety of genes associated with B cell activation (Cambier et al., 1994; Reth and Wienands, 1997; Dal Porto et al., 2004; Hou et al., 2006). We now understand the biochemical nature of the BCR’s signaling pathway beginning with phosphorylation of the BCR by the first kinase in the signaling cascade, the membrane-associated Lyn, in considerable detail. However, what remains only poorly understood are the very earliest events that follow antigen-induced clustering of the BCRs that lead to association of the BCR with Lyn and triggering of the signaling cascade. Of particular interest are the potential roles of plasma membrane lipid heterogeneities and the local lipid microenvironment of the BCR in the initiation of signaling. Indeed, Lyn is acylated by both myristoylation and palmitoylation that both dictate Lyn’s membrane localization and are essential for Lyn’s function (Kovarova et al., 2001). The results of previous biochemical studies using detergent solubility to identify membrane microenvironments suggested that lipid heterogeneities may play an important role in the initiation of B cell signaling by regulating access of the BCR to Lyn (Cheng et al., 1999; Aman and Ravichandran, 2000; Guo et al., 2000). These studies provided evidence that detergent-insoluble, sphingolipid-rich, and cholesterol-rich membrane microdomains termed lipid rafts concentrate the membrane-tethered dually acylated Lyn kinase and, in so doing, potentially provide a platform for BCR signaling. Subsequently, using fluorescence resonance energy transfer (FRET) confocal microscopy in live B cells, we showed that within seconds of the B cell’s encounter with soluble antigens, the BCR transiently associated with a lipid raft probe, a myristoylated and palmitoylated fluorescent protein present in the detergent-insoluble lipid raft fraction of the plasma membrane (Sohn et al., 2006). This interaction was selective and was not observed with fluorescent proteins that were tethered to the detergent-soluble regions of the membrane by geranylgeranylation or myristoylation and preceded by several seconds the induction of a Ca²⁺ flux. These results are consistent with recent revised models of the original raft hypothesis (Simons and Ikonen, 1997; Edidin, 2003) that take into account the dominant role for plasma membrane proteins in capturing and stabilizing intrinsically unstable lipid domains (Hancock, 2006).
Figure 1. Time-lapse FRET imaging using TIRFM reveals the spatial and temporal dynamics of the change in the lipid environment of the BCR during antigen contact. (A) Time-lapse TIRFM images of CH27 B cells expressing Igα-YFP and Lyn16-CFP contacting a planar lipid bilayer containing only ICAM-1 or ICAM-1 and the antigen PC10-BSA. Three-channel TIRFM images were acquired using an electron-multiplier CCD camera. The CFP and YFP images are shown. FRET was calculated by sensitized acceptor emission as described in Materials and methods. Corrected net FRET $F_c = F_{C} - \beta \times D - \gamma \times A$ and

|            | ICAM-1 | ICAM-1 + Antigen |
|------------|---------|------------------|
|            | 4 s     | 60 s             | 600 s            |
|            | 0 s     | 4 s              | 10 s             | 60 s | 120 s | 600 s |
| Lyn16-CFP  | ![Image](image1.png) | ![Image](image2.png) |                 |
| Igα-YFP    | ![Image](image3.png) | ![Image](image4.png) |                 |
| Fc         | ![Image](image5.png) | ![Image](image6.png) |                 |
| Ea         | ![Image](image7.png) | ![Image](image8.png) |                 |
| Merge      | ![Image](image9.png) | ![Image](image10.png) |                 |

![Graph A](image11.png)

![Graph B](image12.png)

![Graph C](image13.png)
The finding that the antigen-clustered BCR associated with the lipid raft probe predicted that the association with lipid rafts would lead to interaction of the BCR with Lyn kinase itself. However, this prediction was not tested directly. In addition, these results were acquired by investigating the response of B cells to soluble antigens, and several recent studies provided evidence that the relevant mode of antigen recognition by B cells in vivo may be on the surfaces of antigen-presenting cells (APCs). Indeed, results from a study using intravital two-photon imaging suggest that B cells contact antigen not in solution but rather on the surfaces of APCs in lymphoid organs (Qi et al., 2006). Studies in vitro showed that B cells encountering antigen on the surface of an APC or on a planar lipid bilayer, approximating an APC surface, form an immune synapse (Batista et al., 2001; Carrasco and Batista, 2006; Fleire et al., 2006), a structure associated with B cell activation. In addition, results of a recent study indicate that the requirements for B cell responses to membrane-bound antigens are significantly different from those for responses to soluble antigens (Depoil et al., 2008). Indeed, unlike BCR signaling in response to soluble antigens that is initiated independently of the B cell coreceptor, CD19, response to membrane antigen was defective in the absence of CD19.

To capture the earliest events in the interaction of BCR with the lipid rafts and the membrane-tethered Lyn kinase after contact with antigen in a planar membrane, we took advantage of FRET in conjunction with total internal reflection fluorescence microscopy (TIRFM). TIRFM provided high resolution images that allowed us to observe the formation of individual BCR microclusters and their interaction with a raft lipid probe. FRET between Igα-YFP and Lyn16-CFP (Fig. 1A) at no point in time was there significant FRET between Igα-YFP and Lyn16-CFP. Thus, molecular interactions of the BCR with raft lipids did not occur in the absence of antigen to engage the BCR.

B cells placed on a bilayer that contained ICAM-1 alone failed to spread and contacted the bilayer over a relatively small area of ~20 μm² (unpublished data). In contrast, on ICAM-1- and antigen-containing bilayers, the B cells spread, forming contact areas of ~80 μm². Time-lapse imaging showed that the first contact points of the B cell membrane with the ICAM-1–only bilayer occurred in discrete membrane protrusions that contained both Igα-YFP and Lyn16-CFP (Fig. 1A). The area of contact grew with time, and although the relative fluorescence intensities (Fls) of Igα-YFP and Lyn16-CFP were similar over the contact area (Fig. 1B) and overlapped extensively at each time point (Fig. 1A), at no point in time was there significant FRET between Igα-YFP and Lyn16-CFP. Thus, molecular interactions of the BCR with raft lipids did not occur in the absence of antigen to engage the BCR.

B cells also first contacted the ICAM-1– and antigen-containing lipid bilayers through multiple, small points (Fig. 1A). Both Lyn16-CFP and Igα-YFP were visible in these early contact points, and FRET was detected between Igα-YFP and Lyn16-CFP, indicating that the earliest antigen-induced BCR microclusters associated with the lipid raft probe. With time, the B cells spread on the bilayer, concentrating the BCR Igα-YFP.

Results

Molecular interactions of antigen-induced BCR microclusters with a lipid raft probe

To analyze the BCR’s interaction with lipid rafts, B cell lines were generated that were specific for the antigen phosphorylcholine (PC) and stably expressed a chimeric BCR Igα chain that contained the FRET acceptor YFP in its cytoplasmic domain (Igα-YFP) and a FRET donor CFP that contained the first 16 amino acids of the Src family kinase Lyn (Lyn16-CFP), resulting in its myristoylation and palmitoylation and association with the detergent-insoluble fractions of the plasma membrane (Sohn et al., 2006). Previous studies showed that cell lines expressing Igα-YFP and Lyn16-CFP were functional and responded to antigens and established the validity of using FRET measurements to detect molecular interactions between antigen-clustered BCRs and lipid raft probes (Sohn et al., 2006). B cells were placed on a planar lipid bilayer that contained biotinylated forms of either the antigen, PC or BSA, and the adhesion molecule, intercellular adhesion molecule-1 (ICAM-1), or ICAM-1 alone and were tethered to the lipid bilayer by streptavidin and biotinylated 1,2-diOleoyl-sn-glycero-3-phosphoethanolamine. Three-channel TIRF microscope images were acquired (CFP, FRET, and YFP) at 2- or 4-s intervals for 15 min. Influences of the relative concentration of YFP and CFP on the FRET measurements were addressed by calibrating the bleed-through of the donor fluorescence in the acceptor detection channel and the amount of directly excited acceptor fluorescence as previously described (van Rheenen et al., 2004; Zal and Gascoigne, 2004) by measuring the donor and acceptor emissions of cells that contained only the acceptor or donor fluorescent proteins in the same field as the experimental cells. FRET was calculated by sensitized acceptor emission and expressed as either corrected FRET values (Fc) or as FRET efficiency normalized for the acceptor (Ea) as detailed in the Materials and methods section.

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B cells also first contacted the ICAM-1– and antigen-containing lipid bilayers through multiple, small points (Fig. 1A). Both Lyn16-CFP and Igα-YFP were visible in these early contact points, and FRET was detected between Igα-YFP and Lyn16-CFP, indicating that the earliest antigen-induced BCR microclusters associated with the lipid raft probe. With time, the B cells spread on the bilayer, concentrating the BCR Igα-YFP.
The interactions of BCR clusters and the raft lipid probe are not dependent on the initiation of BCR signaling or on the actin cytoskeleton. (A) CFP, YFP, and Ea time-lapse images (acquired as in Fig. 1) of CH27 B cells expressing Igα-YFP and the raft lipid probe, Lyn16-CFP, at 4, 60, and 600 s after...
and Lyn16-CFP in the center of the contact area and subsequently contracting, forming a mature synapse. As BCR microclusters were concentrated in the synapse, new B cell clusters continued to form in the periphery of the B cells’ contact area and then moved to the center synapse. Notably, the highest FRET values detected were not in the center of the synapse where Igα-YFP and Lyn16-CFP were most concentrated, but rather FRET was highest in the periphery of the B cell’s contact area with the bilayer, where there was relatively little accumulation of Igα-YFP and Lyn16-CFP but where new BCR-antigen clusters continued to form (Fig. 1 A). Thus, the difference in FRET values in the cell’s periphery versus in the synapse could not be attributed to the concentration of Igα-YFP relative to Lyn16-CFP. Similarly, the relative FIs of Igα-YFP and Lyn16-CFP over the contact area were similar with time for cells engaging either ICAM-1 alone or ICAM-1 and antigen, yet FRET was only observed in cells contacting ICAM-1 and antigen (Fig. 1 B). Moreover, the FRET between Igα-YFP and Lyn16-CFP was specific and did not occur between Igα-YFP and Ger-CFP (Fig. 1 C) that contained the C-terminal polybasic12 residues of K-ras and four residues of rap1B, resulting in CFP’s geranylgeranylation and, as previously shown, targeting to nonraft detergent-soluble membranes (Pyenta et al., 2001; Sohn et al., 2006). Collectively, these data provide evidence for a selective molecular interaction between the antigen-clustered BCRs and the lipid raft probe.

**Association of the antigen-clustered BCRs with raft lipids is independent of signaling and association with the actin cytoskeleton**

To determine whether association of the clustered BCRs with the raft lipid probe was dependent on either signaling or the function of the actin cytoskeleton, B cells were treated with either PP2 to inhibit Src family kinases (Hanke et al., 1996) or with latrunculin B to disassemble the actin cytoskeleton (Brown and Song, 2001) before exposure to ICAM-1- and antigen-containing bilayers. Our previous study showed that PP2 blocked FRET between Igα-CFP and Lyn16-YFP in cells encountering antigen in solution (Sohn et al., 2006). Here, we found that although PP2 affected the ability of B cells to both spread on the bilayer and organize the BCR in a synapse, it did not block FRET between Igα-YFP and Lyn16-CFP after antigen binding (Fig. 2 A). Thus, association of the BCR with raft lipids showed different requirements for Src family kinase activity when the B cell engaged antigen in solution versus on a membrane. The requirement of Src family kinase activity when antigen is bound from solution may reflect a role of these kinases in maintaining some feature of the cell membrane or local membrane topology, requirements that contact between the B cell membrane and the antigen-containing bilayer overcomes. In addition, although PP2 does not block FRET between Igα-YFP and Lyn16-CFP, both the FRET spatial and kinetic patterns were altered by treatment with PP2 (Fig. 2, A and B), presumably reflecting a requirement for Src family kinase activity in these downstream processes. Similar results were obtained in cells treated with latrunculin B. FRET between Igα-YFP and Lyn16-CFP was observed after antigen-induced BCR clustering, but both the kinetics and spatial pattern of FRET were affected as compared with untreated cells. Collectively, these observations indicate that association of the BCRs with raft lipids that occurs within the first several seconds of BCR-antigen engagement is independent of signaling and association with the actin cytoskeleton. However, importantly, failure to either signal or associate with the actin cytoskeleton significantly affected both the spatial distribution and kinetics of the BCR–lipid raft probe interactions by mechanisms that remain to be elucidated.

To independently confirm that BCR signaling was not required for the association of BCR clusters with raft lipids, we analyzed two previously described NIP (4-hydroxy-5-iodo-3-nitrophenyl acetyl)-specific J558L B cell lines expressing Lyn16-CFP and either a wild-type BCR or a signaling-incompetent BCR in which the two tyrosines within the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) of Igα and Igβ chains were mutated to phenylalanine (Tolar et al., 2005). The B cells expressing the ITAM mutant BCR failed to spread on the antigen- and ICAM-1–containing bilayer (Fig. 2 C) and formed fewer BCR microclusters as compared with the wild-type receptor (Fig. 2 D). However, the BCR Igβ-YFP clusters that formed showed FRET with Lyn16-CFP that was comparable with that of the wild-type BCR clusters (Fig. 2 E). Collectively, these results indicate that the interaction of the clustered BCRs with the lipid raft probe did not require signaling or the actin cytoskeleton but rather appeared to rely on an intrinsic property of the clustered BCR.

**FRET between Igα-YFP and Lyn16-CFP correlates temporally and spatially with BCR activation**

Previous studies using FRET confocal microscopy provided evidence that antigen binding from solution resulted in a
Conformational change in the BCR cytoplasmic domains from a “closed” to an “open” form and simultaneous phosphorylation of the BCR (Tolar et al., 2005). We observed that when clustered by antigen, BCRs containing a membrane Ig–CFP and Igα–YFP showed an initial increase in FRET, reflecting the close molecular proximity of the cytoplasmic domains of the clustered BCRs, and then a drop in FRET, indicating that the cytoplasmic domains within the BCR clusters moved apart or opened. To determine whether the observed FRET between Igα–YFP and Lyn16–CFP correlated either temporally or spatially with the antigen-induced transition in the BCR to an open form, we measured FRET in a J558L cell line expressing an NIP-specific BCR containing membrane Ig–CFP and Igα–YFP as the B cells encountered a bilayer containing NIP and ICAM-1 (Fig. 3). Tracking the initial BCR microclusters individually from their formation to the generation of the synapse showed that within the first few seconds of formation, FRET in the microclusters sharply increased, indicating the induced close molecular proximity of the cytoplasmic domains of the BCRs within the microclusters (Fig. 3 A). Despite the continued accumulation of BCRs in the clusters, the FRET level reached a peak and then dropped, which is consistent with a synchronized opening of the cytoplasmic domains of the BCR clusters as previously described (Tolar et al., 2005).

Calculation of FRET ratio images showed that the increase in FRET in the BCR clusters occurred in the initial point of contact and in the cell’s periphery (Fig. 3 B). In contrast, the clustered BCRs accumulated in the synapse showed lower FRET, indicating an open conformation. Thus, transition of the BCR from the closed to open form correlated with FRET between Igα–YFP and Lyn16–CFP both spatially, occurring in the initial contact points and in the periphery of the B cell’s contact area, and temporally, occurring within the first several seconds of encounter with the antigen bilayer.
The antigen-induced interactions of the BCR with Lyn

Association of the antigen-induced BCR microclusters with the lipid raft probe has been suggested to play a role in facilitating the association of clustered BCR with the Lyn kinase. To directly characterize the interaction of the BCR with Lyn, CH27 B cells were analyzed that expressed Igα-YFP and full-length Lyn (LynFL) linked by six amino acids at the C terminus to CFP (LynFL-CFP). Images of B cells engaging a bilayer containing only ICAM-1 showed extensive colocalization of Igα-YFP with LynFL-CFP but no FRET (Fig. 4 A). In contrast, images of B cells engaging an ICAM-1– and antigen-containing bilayer showed significant FRET at the first points of contact where LynFL-CFP colocalized with Igα-YFP (Fig. 4 A). FRET between LynFL-CFP and Igα-YFP persisted as the BCR microclusters formed a central synapse. Quantification of the FIs and FRET over the contact area with time showed that although the relative FIs of Igα-YFP and LynFL-CFP were similar with time in the presence or absence of antigen, FRET was only detected when antigen was present (Fig. 4 B). Thus, the FRET cannot be attributed to a change in the ratios of YFP and CFP. The concentration of FRET between the BCR and LynFL in the center synapse was in contrast to the observation for the lipid raft probe and BCR, in which case FRET was highest in the cell’s periphery. The antigen-induced association of Lyn kinase with BCR. (A) CFP, YFP, Ea, and merged time-lapse images of CH27 B cells expressing LynFL-CFP and Igα-YFP 0–600 s after the encounter of either ICAM-1 alone or ICAM-1– and antigen-containing bilayers. CFP and YFP FIs and Ea across the contact area indicated by red lines (scale, 20 μm) are given. (B) Quantification of the Igα-YFP and LynFL-CFP FIs and Ea plotted against time for cells imaged in A.

Figure 4.
FRET peak between Ig during the initial contact phase (Fig. 5A). The initial increase in FRET that was significantly greater in magnitude by a peak in FRET that was significantly greater in magnitude was detected and colocalized with LynFL-CFP ∼20–28 s before FRET was detected at 28–44 s (Fig. 6A). Thus, BCR clustering preceded by several seconds the close molecular association of BCR with the lipid raft probe and with Lyn. In addition, these results showed no evidence that the lipid raft probes and Lyn are in preformed structures, but rather both appeared to coalesce around the BCR microclusters.

Individual BCR microclusters associate with the lipid raft probe and with Lyn several seconds after forming

To better understand the dynamics between the formation of BCR microclusters and their association with Lyn and the lipid raft probe, we compared the pattern of FRET between either Lyn16-CFP or LynFL-CFP and Igα-YFP in individual BCR microclusters as the clusters first formed in membrane protrusions during the first 44 s of contact of the B cell with the antigen-containing bilayer (Fig. 6A). BCR microclusters appeared ∼12–20 s before Lyn16-CFP colocalized with the BCR clusters and FRET between Lyn16-CFP and Igα-YFP was detected (Fig. 6A). In these microclusters, the FRET appeared to increase, peak, and then decrease. Similarly, BCR clusters were detected and colocalized with LynFL-CFP ∼20–28 s before FRET was detected at 28–44 s (Fig. 6A). Thus, BCR clustering preceded by several seconds the close molecular association of BCR with the lipid raft probe and with Lyn. In addition, these results showed no evidence that the lipid raft probes and Lyn are in preformed structures, but rather both appeared to coalesce around the BCR microclusters.

Association of the BCR with Lyn facilitates directional movement to the synapse

It was of interest to determine the repercussions of the association of the BCR microclusters with the lipid raft probe as compared with Lyn itself in terms of the movement of the microclusters from the cell’s periphery to the cell’s center to form an immune synapse. To do so, the trajectories of individual BCR microclusters were analyzed as they formed in the periphery and moved to the synapse to determine when microclusters associated with the raft probe versus Lyn (Fig. S2 and Video 9, available at http://www.jcb.org/cgi/content/full/jcb.200802007/DC1). The trajectories of the BCR clusters formed in the periphery of the contact area showed three patterns: an immediate movement toward the center of the contact area, a delayed movement to the center, and a random nondirectional movement in the periphery. FRET between BCR clusters and the lipid raft probe moved in cells expressing either Igα-YFP and Lyn16-CFP or Igα-YFP and LynFL-CFP. An analysis of the time the BCR clusters moved randomly before moving in a directed trajectory to the center of the contact area showed that the clusters associated with Lyn16-CFP spent considerably more time in a random movement (an average of 25 s) before moving to the center as compared with those associated with LynFL-CFP (an average of 8 s; Fig. 6B). This observation suggests that association of the BCR with Lyn facilitates directional movement to the synapse.
Figure 6. **BCR clustering precedes by several seconds the association of clustered BCR with raft lipids and Lyn kinase.** (A) The merged TIRFM images of CFP (red), YFP (blue), and Ea (green; top) and quantification of the fold increases of CFP and YFP Fls and Ea for the BCR microclusters (bottom). The images were taken at 8-s intervals of CH27 B cells expressing either Igα-YFP and the lipid raft probe, Lyn16-CFP (top row), or Igα-YFP and LynFL kinase, LynFL-CFP (bottom row), as the cell first encounters an ICAM-1– and antigen-containing bilayer. The clusters marked by white arrows were quantified as the fold intensities relative to the intensities of the first frame in each channel showing a signal over background over a 4-μm² region of interest surrounding the clusters. Bars, 2 μm. (B) TIRFM time-lapse images were taken of CH27 B cells expressing either Igα-YFP and Lyn16-CFP or Igα-YFP and LynFL-CFP as they contacted PC₁₀:BSA– and ICAM-1– containing bilayers. Individual BCR clusters that formed in the periphery of the contact area and moved to the central synapse were tracked, and the pattern of movement of single BCR clusters was analyzed by time-lapse Ea images as described in Materials and methods. The time delay before a cluster that formed in the periphery moved to the center was determined. A total of ~200 clusters from cells expressing either both Igα-YFP and lyn16-CFP (four cells) or both Igα-YFP and lynFL-CFP (three cells) were included in the analyses. Error bars represent SEM.
Discussion

The use of live cell imaging allowed an investigation into the earliest events in the initiation of BCR signaling that precede the interaction of the BCR with Lyn kinase and the formation of an immunological synapse (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200802007/DC1). We provide evidence for an ordered series of events that begins with the antigen-induced clustering of the BCRs. The newly clustered BCRs associate with a lipid raft probe in the periphery of the contact area of the B cell with an antigen-containing lipid bilayer. Association of the antigen-clustered BCRs with the lipid raft probe is highest in the cells’ periphery and weaker as the BCR clusters move to the center of the contact area to form a synapse. BCR clusters associated with a lipid raft probe appear to move randomly in the peripheral contact area, and it is not until the clustered BCRs stably associate with Lyn that the random motion ceases and the BCR clusters move directionally to the center of the contact area to form a synapse.

In interpreting FRET data generated using intermolecular reporters, as described here, it is important to control for potential artifacts that can result from relative differences in the concentrations of the two reporters. The influence of the concentration on artifacts in the FRET measurements can largely be avoided by calibrating the bleed-through of the donor fluorescence in the acceptor detection channel and the amount of directly excited acceptor fluorescence as described previously (van Rheenen et al., 2004; Zal and Gascoigne, 2004). In the studies reported here, calibration involved the use of control cells in the same field as the experimental cells, that each contained only the acceptor or the donor fluorescent protein and measurements at the donor and acceptor emission wavelengths. Nevertheless, it is possible that an effect of the changes in concentration of the BCR as it clusters was directly responsible for the changes in measured FRET.

We have addressed this issue by providing data demonstrating that although the ratio of FIs of the CFP and YFP did not change significantly over time on cells engaging bilayers containing ICAM alone or ICAM plus the antigen, FRET was only observed in cells engaging antigen. Moreover, the spatial pattern of FRET did not correlate with the highest intensities of YFP and CFP, a correlation that should occur if FRET was simply the result of the concentration of the clustered BCR. We also provided data demonstrating that FRET between Igα-YFP and Lyn16-CFP was specific and did not occur between Igα-YFP and a different lipid probe, namely geranylgeranyl-CFP, even though the FIs were similar in both cases for CFP and YFP. Collectively, these results provide strong evidence that the FRET measurements reported here reflect a genuine, specific molecular interaction between Igα-CFP and Lyn16-YFP and LynFL-YFP.

The view of lipid rafts and their interactions with immune receptors during antigen engagement provided by these studies differ in the molecular details from earlier views as first articulated in the raft hypothesis (Simons and Ikonen, 1997). Lipid rafts were initially viewed as freely diffusing, stable, lateral assemblies of sphingolipids and cholesterol that formed signaling platforms. In the intervening years, results of studies of both model membranes and living cells along with computational modeling led to an updated model of lipid rafts that takes into account a more dominant role for membrane proteins in capturing and stabilizing intrinsically unstable liquid-ordered membrane microdomains (Hancock, 2006). Here, we provide evidence consistent with this updated version of the raft hypothesis. The raft lipids do not appear to form stable microscopic structures either in resting or activated cells. Indeed, we were unable to detect FRET between Lyn16-YFP and Lyn16-CFP in cells that expressed both (unpublished data), suggesting that stable raft microdomains, if they exist, must be small as indicated previously (Sharma et al., 2004). Second, the results provided here indicate that the BCRs first cluster and then condense raft lipids around them. These clustered BCR-raft lipid interactions are dynamic, weak, and transient. The interaction of lipid rafts with the clustered BCRs is not dependent on the initiation of signaling or on the actin cytoskeleton and thus appears to be an intrinsic property of the clustered BCR that prefers the microenvironment of raft lipids.

In contrast to the ephemeral interactions of the clustered BCRs with raft lipids that predominate early after BCR clustering, the BCR forms more stable protein–protein interactions with Lyn that dominate later and are dependent on a kinase-active Lyn. Indeed, association of the clustered BCR with Lyn predicted the directional movement of the BCRs to the synapse. These observations are similar to those of Larson et al. (2005), who showed that LynFL codiffuses with the clustered IgE receptor, but a minimal raft probe did not. Similarly, Douglass and Vale (2005) used TIRFM to observe the diffusion of single molecules on the surface of T cells during T cell activation by antigen and provided evidence for membrane microdomains created by protein–protein networks that exclude or trap signaling molecules on the T cells’ membrane. They provided evidence that the full-length Src family kinase, Lck, stably associated with T cell receptor signaling domains, but the minimal lipid probe did not. However, these domains were large and formed relatively late after antigen engagement, and the authors suggested that lipid raft–receptor interactions might precede the formation of these domains and play an organizational role within a receptor cluster. Here, we provide evidence that this may indeed be the case for signaling through the BCR. Collectively, these studies illustrate the importance of both lipid–protein and protein–protein interactions in the initiation of signaling.

The results presented here characterizing the interaction of BCR microclusters and a lipid raft probe during contact of the B cell with antigen on a membrane differ from our previous results characterizing the same process in B cells responding to antigen in solution (Sohn et al., 2006). In response to soluble antigen, BCR–lipid raft probe interactions were blocked by PP2, a Src family kinase inhibitor (Sohn et al., 2006). In contrast, we show here that in response to antigen on membranes, PP2 does not block interactions of the BCR with the lipid raft probe, although PP2 influences the dynamics of the interactions. In addition, we show here that a signaling-deficient BCR associates with the lipid raft probe upon clustering in response to membrane-bound antigen. These findings raise the question of how the B cells’ encounter with antigens on membrane differs from that with antigen in solution. When interacting with antigens on membranes, the topology of the B cell membrane changes dramatically.
The initial contacts are through small membrane protrusions that may concentrate or restrict BCR movement, facilitating clustering. These interactions trigger spreading of the BCR over the antigen-membrane and then contraction. It may be that Src kinase activity is required for B cells in solution to provide a restriction on BCR membrane movements that is replaced or overcome by membrane topology in B cells encountering membrane-bound antigens. Clearly, additional studies will be required to fully delineate the mechanisms at play in these early events.

The observation that BCR clusters associate with lipid rafts raises the following question: what function do lipid rafts provide? Using FRET confocal microscopy, we previously showed that the cytoplasmic domains of antigen-clustered BCRs undergo a conformational change from a closed to an open form (Tolar et al., 2005). We show here that the transition of the clustered BCR from the closed to an open form correlates both temporally and spatially with condensing of the raft lipid probe around the clustered BCR. By several criteria, the open form of the BCR was signaling active and phosphorylated by Lyn. We speculate that the well-characterized local thickening of raft membranes (McIntosh et al., 2003) or the curvature of the raft membrane (Reynwar et al., 2007) around the clustered BCR may induce the observed conformational change in the clustered BCR cytoplasmic domains. The open BCR cluster would then be phosphorylated by Lyn that was also condensed around the clustered BCR by virtue of its lipid anchor to the plasma membrane. Thus, lipid rafts may provide two distinct functions: namely, to segregate the BCR and Lyn in the plane of the membrane in resting cells and facilitate their interactions after BCR clustering and to alter the character of the membrane surrounding the clustered BCR, inducing an alteration in the conformation of the cluster to accommodate the membrane change. Thus, interaction of the BCR with membrane lipids may play a critical role in the initiation of signaling.

Materials and methods

Cell lines, antigens, and reagents

The CH27 mouse B cells that stably express the recombinant chimeric protein Igα-YFP (Igα fused to the N terminus of YFP) alone or Igα-YFP and Lyn16-CFP (containing the first 16 amino acids of Lyn, including the myristoylation and palmitoylation sequences on the N terminus of the monomeric form of CFP) were described previously (Sohn et al., 2006). The J558 B cell line, which stably expresses the Igα-specific B1-8 μ heavy chain, was maintained as previously described (Tolar et al., 2005). PC conjugated with BSA containing 10 PC per BSA (PC10:BSA; Biosearch Technologies) was used as an antigen for the PC-specific CH27 cell line, and NIP10 or NIP16-BSA containing 16 or 14 NIP groups per BSA molecule (Dal Porto et al., 1998) was used as the antigen for the NIP-specific J558 cell line. PP2 and latrunculin B were purchased from EMD, and 50 μM PP2 dimethyl sulfoxide was added onto the lipid bilayers in PBS for 10 min. After washing with 20 ml PBS, the bilayer was incubated with 25 μg/ml streptavidin for 10 min, and excess streptavidin was removed by washing with 20 ml PBS. The bilayers were incubated for 20 min with 0.5 μg/ml of biotinylated mouse ICAM-1, and excess ICAM-1 was removed by washing. The streptavidin- and ICAM-1–containing planar lipid bilayers were incubated with 0.75 μg/ml of biotinylated PC-BSA or NIP-BSA. The unbound excess of antigen was removed by washing with 20 ml PBS. The mobility of ICAM-1 and antigens in the lipid bilayers was confirmed by analyses of the proteins labeled with fluorescent dyes. Alternatively, NIP- and ICAM-1–containing planar lipid bilayers were prepared by fusing small unilamellar lipid vesicles with a clean glass coverslip coated previously (Lair and McConnell, 1992) using 1,2-dioleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-[N-(5-amino-1-carboxypentyl iminodiacetic acid) succinyl] in nickel salt (Avanti Polar Lipids, Inc.) at a 10:1 ratio. Small unilamellar vesicles were obtained by sonication and clarified by ultracentrifugation and filtering. Glass coverslips were cleaned in Naonstrip (CyanTek), washed, and dried. Lipid bilayers were prepared from 0.1-mM lipid solution on the coverslips attached to the bottom of Lab-Tek imaging chambers. After excess lipids were washed away, histidine-tagged antigens and ICAM-1 were bound. Before imaging, chambers were washed with HBSS supplemented with 1% FCS. NIP10-BSA was prepared as described previously (Tolar et al., 2005) and conjugated to a cysteine-containing peptide terminated with a 12-histidine tag (ASTGATASACTGASTGSH12) using SMCC (Thermo Fisher Scientific) according to the manufacturer’s protocols. Recombinant ICAM-1 tagged with a 12-histidine tag was a gift from J. Hupa (Stanford University, Palo Alto, CA). Conjugation of NIP10-BSA to succinimidyl AlexaFluor647 and ICAM-1-H12 to AlexaFluor488 (both obtained from Invitrogen) was performed according to the manufacturer’s protocols.

TIRFM imaging and image analysis

Through-lens TIRFM (Axelrod, 1981) was performed on an inverted microscope (IX-81; Olympus) equipped with 40× 1.45 NA and 100× 1.45 NA objectives (Olympus). For Figs. 1 C and 5 A, 100× 1.45 NA objectives were used for the image acquisition. Cells expressing CFP and/or YFP were attached to the lipid bilayer on a glass cover slip. TIRFM imaging was performed at 37°C using a heated chamber. A 442-nm laser (Melles Griot) was used for CFP excitation, and CFP and FRET images were acquired simultaneously using a dual image splitter (MAGS Biosystems) equipped with a 505 dichroic beamsplitter and HQ485/30 (CFP) and HQ560/50 (FRET) emission filters (Chroma Technology Corp.). The 514-nm line from an argon gas laser was used to excite YFP, and images were acquired through the same image splitter with the low-pass filter (HQ560/50 filter). CFP-FRET and YFP dual-view images were sequentially acquired in each time point through the alternative switch of each laser line. Images were captured into 16-bit grayscale with no binning and no averaging as 512 × 512 pixels by an electron-multiplying charge-coupled device camera (Cascade II; Photometrics) under management by MetaMorph software (MDS Analytical Technologies). Otherwise, a 1,024 × 1,024–pixel size of images recorded into 10-bit grayscale with an intensifier.
 CCD camera (XR/MEGA-10; Stanford Photonics) was obtained under control by QED software (Media Cybernetics, Inc.) for Figs. 6 A (top row) and S2. FRET images obtained by TIRFM were analyzed by the sensitized acceptor emission method as described in detail previously (van Rheenen et al., 2004; Tolar et al., 2005; Sohn et al., 2006). The FRET efficiency normalized for the acceptor (Ea) predominantly used here was described in a previous paper in detail (Tolar et al., 2005). In brief, first, three CFP (D), FRET (F), and YFP (A) images were obtained by splitting CFP-FRET and YFP dual-view images into each CFP, FRET, and YFP image after alignment between two images using the custom-made macro function in the Image Pro Plus software package (Media Cybernetics, Inc.). CFP-FRET dual-view images from 1.0 μm of blue-green fluorescent polystyrene microspheres (excitation/emission of 430/465) that fluoresce in both the CFP and FRET channels (Invitrogen) were used as an alignment reference in each experiment. Second, the CFP, FRET, and YFP images were background subtracted, flattened for background, and smoothed by a Gauss filter method using Image Pro Plus software. FRET efficiency (Ea) was calculated by the following equation: Ea = (F – β × D – γ × A)/(A × Kd), as described previously (Tolar et al., 2005). Correction factors for donor (CFP) bleed-through (β) and acceptor (YFP) cross talk (γ) in the FRET channel were obtained from single CFP- or YFP-expressing cells present in the same image fields with the experimental cells to eliminate the bias among different fields or times. The β factor was 0.7 ± 0.05, and the γ factor was 0.6 ± 0.1. In our TIRF microscope system, the bleed-through of YFP emission in FRET channel to the CFP channel during 424-nm excitation (β factor) from the YFP single positive cell was negligible. The Kd constant was obtained as described previously (Tolar et al., 2005) by acquiring TIRF as well as epifluorescence images of Daudi, human B cells expressing lyn16-CFP-YFP fusion proteins (Sohn et al., 2006). The Ebleaching value from the control cells was mainly obtained from the epifluorescence images before and after bleaching YFP because of the ease of bleaching. The Ebleaching and Kd values were 0.5 ± 0.5 and 5 ± 0.2, respectively, in our setting condition.

For the quantification of FRET at the single-cell level with time, the mean Fls from the background-subtracted images of each CFP, FRET, and YFP channel were calculated from a region of interest over background levels using the auto-tracking mode of Image Pro Plus software. FRET efficiencies (Ea) were calculated by the aforementioned equation, and data are shown as the mean ± SEM. For counting the number of BCR microclusters per cell in Fig. 2D, YFP-roded IgM clusters that appeared on the TIRF images were manually counted in each cell after bandpass filtering the images using Matlab software (The Mathworks, Inc.). FRET between BCR subunits in Fig. 3, in which the stoichiometry of donor and acceptor is constant, was calculated only from CFP and FRET images as

$$R - \beta - \frac{K_d}{\text{nA}}$$

where R is the CFP/FRET fluorescence ratio and n is the CFP·YFP stoichiometry (n = 2). Fl of the BCR was calculated as $f = \text{CFP}/1 - E$. For image figures, all images shown were converted to the eight-bit scale from original 10- or 16-bit grayscale recorded in the nonsaturated level.

**Single-particle-tracking analysis from time-lapse TIRF images**

The time delay before movement of single BCR clusters associated with the lipid raft probe or with the lyn in Fig. 6 B or in Fig. S2 was analyzed. Time-lapse TIRF FRET images were acquired as described in the previous section from CH27 cells expressing either IgIg-YFP and lyn16-CFP or IgIg-YFP and LynFL-CFP. The images were analyzed by single-particle tracking using Image Pro Plus software. Tracking was performed by autotracking particles detected by centroid lipid-aid analysis above 0.5 μm² that moved within a distance of 1 μm at each time interval with one image frame skip. Clusters that merged or split were included in the tracking analysis. To eliminate artifacts of autotracking, the analyses were manually confirmed. About 200 particles from three to four cells were analyzed, and t tests were used to determine the significance of the differences between IgIg-YFP·lyn16-CFP clusters and IgIg-YFP·LynFL-CFP clusters. The confine index (Schwickert et al., 2007) was calculated as the ratio of the lateral distance traveled to the accumulated cumulative distance and was used as a criteria for the classification of particle movements: >0.8, directional; 0.4-0.8, delayed directional; <0.4, random. When a cluster transitioned from random to directional movement, the time spent in random movement was calculated and expressed as the time delay. For Videos 9, IgIg-YFP clusters were tracked using the following custom macros. In brief, the BCR clusters were first bandpass filtered and, clusters above a selected threshold were chosen for single-cluster tracking using custom-made Matlab script. Single-cluster tracking was performed at the same settings used in Image Pro Plus software analysis (Figs. 6 B and S2) with the exception that the Gaussian-fitted position of the clusters was determined using Matlab script. In all cases, the accuracy of the tracking algorithm was checked visually, and, when needed, the algorithm parameters were adjusted to provide optimal tracking. Cluster trajectories >100 s during the contraction phase were used. The track of each cluster was confirmed manually. Several representative BCR clusters showing directional (Video 9, red circles), delayed directional (Video 9, green circles), and random (Video 9, blue circles) movements were color assigned, and the video was made at 10 frames per second using Matlab script. For calculation of the average molar ratio of YFP to CFP over the contact area of cells to the lipid bilayer, CFP and YFP Fls were obtained at each time point and compared with the CFP and YFP Fls obtained from Daudi B cells expressing lyn16-mCFP·YFP fusion protein given a 1:1 molar ratio as a reference. In reference cells, CFP intensity was obtained after YFP photobleaching. In our setting, a 1:1 ratio of CFP and YFP Fls gives a 10:1 molar ratio.

**Online supplemental material**

Video S1 shows that the association of antigen-clustered BCR with raft lipid probe differs from that with Lyn kinase dramatically using time-lapse images. Fig. S2 shows the dynamic movement of BCR clusters as they associate with raft lipids and move to the synapse using single-cluster tracking. Fig. S3 shows a model for the role of raft lipids in B cell activation. Videos 1–8 are the videos from which the eight still images shown in Fig. S1 were taken. Video 9 shows the dynamic movement of BCR clusters to the synapse during the contraction phase after antigen binding on the lipid bilayer. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200802007/DC1.

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