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Visualizing Lipid Raft Dynamics and Early Signaling Events during Antigen Receptor-mediated B-Lymphocyte Activation[V]

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Recent biochemical evidence indicates that an early event in signal transduction by the B-cell antigen receptor (BCR) is its translocation to specialized membrane subdomains known as lipid rafts. We have taken a microscopic approach to image lipid rafts and early events associated with BCR signal transduction. Lipid rafts were visualized on primary splenic B lymphocytes from wild-type or anti-hen egg lysozyme BCR transgenic mice, and on a mature mouse B-cell line Bal 17 by using fluorescent conjugates of cholera toxin B subunit or a Lyn-based chimeric protein, which targets green fluorescent protein to the lipid raft compartment. Time-lapse imaging of B cells stimulated via the BCR with the antigen hen egg lysozyme, or surrogate for antigen anti-IgM, demonstrated that lipid rafts are highly dynamic entities, which move laterally on the surface of these cells and coalesce into large regions. These regions of aggregated lipid rafts colocalized with the BCR and tyrosine-phosphorylated proteins. Microscopic imaging of live B cells also revealed an inducible colocalization of lipid rafts with the tyrosine kinase Syk and the receptor tyrosine phosphatase CD45. These two proteins play indispensable roles in BCR-mediated signaling but are not detectable in biochemically purified lipid raft fractions. Strikingly, BCR stimulation also induced the formation of long, thread-like filopodial projections, similar to previously described structures called cytonemes. These B-cell cytonemes are rich in lipid rafts and actin filaments, suggesting that they might play a role in long-range communication and/or transportation of signaling molecules during an immune response. These results provide a window into the morphological and molecular organization of the B-cell membrane during the early phase of BCR signaling.

INTRODUCTION

Lipid rafts are plasma membrane microdomains that are implicated in the assembly of diverse signaling pathways such as those mediated by growth factors, morphogens, integrins (Simons, 2000), and antigen receptors on immune cells (Field, 1995; Xavier, 1998; Miceli, 2001; Cheng, 2001a; Holowka, 2001). These domains are rich in glycosphingolipids and cholesterol, which together create a liquid-ordered phase within the plasma membrane. They are fluid at physiological temperatures, allowing lateral diffusion of proteins and lipids within the plane of the membrane (Brown, 1998; Kurzchalia, 1999). In addition, lipid rafts are constitutively enriched in certain types of proteins such as GPI-linked proteins and lipid chain-modified proteins, including the Src kinases, Ras, and heterotrimeric G proteins (Melkonian, 1999).

The importance of lipid rafts has been underscored by recent studies implicating lipid rafts in various disease processes. For example, these microdomains have been shown to act as sites for budding of viruses such as human immunodeficiency virus, Ebola, and Marburg (Bavari, 2002; Ono, 2001; van der Goot, 2001). There is some evidence that the nonpathogenic prion proteins are converted into the pathogenic scrapie form in lipid rafts (Naslavsky, 1997) and that in Alzheimer's disease, the amyloid precursor protein might be processed in lipid rafts (Lee, 1998). They also act as portals for entry of bacterial toxins such as cholera toxin, the B

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Abbreviations used: BCR, B-cell receptor; CTB, cholera toxin B subunit; GFP, green fluorescent protein; HEL, hen egg lysozyme; ITAMs, immunoreceptor tyrosine-based activation motif; TRITC, tetramethyl rhodamine B isothiocyanate.

subunit of which binds to the lipid raft-enriched GM1 ganglioside (Wolf, 2002). Indeed, this property of cholera toxin B subunit has been widely exploited to visualize lipid rafts on a variety of cell types.

Lipid rafts were recently shown to participate in BCR-mediated signal transduction. Engagement of the BCR by anti-Ig antibodies, used as surrogate for antigen, induced its translocation into lipid rafts, as assessed by Western blotting of biochemically purified raft fractions (Cheng, 1999; Petrie, 2000). Furthermore, disruption of lipid rafts by pretreatment of B cells with the antibiotic filipin results in complete loss of BCR-dependent calcium flux, suggesting that the integrity of lipid rafts is critical for signal transduction through the BCR (Aman, 2000). Until recently, the most proximal event in the cascade of signaling mediated by the BCR was thought to be its own phosphorylation by members of the Src family of tyrosine kinases (Kurosaki, 1999). However, experiments using Src kinase inhibitors and actin-depolymerizing agents indicate that BCR translocation to the lipid raft fraction does not require receptor phosphorylation or association with the actin cytoskeletal assembly but rather may be a direct consequence of receptor oligomerization by antigen (Cheng, 2001b). Interestingly, the developmental stage of the B cell influences the ability of the BCR to enter lipid rafts and the outcome of signaling through the receptor. A fraction of the pre-BCR was found to be constitutively associated with lipid rafts (Guo, 2000), which may reflect constitutive signaling that promotes the transition to the next stage in the development. On the other hand, the BCR does not mobilize to lipid rafts upon receptor engagement in immature B cells, and this may contribute to the very different cellular response to antigen in immature and mature B cells (Sproul, 2000; Chung, 2001).

Although biochemical isolation procedures have indicated that the BCR translocates to lipid rafts after receptor engagement, there is some concern that the isolation procedure may not accurately reveal the events occurring in the cell. Certain protein–protein interactions may be lost or conceivably enhanced by the detergent extraction and prolonged isolation protocol. In this study, we have used a microscopic visualization approach to examine lipid raft dynamics and the colocalization of signaling molecules with lipid rafts in live B cells. We report herein that a proportion of the BCR becomes associated with lipid rafts upon receptor cross-linking, in confirmation of previous biochemical fractionation results. We also visualized two key early signaling components, the tyrosine kinase Syk and the tyrosine phosphatase CD45 that cannot be detected in biochemically purified lipid rafts from anti-IgM–stimulated B cells. Current understanding of antigen receptor signaling pathway predicts that these components should be accessible to antigen receptor and Src family tyrosine kinases in lipid rafts. Indeed, both of these proteins were readily visualized colocalizing with lipid rafts in live B cells consistent with their participation in BCR signaling. Fluorescence microscopy also revealed that lipid rafts in B cells are dynamic structures that coalesce into large patches on the cell surface after BCR stimulation. In addition, real-time imaging of B cells stained for lipid rafts revealed that BCR stimulation induces the formation of lipid raft-containing, long thread-like filopodial outgrowths of the plasma membrane, known as cytonemes.

MATERIALS AND METHODS

Cells and Reagents

The mature mouse B-cell lymphoma line Bal 17 was cultured in B-cell medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, and 2 mM sodium pyruvate. Primary B cells were purified from spleens of 8- to 12-wk-old male C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or MD4 (anti-HEL Ig transgenic, obtained from Dr. J.G. Cyster, University of California, San Francisco, San Francisco, CA) (Mason, 1992) mice by hypotonic lysis of the red blood cells followed by incubation of the cells with anti-CD43–coated MicroBeads (Miltenyi Biotec, Auburn, CA), which bind to all splenic cells with the exception of resting mature B cells. The B cells were then obtained by passing the cells through a negative depletion column attached to a VarioMACS magnet (Miltenyi Biotec). The B cells purified by this protocol were 95% pure as determined by flow cytometry with anti-CD19 staining. These cells were rested in B-cell medium for 1 h at 37°C before stimulation. Unconjugated and rhodamine conjugates of affinity-purified $F(ab')_2$ or Fab fragments of goat anti-mouse IgM were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC) conjugate of anti-phosphotyrosine monoclonal antibody (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Purified HEL and cholera toxin B subunit (CTB) conjugated to FITC were obtained from Sigma-Aldrich (St. Louis, MO), and the CTB-tetramethylrhodamine B isothiocyanate (TRITC) conjugate was from List Biological Laboratories (Campbell, CA). Anti-CD45, anti-CD71, and Fc receptor blocking antibody (clone 2.4G2) were from BD Biosciences PharMingen (San Diego, CA). The Src family kinase inhibitor PP1 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Green Fluorescent Protein (GFP) Fusion Constructs

A 156-base pair *Bam*HI/*Sna*B I fragment, encoding the N-terminal 24 amino acids of murine Lyn, designated as Lyn24, was excised out of pBS-LynF2.4 (Chan, 1997) and subcloned into the *Bam*HI and *Eco*R V sites of a GFP expression vector pQBI-fN2 (Quantum Biotechnologies, Montreal, Quebec, Canada). The resulting fusion protein, Lyn24-GFP, was composed of 24 amino acids of Lyn followed by GFP (Figure 1A). A plasmid encoding a Syk-GFP fusion protein was obtained from Dr. Robert Geahlen (Purdue University, West Lafayette, IN) and has been described previously (Ma, 2001). An expression construct encoding an actin-GFP fusion protein was purchased from BD Biosciences Clonetech (Palo Alto, CA).

Microscopy and Data Processing

Ten million Bal 17 cells were transiently transfected by electroporation (300 V, 975 μF) with 20 μg of the Lyn24-GFP- or Syk-GFP–encoding recombinant plasmids, 24 h before examination. The cells were stained with 25 μg/ml CTB-TRITC for 20 min on ice, and the unbound reagent was washed away by centrifugation. Five million cells/500 μl of RPMI 1640 medium were adhered to poly-L-lysine–coated Delta T glass dishes (Bioptechs, Butler, PA) or left in suspension (in 100 μl of RPMI 1640) followed by stimulation with 25 μg/ml $F(ab')_2$ fragments of goat anti-mouse IgM at room temperature. The suspension cells were mixed rapidly by pipetting and 12 μl was transferred to slides for imaging. For cells that were adhered to the glass dishes, 50 μg/ml anti-mouse IgM $F(ab')_2$ was added to the medium, mixed by pipetting, and time-lapse imaging was started instantly. For BCR and lipid raft colocalization experiments, 5×10^6 Bal 17 cells in 100 μl were stained with 25 μg/ml CTB-FITC, followed by stimulation with 25 μg/ml rhodamine conjugates of Fab or $F(ab')_2$ fragments of anti-mouse IgM. B cells derived from MD4 mice were stimulated with plate-bound HEL (plates coated by incubating for 1 h with 10 mg/ml HEL) for indicated periods at

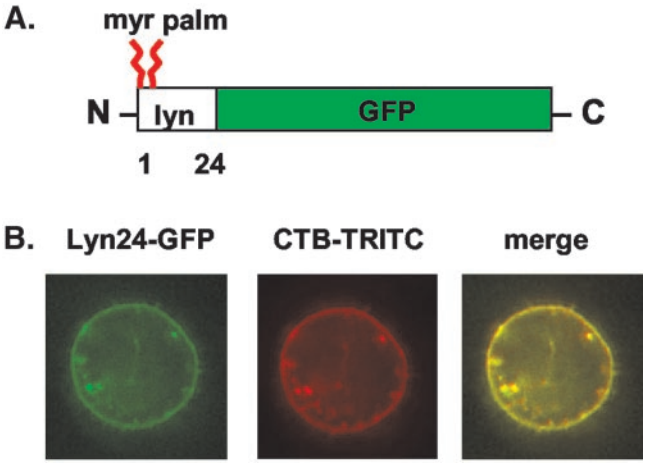

Figure 1. Correlation between lipid raft staining with cholera toxin B and Lyn24GFP. (A) A marker specific to lipid rafts was constructed by fusing the N-terminal 24 amino acids of the Src family tyrosine kinase Lyn, which contain N-terminal myristylation (myr) and palmitoylation (palm) sites, to the N terminus of green fluorescent protein (see MATERIALS AND METHODS). The resulting chimera was termed Lyn24GFP. (B) Bal 17 cells were transiently transfected with the Lyn24GFP construct and after overnight expression stained with 25 μg/ml CTB-TRITC. Images were acquired of surface planar sections simultaneously using the fluorescein and rhodamine filters, and single-color and overlay images are shown as indicated.

37°C followed by fixation with 4% paraformaldehyde (PFA) and staining with CTB-FITC and anti-mouse IgM-TRITC. To test whether the above-mentioned method of activation was able to induce early signaling events such as tyrosine phosphorylation in MD4 B cells, lysates of unstimulated cells and those stimulated with plate-bound HEL were subjected to Western blotting with an anti-phosphotyrosine antibody. Several phosphoproteins were found to be induced in HEL-stimulated B cells, confirming that stimulation with 10 mg/ml plate-bound HEL for 10 min was sufficient to transduce an activation signal (our unpublished data). Images were taken at 10 min poststimulation by using the fluorescein and rhodamine filters. For anti-phosphotyrosine staining, 1×10^7 Bal 17 cells in 200 μl were stimulated as described above, fixed with 4% PFA for 30 min at room temperature, washed, and stained with FITC-conjugated anti-phosphotyrosine antibody (clone 4G10) in 100 μl of permeabilization buffer (0.5% saponin and 0.1% bovine serum albumin in phosphate-buffered saline [PBS]) for 1 h in the dark at room temperature. After washing twice with PBS, the cells were resuspended in 50 μl of PBS, of which 12 μl was transferred to slides and examined by microscopy. Inhibition of Src family kinase activity was performed by preincubating the cells with 10 μM PP1 for 15 min at 37°C. For time-lapse imaging of lipid rafts, 5×10^6 Bal 17 cells were adhered to poly-L-lysine–coated Delta T glass dishes for 1 h, and the cells were rinsed with RPMI 1640 medium and stimulated with 50 μg/ml F(ab')$_2$ goat anti-mouse IgM. Time-lapse movie recording was started simultaneously and images were collected every 15 s for a period of 15 min. All images were acquired using a Deltavision microscope (Applied Precision, Issaquah, WA) with an attached charge-coupled device camera (Nikon, Tokyo, Japan). The images were digitally deconvolved using the Softworx program associated with the microscope and processed with Adobe Photoshop 5.5, Microsoft PowerPoint, and Quicktime Pro programs.

RESULTS

Lipid Raft Staining on Resting B Lymphocytes

To study the distribution of lipid rafts in B cells before and during activation, we made use of CTB, which binds to the GM1 ganglioside, a component of lipid raft membrane microdomains in the outer leaflet of the plasma membrane (Brown, 1998). Biotinylated CTB was able to detect GM1 by Western blotting and, by this criterion, GM1 was highly enriched in lipid raft preparations from Bal 17 B cells (our unpublished data) as previously demonstrated in other cell types (Field, 1995; Xavier, 1998; Cheng, 1999). Therefore, TRITC-conjugated CTB (CTB-TRITC) was used to detect and visualize the GM1-containing lipid rafts on mouse splenic B cells or on a mouse B-cell line Bal 17. However, proteins such as Src family kinases are associated with lipid rafts in the inner leaflet of the plasma membrane and transduce signals to downstream proteins from this face of the membrane. To test the correlation of outer leaflet lipid rafts with those in the inner leaflet, and provide an independent raft visualization approach, a chimeric fusion construct was created by fusing the N-terminal 24 amino acids of Lyn, which contain the lipid raft targeting myristoylation and palmitoylation modifications, and GFP. The resultant fusion protein was termed Lyn24-GFP (Figure 1A). As illustrated in Figure 1B, the CTB staining of lipid rafts was characteristically punctate and randomly distributed across the plasma membrane. Microscopic visualization of the Lyn24-GFP fusion protein in transiently transfected Bal 17 cells revealed a similar punctate appearance that showed striking colocalization with cholera toxin B-TRITC staining (Figure 1B). Thus, both methods seemed to preferentially visualize lipid rafts in the plasma membrane of B cells and independently confirm the punctate staining pattern of these structures. The correlation between the two visualization methods (Figure 1B, right) provides further support for the assumption that CTB is not only used to mark outer leaflet rafts but also lipid rafts on the inner leaflet of the plasma membrane.

BCR Cross-Linking Induces Lipid Raft Aggregation

It has been suggested that the size of individual lipid rafts might depend on the activation state of the cell (Brown, 1998); therefore, we wanted to test whether the size and distribution of the lipid rafts on B cells was affected by BCR cross-linking. Images were acquired of 50 focal planes (Z-stack), each separated from the adjacent plane by 0.2 μm, through Bal 17 cells that were unstimulated or stimulated with anti-BCR F(ab')$_2$ antibodies. These images were digitally deconvolved and combined to generate a three-dimensional reconstruction by using the Softworx software. The resulting image (Figure 2A, left) showed lipid rafts as small patches scattered randomly all over the surface of the unstimulated cells. However, the three-dimensional image of Bal 17 cells stimulated with anti-IgM revealed that in response to stimulation, lipid rafts coalesced into one or a few large aggregated patches (Figure 2A, right). To make sure that the observed lipid raft coalescence was not a peculiarity of the cell line used, we purified mature resting B cells from the spleens of mice and subjected them to BCR cross-linking. Similar to our observations in Bal 17 cells, splenic B cells also polarized lipid rafts as large patches on one side of the cell (Figure 2B). Together, these images demonstrate that smaller

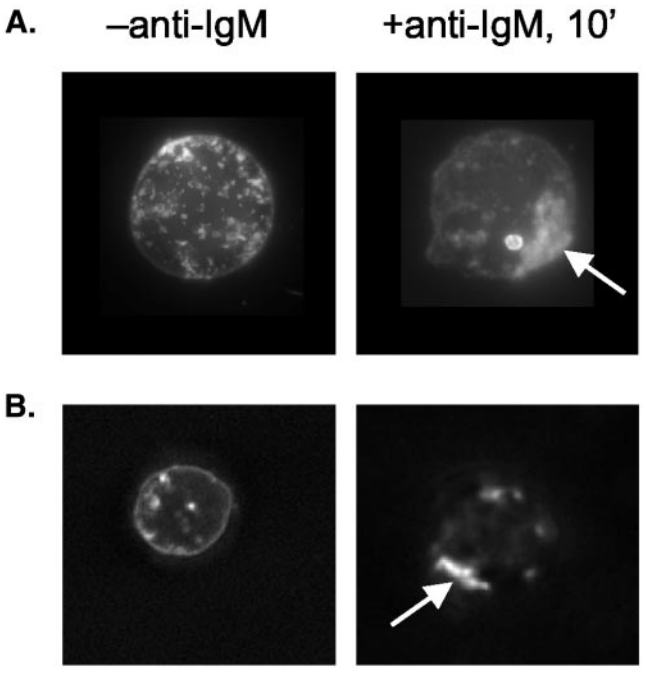

Figure 2. Lipid rafts coalesce and polarize upon BCR ligation. Bal 17 cells (A) or splenic B cells from C57BL/6 mice (B) were left unstimulated (left) or stimulated with 25 μg/ml anti-mouse IgM $F(ab')_2$ for 10 min (right), fixed with 4% PFA, and stained with CTB-TRITC. The cell suspension was placed on a glass slide, covered with a coverslip, and images were acquired, using the rhodamine-phycoerythrin-Texas Red filter, of a surface planar section for splenic B cells (B), or a Z-stack of 50 adjacent focal planes for Bal 17 cells followed by three-dimensional reconstruction by using the Softworx program (A). The areas of the cell that are enriched for lipid rafts are indicated by white arrows.

lipid raft moieties coalesce in one or a few regions of a stimulated B cell as patches.

Cross-linked BCR Colocalizes with Lipid Rafts

Biochemical evidence from purified lipid rafts has indicated that the BCR in both mouse and human B cells rapidly translocates to the lipid raft fraction upon receptor cross-linking (Cheng, 1999; Petrie, 2000). To examine this phenomenon visually, we sought to determine the distribution of the BCR on live B cells. Bal 17 cells were stained with CTB-FITC and treated for 10 min at room temperature with either nonstimulatory Fab fragments or stimulatory $F(ab')_2$ fragments of rhodamine-conjugated anti-mouse IgM antibodies. Images of single focal planes were acquired in both green and red channels simultaneously. Cells that were treated with monovalent anti-IgM (Fab) fragments showed a uniform distribution of the receptor with characteristic punctate lipid raft staining. There was little colocalization of the BCR with lipid rafts (Figure 3, first row). In contrast, the $F(ab')_2$ fragment of anti-IgM cross-linked the BCR into small and large patches and induced coalescence of lipid rafts into several large patches. Overlay of the two stains revealed that a significant fraction of the receptor was colocalized with the coalesced lipid rafts at 10 min after stimulation. Interestingly, a significant population of the cross-linked receptor was also found to be located outside the lipid rafts at this time, supporting the biochemical evidence and suggesting that not all of the receptor mobilizes to lipid rafts, at least not within 10 min (Figure 3, second row). However, at later time points (20 min post cross-linking) almost all the BCR was found to colocalize with lipid rafts (our unpublished data). A quantitation of the fraction of B cells that exhibited lipid raft coalescence and BCR colocalization after 5 and 10 min of BCR ligation is presented in Table 1. To test whether the association of BCR with lipid rafts is dependent on the type of stimulus or state of the cell, we sought to address this phenomenon by using real antigen. Mature, resting B cells from the spleens of MD4 (expressing anti-HEL transgenic BCR) (Mason, 1992) were stimulated with plate-bound HEL or not, for 30 min; fixed; and stained with CTB-FITC and anti-IgM-TRITC. Unstimulated MD4 B cells exhibited uniform plasma membrane staining for BCR and punctate staining for lipid rafts (Figure 3, third row), whereas plate-bound HEL stimulation induced the polarization of the BCR. Furthermore, the BCR was mobilized to the lipid raft compartment, as seen by the colocalization of the BCR cap with lipid raft patches in the two representative cells shown in Figure 3 (fourth and fifth rows, right).

CD45 Colocalizes with Lipid Rafts upon BCR Engagement

Tyrosine phosphorylation of the BCR depends on the activation of Src family tyrosine kinases, which are strongly enriched in lipid rafts (Kurosaki, 1999). The activation of these kinases is in turn regulated by the phosphorylation state of their C-terminal negative regulatory tyrosine, which is tyrosine phosphorylated by the kinase Csk and dephosphorylated by the receptor tyrosine phosphatase CD45 (Yanagi, 1996; Thomas, 1999). Consequently, mice deficient in CD45 are compromised in their ability to signal through the T-cell receptor (TCR) and BCR (Chan, 1994). For CD45 to promote activity of Src kinases, it is expected that during lymphocyte activation CD45 and the Src kinases would be in proximity, perhaps in lipid rafts. Surprisingly, CD45 is absent from biochemically purified lipid rafts of antigen receptor-stimulated T cells (Xavier, 1998) and B cells (Cheng, 1999). Thus, we decided to examine the microscopic distribution of CD45 with respect to lipid rafts in the absence and presence of BCR stimulation in live B cells. In unstimulated Bal 17 cells, CD45 was found to be uniformly distributed, except in 10% of the cells where there was some degree of colocalization between lipid rafts and CD45. It was interesting to note that in this population of cells, the colocalization was visible with those rafts whose diameter was at least 500 nm. The other 90% of the cells did not show any overlap between CD45 and lipid rafts (Figure 4A, first row, and Table 1). On BCR cross-linking there was a cumulative increase in the association of CD45 with larger patches of lipid rafts (Figure 4, A, second row, and B; and Movie 1 in supplementary data). However, not all of the CD45 in the cell becomes associated with lipid rafts, probably because of the abundant nature of this protein. The induced association of CD45 with the lipid raft fraction was also consistently observed in MD4 B cells that were stimulated with plate-

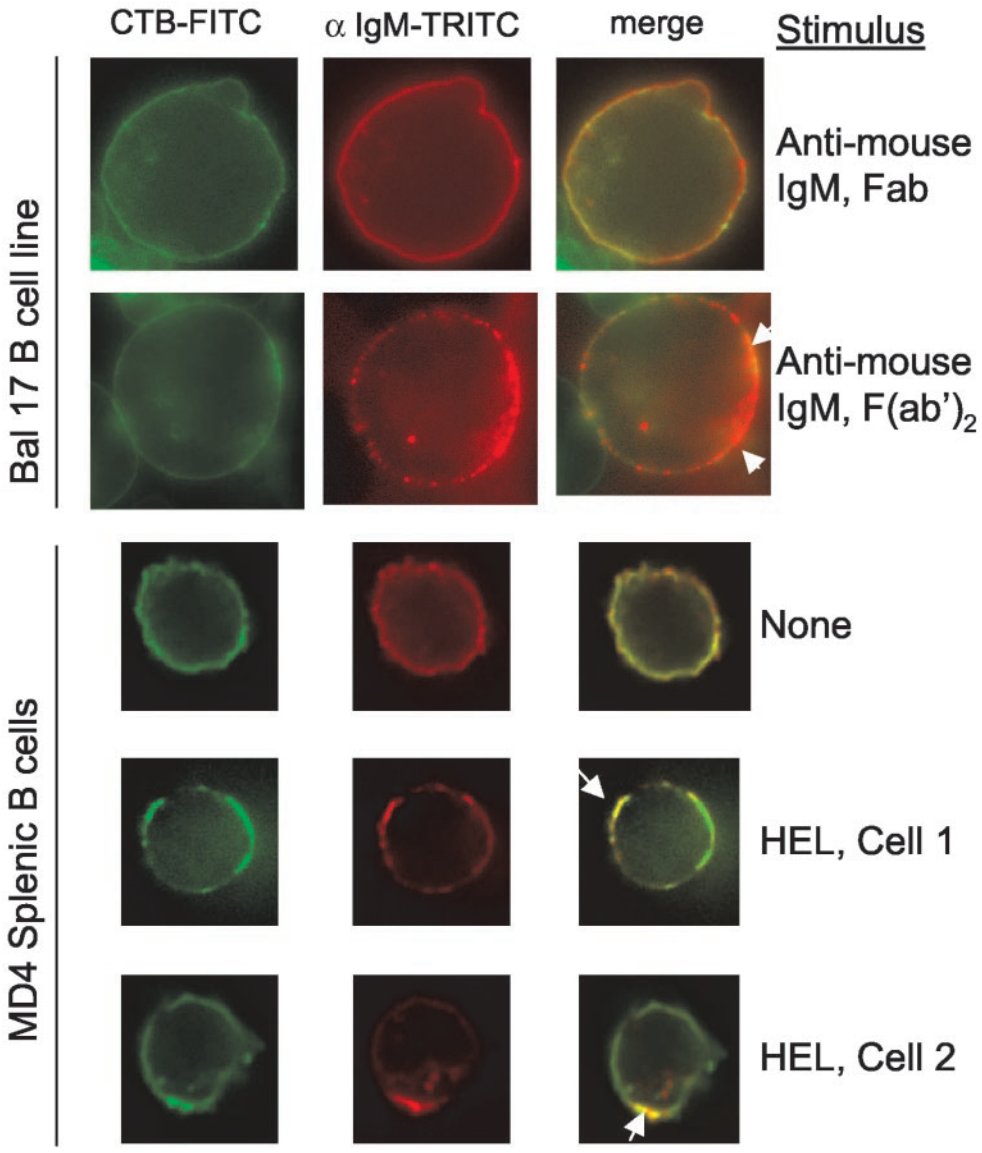

Figure 3. BCR colocalizes with lipid rafts upon ligation. Bal 17 cells were stained with CTB-FITC as described in text, followed by treatment with 25 μg/ml rhodamine conjugates of anti-IgM Fab fragment or anti-IgM F(ab')$_2$ fragment at room temperature. MD4 B cells were stimulated with 10 mg/ml plate-bound HEL. Images were collected after 10 min, by using the fluorescein and rhodamine filters of the Deltavision microscope. Lipid raft staining is shown in green, BCR in red and the colocalization regions in yellow as indicated by the white arrows.

bound HEL for 10 min (Figure 4A, third, fourth, and fifth rows).

To contrast the localization of CD45 with that of nonraft-associated cell surface proteins, we examined the localization of transferrin receptor (CD71). The transferrin receptor is a surface molecule that rapidly shuttles between the plasma membrane and endosomes and therefore has been previously used as a marker for endosomes and the nonraft fraction of the plasma membrane (Harder, 1998). It was also found to be extracted by Triton X-100 under conditions that leave lipid rafts intact (our unpublished data). Bal 17 cells were either left unstimulated or stimulated with anti-BCR antibodies for 10 min. Fixed cells that had been subjected to a prior treatment with Fc receptor-blocking antibody 2.4G2 were stained with FITC-conjugated anti-CD71 antibodies and CTB-TRITC. Although the lipid rafts coalesced upon BCR cross-linking, as shown in Figure 5, no colocalization was evident between the transferrin receptor and lipid rafts before or after stimulation. Similar results were obtained for MD4 cells stimulated with plate-bound HEL (our unpublished data). Therefore, the transferrin receptor is an example of a plasma membrane protein that is not associated with lipid rafts in stimulated or unstimulated B cells and serves as a negative control for these colocalization experiments.

Syk Kinase Migrates to Lipid Rafts upon BCR Cross-Linking

Cross-linking of the BCR induces phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) found on the Igα/Igβ signal-transducing chains of the BCR, creating a binding site for the tyrosine kinase Syk. Recruitment of Syk to the engaged BCRs and its enzymatic activation are likely to play a critical

Table 1. Quantitation of B cells exhibiting colocalization of signaling molecules with lipid rafts

Event[a]	% Cells positive[b,c] 0 min	5 min	10 min
Lipid raft coalescence	0	10	95
BCR and lipid raft colocalization	0	90	95
CD45 and lipid raft colocalization[d]	10	10	90
Syk and lipid raft colocalization	50[e]	90	90
pY and lipid raft colocalization	0	90	90
CD71 and lipid raft colocalization	0	0	0

[a] Signaling event occurring upon BCR cross-linking.

[b] Percentage of cells showing colocalization of lipid rafts with the BCR, CD45, Syk, pY, or CD71 out of the total number of cells scored ($n \geq 80$). During calculation of percentages, the fractions were rounded off to the nearest five.

[c] Degree of colocalization varied according to time of cross-linking and the signaling molecule looked at, e.g., only 50% of the BCR in 90% of the cells is colocalized with lipid rafts at 10 min, but ~95% of it was cocapped with lipid rafts at 20 min.

[d] In ~10% of the unstimulated cells, there was some colocalization of CD45 with lipid rafts; however, note that in these cells, the lipid rafts that were associated with CD45 were present as bigger patches than the usual punctate staining. Figure 4A (first row) shows an unstimulated cell representing the other 90% of the population that do not show any colocalization between CD45 and lipid rafts.

[e] Some colocalization was observed between Syk and lipid rafts even in unstimulated B cells; however, because these cells were adhered to glass using poly-L-lysine it is possible that lipid rafts are recruited to the site of attachment and this in turn may have led to Syk recruitment. Nonetheless, recruitment of Syk to the surface upon BCR engagement, and colocalization with plasma membrane lipid rafts was an inducible event.

role in antigen receptor signaling (Kurosaki, 1999). In agreement with this view, it has been demonstrated that anti-IgM stimulation mobilizes Syk from the cytosol to the cross-linked BCR at the membrane (Hutchcroft, 1992; Law, 1993; Ma, 2001). To examine this redistribution relative to lipid rafts, we carried out real-time microscopic imaging of the mobilization of Syk to the plasma membrane. Stable transfectants of Syk-GFP in Bal 17 cells were stimulated with anti-IgM in suspension, transferred to glass slides, and visualized by time-lapse image acquisition. Figure 6A shows the progressive accumulation of cytosolic Syk-GFP at the surface after which it patches in one region of the cell at 10 min (time-lapse Movie 2 in supplementary data). This distribution mirrors patching and capping of the BCR upon cross-linking by antigen, suggesting that Syk might associate with the BCR in lipid rafts. However, when lipid rafts were biochemically isolated from anti-IgM–stimulated B cells, Syk was not detected in the lipid raft fractions (our unpublished data). Therefore, we used microscopy to examine whether Syk translocates to lipid rafts upon BCR engagement. For these experiments, we transiently transfected Bal 17 cells with a plasmid encoding a Syk-GFP fusion protein, adhered them to poly-L-lysine–coated Delta T dishes for 1 h, and then stained the rafts with CTB-TRITC. The dishes were placed on a microscope stage adapter and after focusing on a cell that was positive for both Syk-GFP and CTB-TRITC, anti-mouse IgM was added to the dish. At time 0, Syk-GFP was localized mostly in the cytoplasm, although a small amount of colocalization with the surface lipid rafts was visible in these unstimulated cells (Figure 6B, top, and Table 1). Because the Bal 17 cells were adhered to glass through poly-L-lysine, it is possible that lipid rafts get recruited to the site of attachment. Syk has been shown to play a role in integrin-mediated signaling and adhesion of lymphoid and myeloid cells (Miller, 1999; Stupack, 1999; Mocasi, 2000); thus, the fraction of Syk that colocalizes with lipid rafts in unstimulated Bal 17 cells might participate in attachment-dependent signaling. As seen in the bottom panels of Figure 6B, after 10 min of BCR cross-linking, Syk was recruited to the cell surface and upon overlaying with CTB-TRITC staining, it showed substantial colocalization with the lipid rafts. It is to be noted that for these experiments Syk-GFP was overexpressed in B cells and together with the endogenous complement of Syk, is probably in excess over other signaling components. Perhaps for this reason, we do not find all of Syk getting recruited to the cell surface upon BCR ligation and there is still a substantial amount of Syk present in the cytosol. Our results are consistent with the hypothesis that ligand-induced clustering of the BCR promotes its movement to lipid rafts, where it becomes tyrosine phosphorylated on its ITAMs and subsequently recruits Syk as an intermediate step in the signaling cascade. In addition, our data demonstrate the advantage of detecting relocation of molecules into lipid rafts by microscopic imaging techniques, compared with the biochemical purification approach, which failed to detect Syk in the lipid raft fraction upon BCR cross-linking, presumably due to dissociation during the prolonged isolation procedure.

Once Syk is recruited to the BCR, it gets tyrosine phosphorylated, which increases its enzymatic activity (Kurosaki, 1999). After its own activation, Syk in turn phosphorylates downstream signaling molecules such as BLNK and phospholipase Cγ2, in association with their recruitment to the BCR signaling complex. To examine the association of tyrosine-phosphorylated proteins with lipid rafts, we stimulated Bal 17 cells with $F(ab')_2$ anti-IgM for 1 or 5 min, and purified lipid rafts from these cells. Western blot analysis of purified lipid raft fractions from unstimulated and BCR-stimulated cells revealed a number of tyrosine phosphorylated protein bands, including those at ~110, 65–75, 50–60 (including members of the Src family of tyrosine kinases), 40, and 34 kDa (our unpublished data). An appreciable portion of the induced tyrosine phosphoproteins has also been found in the Triton X-100–soluble and high-density–insoluble fractions after BCR ligation (Cheng, 1999; Petrie, 2000). To circumvent the problem of dissociation of phosphorylated proteins from lipid rafts during biochemical purification, we looked for the distribution of tyrosine-phosphorylated proteins with respect to lipid rafts microscopically. In unstimulated Bal 17 cells, basal tyrosine phosphorylation was seen largely dispersed in the cell, although some concentration was detected at the membrane (Figure 6C, top row, middle). In contrast, in cells stimulated through the BCR for 10 min tyrosine phosphorylation was induced and was largely localized to a large concentrated patch colocalizing with lipid rafts (Figure 6C, middle row). To address the role of tyrosine phosphorylation by Src family kinases in BCR-dependent lipid raft aggregation, the cells

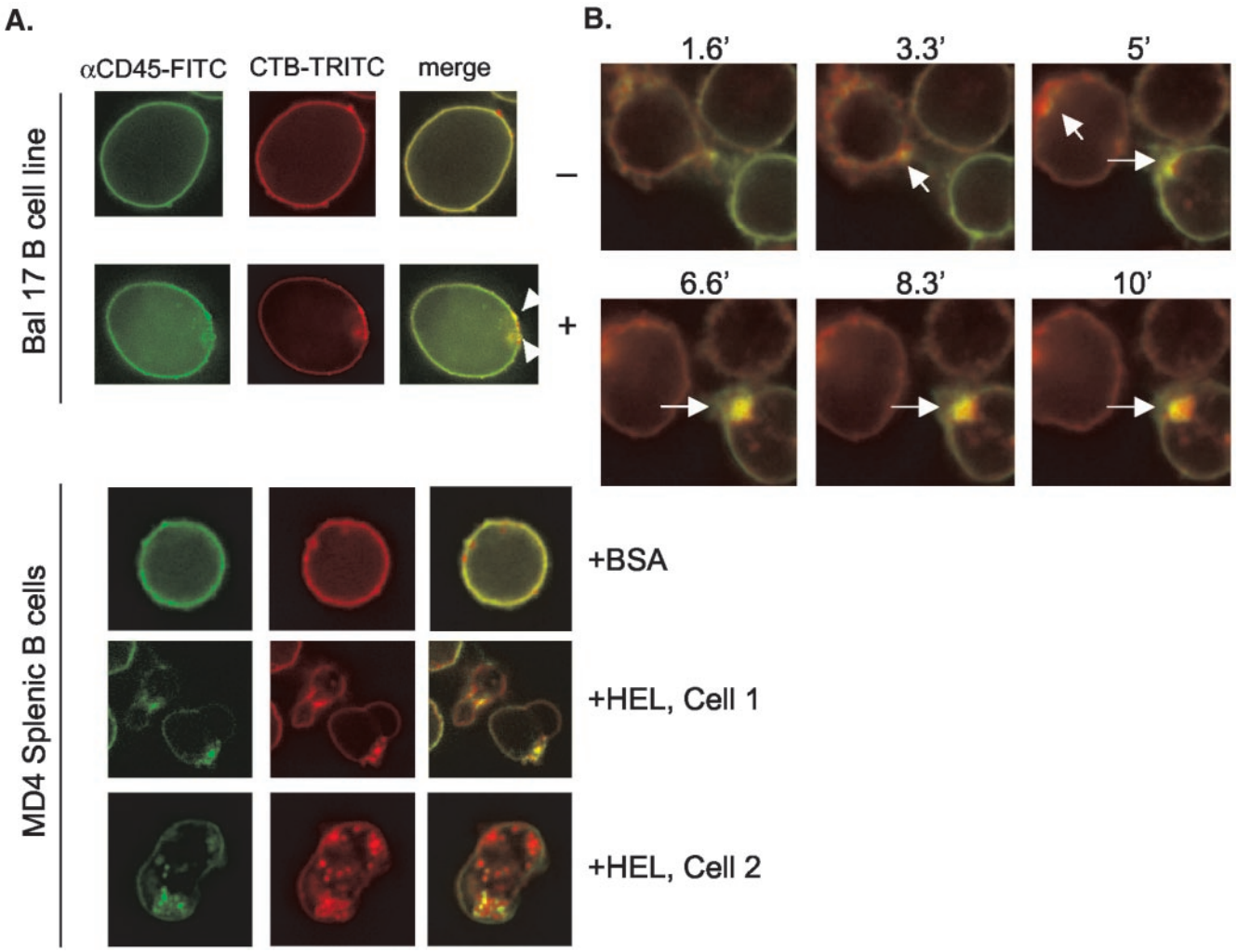

Figure 4. CD45 colocalizes with lipid rafts after BCR cross-linking. (A) Bal 17 cells were stimulated (+) or not (−) with 25 μg/ml anti-IgM F(ab′)$_2$ for 10 min at 37°C. MD4 B cells were stimulated with plate-bound HEL for 10 min at 37°C. The stimulation was stopped by fixing the cells with 4% PFA followed by staining lipid rafts with CTB-TRITC. The cells were washed and preblocked with the Fc blocking antibody 2.4G2 for 10 min followed by washing and staining with anti-CD45-FITC for 30 min at 4°C. After washing the cells were visualized using the fluorescein and rhodamine filters of the Deltavision microscope. The panels showing CD45 and CTB staining in unstimulated cells are representative of 90% of the population in which no colocalization was observed (refer to Table 1 for quantitation data). In the other 10% of cells, some colocalization was seen. (B) Bal 17 cells were adhered onto a poly-L-lysine–coated glass dish for 1 h and after blocking Fc receptor binding by treating with 2.4G2 for 10 min, the cells were stained with FITC-conjugated anti-CD45 for 30 min at 4°C. The cells were washed and subsequently stained with CTB-TRITC. The dish was fixed on a stage adapter, the microscope focused on the cells and images acquired using the fluorescein and rhodamine filters in the unstimulated state (top). Anti-IgM F(ab′)$_2$ (50 μg/ml) was added to the dish and image acquisition was started simultaneously. Images were collected over a period of 10 min with a time lapse of 15 s. Surface planar views of the cells are shown, with CD45 in green, lipid rafts in red and regions of colocalization in yellow indicated by white arrows. The time course of CD45 colocalization, after BCR stimulation, can be viewed in movie format (Movie 1) in the supplementary information section.

were preincubated with 10 μM PP1, a Src family kinase-specific inhibitor, before BCR cross-linking. As seen in Figure 6C (bottom row), BCR stimulation induced lipid raft coalescence, however, tyrosine phosphorylation was not induced under these conditions. These results suggest that a substantial fraction of BCR-induced tyrosine phosphorylation occurs at or near lipid raft regions of the plasma membrane, but Src family-dependent phosphorylation is not required for BCR-dependent lipid raft aggregation.

Dynamics of the Plasma Membrane and Induction of Cytonemes during B-Cell Activation

During the course of these studies, we noticed that BCR stimulation induced dramatic changes in the shape of Bal 17 and primary splenic B cells, including the appearance of plasma membrane outgrowths resembling amoeboid pseudopodia (our unpublished data) and long, filopodia-like structures (Figure 7, A and D). The filopodial structures are

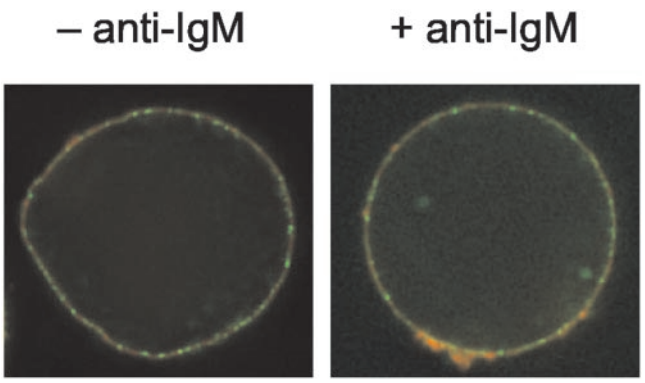

Figure 5. Transferrin receptor (CD71) does not colocalize with lipid rafts either before or after BCR ligation. Bal 17 cells were either left unstimulated (left) or stimulated (right) with 25 μg/ml anti-IgM $F(ab')_2$ fragment for 10 min at 37°C. The reaction was stopped by fixing the cells with 4% PFA for 20 min at room temperature. The cells were washed and stained with CTB-TRITC as described in text, followed by blocking with 2.4G2 antibody for 10 min. After washing, the cells were stained with FITC-conjugated anti-CD71 (anti-transferrin receptor) antibody for 30 min at 4°C. Images were collected using the fluorescein and rhodamine filters of the Deltavision microscope. Lipid raft staining is shown in red, CD71 in green, and the colocalization regions in yellow.

reminiscent of the thin actin-based extensions, called cytonemes, which were described to be induced during wing development in *Drosophila* (Ramirez-Weber, 1999). The cytonemes observed on B cells were thin and thread-like with a thickness of 0.2–0.4 μm and lengths reaching up to $\geq$80 μm, almost equivalent to 10 cell diameters (Figure 7B). B-cell cytonemes often showed a branched structure with concentrated lipid raft staining at branch points. In addition, they had bulbous tips that were enriched in lipid rafts. Figure 7C shows a three-dimensional image reconstruction of a Bal 17 B cell that had been stimulated with anti-IgM for 30 min and stained with CTB. A long cytoneme can be seen going over the B cell. Besides the overall lipid raft staining, B-cell cytonemes showed a punctate staining for lipid rafts along the length of the threads (Figure 8A). To examine the involvement of cytoskeletal elements in the formation and maintenance of B-cell cytonemes, we expressed an actin-GFP fusion protein in Bal 17 cells. BCR cross-linking resulted in the induction of cytonemes with punctate staining for actin-GFP along the length of the extension (Figure 8B). Thus, the arrangement of the actin in B-cell cytonemes was similar to that observed for lipid rafts. The BCR-induced cytonemes formed as early as 5 min after stimulation; however, they were both shorter and short-lived at early time points. They were found to elongate with time and stabilized between 30 and 40 min after stimulation. It is unknown whether the short processes rapidly extend and retract or simply break off. B-cell cytonemes were extremely fragile and sensitive to strong fixation and movement, similar to the *Drosophila* cytonemes (Ramirez-Weber, 1999). It was difficult to observe them unless the cells were either living or only mildly fixed. Although unstimulated B cells bear numerous, short filopodia on the surface, the number of cytonemes was restricted to a maximum of three per cell. The induction of cytonemes on B cells suggests that they may participate in long-distance communication between the antigen-stimulated B cells and other immune cells in the lymphoid organs such as follicular dendritic cells and T cells.

DISCUSSION

In this study, we have carried out a microscopic visualization of the earliest signaling events that occur after BCR ligation and their relationship to cholesterol-rich plasma membrane microdomains known as lipid rafts. We have shown that upon BCR cross-linking smaller lipid rafts coalesce together to occupy a large area of the activated B-cell surface. A fraction of the engaged antigen receptor associates with the polarized lipid rafts and in turn recruits the tyrosine kinase Syk to lipid rafts. The functional outcome of the recruitment of Syk is evident by the association of tyrosine-phosphorylated proteins with the lipid raft fraction. Thus, these images strongly support the emerging view that lipid rafts represent a favored site of BCR signaling that cross-linked receptors rapidly access concomitant with raft polarization (Pierce, 2002). We also present microscopic evidence for the association of the tyrosine phosphatase CD45 with lipid rafts, which is not detectable by conventional biochemical detection methods. Finally, we report the induction of filopodia-like extensions called cytonemes, on the B-cell surface upon ligation of the BCR. These thread-like projections contain both regions of intense lipid raft staining and polymerized actin filaments.

The coalescence of lipid rafts and entry of crucial signaling molecules into these microdomains upon ligand-mediated cross-linking of the BCR suggests that lipid rafts serve as a site for the clustering and concentration of signaling proteins. Lipid rafts in resting B cells are enriched in certain signaling components such as Lyn (Cheng, 1999), Fyn, Blk (our unpublished observations), and Ras (Melkonian, 1999). Because tyrosine phosphorylation of BCR ITAMs by Src family kinases is the initiating event during BCR signaling, and these kinases are enriched in lipid rafts, ligand-induced mobilization of the BCR to lipid rafts may promote the initiation of BCR signaling. However, the BCR does not enter lipid rafts in immature B cells, which undergo apoptosis rather than activation upon receptor ligation (Sproul, 2000; Chung, 2001). Thus, movement of the BCR to lipid rafts may not only amplify signaling but also change its biological outcome, for example, by allowing activation of signaling events that are particularly dependent on lipid rafts.

Although biochemical purification of lipid rafts has led to the identification of a number of signaling proteins that associate with rafts upon receptor cross-linking, the purification protocols used may fail to detect weak protein–protein and protein–lipid interactions that dissociate during the long preparative centrifugation. An example of such an interaction is that between the signal-transducing chains Igα/Igβ and the tyrosine kinase Syk (DeFranco, 1997). Although we could detect several proteins getting translocated to the biochemically isolated raft fraction upon BCR cross-linking, we failed to detect Syk in these fractions (our unpublished data). However, by using deconvolution microscopy to visualize Syk in live B cells, we could demonstrate that upon anti-IgM stimulation, Syk is mobilized from the cytosol to the membrane and strongly colocalizes with ag-

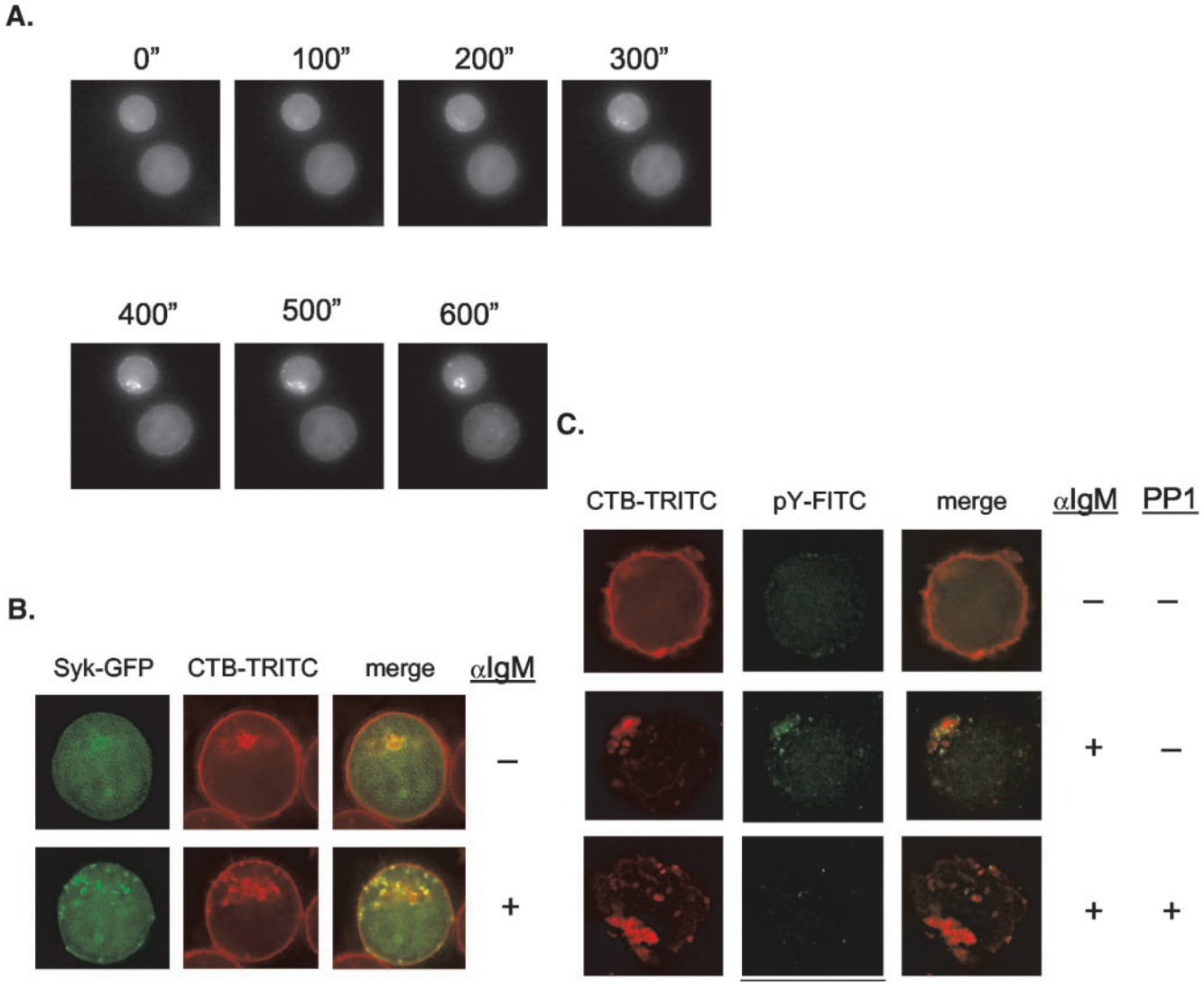

Figure 6. Syk translocates to lipid rafts upon BCR cross-linking with a concomitant association of tyrosine phosphorylated proteins with aggregated lipid rafts. (A) Bal 17 cells stably expressing Syk-GFP were stimulated with 25 μg/ml anti-mouse IgM at room temperature. Next, 12 μl of the cell suspension was placed on a slide and covered with a coverslip. After focusing on cells, image acquisition was started and continued every 10 s for a period of 10 min. All 60 images were saved as a time-lapse movie (supplementary data, Movie 2). Shown are still images at seven time points as indicated. The pattern of patching and capping seen in the upper cell was more commonly observed than the pattern seen for the lower cell, which has patches of Syk concentration at 10 min but has not yet started capping. (B) Bal 17 cells were transfected with 20 μg of Syk-GFP fusion construct. After 24 h, cells were adhered onto a poly-L-lysine–coated glass dish for 1 h and stained with CTB-TRITC. The dish was fixed on a stage adapter, and images were acquired using the fluorescein and rhodamine filters in the unstimulated state (top row). Anti-IgM $F(ab')_2$ (50 μg/ml) was added to the dish and images were acquired again after 10 min of stimulation (bottom row). Surface views of the cells are shown, with Syk-GFP in green, lipid rafts in red, and regions of colocalization in yellow. (C) Bal 17 cells were preincubated (bottom row) or not (top and middle rows) with 10 μM PP1 and either left unstimulated (top row), or stimulated with 25 μg/ml anti-IgM $F(ab')_2$ (middle and bottom rows) for 10 min at 37°C, and the reaction stopped by fixing the cells in 4% PFA. The cells were then stained with CTB-TRITC as described in text, washed twice, permeabilized with 0.5% saponin, stained with anti-phosphotyrosine-FITC, and washed twice. Images were acquired using the fluorescein and rhodamine filters. Phosphotyrosine staining is shown in green, lipid rafts in red, and regions of colocalization in yellow.

gregating lipid rafts (Figure 6, A and B). Moreover, the Syk in lipid rafts seems to be enzymatically active, as judged by the concentration of tyrosine-phosphorylated proteins in the region of aggregrated lipid rafts (Figure 6C, middle).

During Triton X-100 detergent extraction of B-cell membrane, CD45 shows up in the detergent-soluble fraction (Cheng, 1999) (our unpublished data), whereas the B-cell Src family kinases such as Lyn, Fyn, and Blk are largely insol-

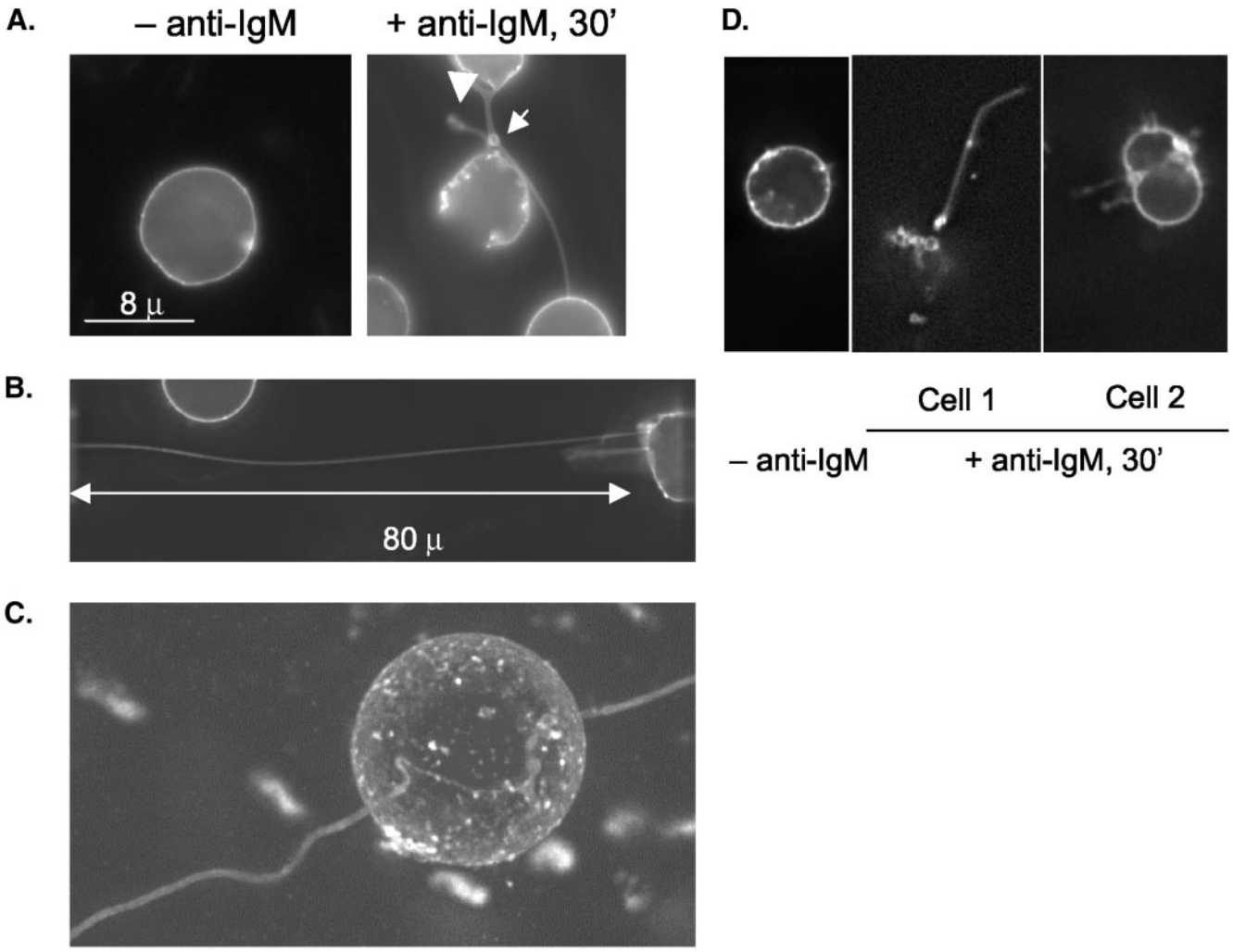

Figure 7. Induction of cytoneme-like extensions of the B-cell membrane upon BCR engagement. (A–C) Bal 17 cells or (D) primary splenic B cells were stained with CTB-TRITC as described in text and images of unstimulated cells (left) were acquired. (A) Cells were stimulated with 25 μg/ml anti-IgM F(ab')$_2$ at room temperature and images were acquired at 30 min. The white arrowhead and arrow indicate the concentrated lipid raft staining at a branch point and the tip of the B-cell cytoneme, respectively. (B) This experiment was similar to A except that the cells were plated sparsely and the image shown is at 40 min after anti-IgM cross-linking. The length of the cytonemes was measured using the Softworx tools and the thickness was measured at the limit of resolution of light microscopy to be approx. 0.2–0.4. (C) In this experiment, images were acquired of a Z-stack of 50 adjacent focal planes of a cell that had been stained with CTB-TRITC and stimulated with anti-IgM for 20 min. (D) Splenic B cells were similarly treated with anti-IgM at room temperature and images acquired at 30 min. The left panel shows a representative unstimulated cell and the middle and right panels show two representative cells with induced cytonemes. In the middle panel, the body of cell 1 is out of focus but the cytoneme stained with CTB-TRITC can be seen clearly. Cell 2 in the right panel shows two protruding cytonemes.

uble and partition into the lipid raft fraction (our unpublished data). This presents a paradox because CD45 is required for the full activation of Src kinases due to its role in the dephosphorylation of the C-terminal negative regulatory tyrosine of these kinases (Yanagi, 1996; Thomas, 1999). To address the possibility that, like Syk, the lack of observed association of CD45 with lipid rafts might be an artifact of the lengthy purification protocol, we visualized lipid rafts and CD45 by deconvolution microscopy in live B cells. Approximately 10% of B cells showed colocalization of CD45 with lipid rafts in the absence of BCR engagement. It is noteworthy, however, that in these cells, CD45 was colocalized with only those lipid raft patches that were $\geq$500 nm in size, possibly making the colocalization easier to visualize. Nonetheless, upon BCR stimulation, there was an increased association of CD45, primarily with aggregating lipid rafts. We speculate that in resting B cells, CD45 associates loosely with lipid rafts and thus has access to the Src family kinases. If this is the case, CD45 could strike a balance with Csk to keep the negative regulatory tyrosine of Src family kinases dephosphorylated and the kinases in a primed state (Thomas, 1999). On antigenic stimulation, as the lipid rafts aggre-

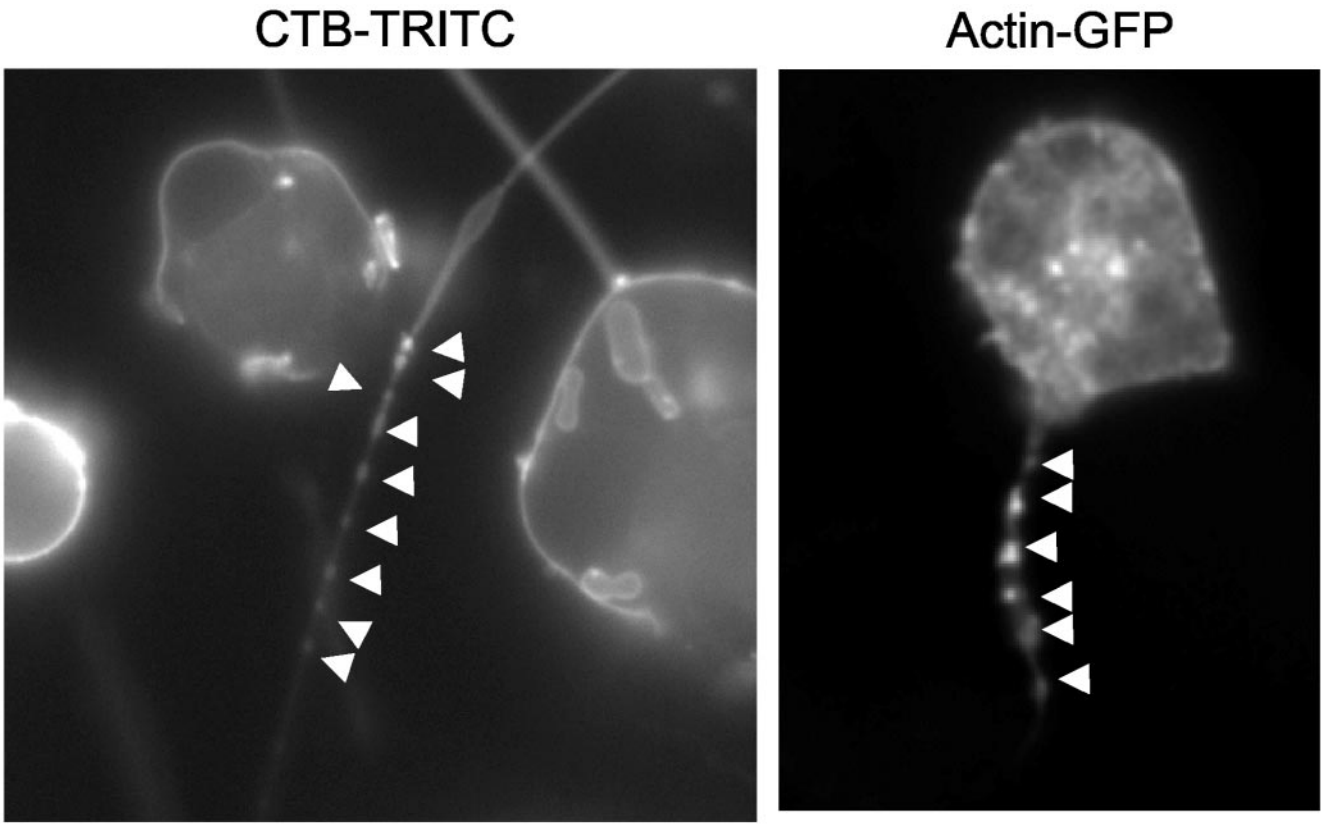

Figure 8. Punctate staining pattern for lipid rafts and actin along the length of B cell-cytonemes. (A) Bal 17 cells were stained with CTB-TRITC and cells were stimulated with 25 μg/ml anti-IgM F(ab′)$_2$ at room temperature and images were acquired at 15 min. (B) Bal 17 cells were transfected with an expression plasmid encoding an actin-GFP fusion protein and after 24 h the transfectants were adhered to glass Delta T dishes. The cells were stimulated with 50 μg/ml anti-IgM F(ab′)$_2$ at room temperature for 15 min and images were acquired using a GFP filter set on the Deltavision microscope. The white arrows indicate the punctate staining pattern for lipid rafts (A) and actin-GFP (B) of the B cell cytonemes of stimulated cells.

gate and the BCR translocates to rafts, the nonphosphorylated Src kinases can transphosphorylate each other on the positive regulatory tyrosine for full activation (Thomas, 1999). However, CD45 would need continuous access to the Src kinases in lipid rafts to keep the negative regulatory tyrosine dephosphorylated. Lipid raft aggregation might serve to trap CD45 in proximity to the Src kinases and yet keep it separated from the receptor signaling complex. Indeed, using confocal microscopy it was demonstrated that CD45 mobilizes to the immunological synapse during TCR activation; however, within the central supramolecular activation cluster it occupies a niche that is distinct from the TCR, suggestive of parallel Src kinase activation and TCR-induced signalosome generation mechanisms operating simultaneously in the central supramolecular activation cluster (Johnson, 2000).

Lipid rafts are thought to vary in size from ~70 nm in diameter to 1 μm, depending on the physiological state of the cell (Brown, 1998). The smallest lipid raft structures are therefore submicroscopic. Indeed, in addition to punctate staining with CTB and the Lyn24-GFP fusion protein, both of these reagents exhibited a weak generalized membrane staining, which we presume indicates the presence of these small lipid rafts. BCR stimulation caused a dramatic polarization of lipid rafts to one or a few regions of the cell. The mechanical forces that bring small rafts together to form larger structures remain to be determined. A recent report suggests that in T cells the protein agrin, which was previously identified as important for organizing synaptic membranes of the neuromuscular junctions in the developing nervous system, is responsible for the aggregation of lipid rafts and clustering of signaling molecules at the T-cell synapse with antigen-presenting cells (Khan, 2001). Alternatively, lipid raft aggregation may be driven by actin filaments and myosin motors (Dustin, 2000; Krummel, 2000).

Irrespective of how lipid rafts aggregate in B cells, the functional significance of lipid raft aggregation might be to enhance BCR signaling. Our findings that BCR stimulation recruited Syk to lipid rafts and induced concentrated protein tyrosine phosphorylation in the proximity of lipid rafts indicate that BCR signaling is occurring primarily in lipid rafts. The presence of BCR-associated early signaling proteins in a concentrated region of the cell might serve to enhance the strength of the signaling reactions. In addition, the concentration of the engaged BCR in patches of lipid rafts might help in the uptake of particulate antigens, such

as viral particles, or interaction with antigen-binding follicular dendritic cells.

Finally, by observing live B cells by using deconvolution microscopy, we found the plasma membrane of activated B cells to be extremely dynamic. In addition to the movement and aggregation of lipid rafts, the contours of the cell changed shape rapidly upon anti-IgM stimulation. Strikingly, BCR engagement led to the induction of long, thread-like projections from the membrane (Figures 7 and 8). These projections are reminiscent of actin-based extensions known as cytonemes that are protruded from wing imaginal disk cells in *Drosophila* during wing development (Ramirez-Weber, 1999). *Drosophila* cytonemes are thought to participate in long-range communication between the cells of the imaginal disk and the signaling center through which morphogens or receptors could be transported. These structures were also detectable in cells from mouse limb buds and chick embryos (Ramirez-Weber, 1999), suggesting that they might be a ubiquitous developmental feature. Although cytonemes are now well established as playing a role in early development, this is the first evidence as far as we know of their existence in lymphocytes. We suspect that cytonemes have been missed in lymphocytes previously because they are rather fragile and are easily destroyed by fixation. Recently, similar structures were also reported to be induced in neutrophils upon adhesion to fibronectin-coated substratum when their spreading was blocked with chemical inhibitors (Galkina, 2001).

B-cell cytonemes showed localized staining with CTB, indicating that they contain lipid raft material. Particularly intense staining was observed at the tips of cytonemes, suggesting that lipid rafts might be concentrated at the ends of cytonemes. The punctate pattern of lipid raft staining along the length of the B-cell cytonemes was similar to the distribution of actin in these extensions (Figure 8). The significance of these projections is not understood, but it is possible that B cells use cytonemes for the transport of signaling molecules and/or receptors to permit rapid long-range communication with other immune cells after BCR-mediated activation. Indeed, the time course of appearance of long, stable cytonemes is consistent with a possible role in presentation of antigen taken up via the BCR to helper T cells (Finkelman, 1992). The processed peptides loaded on class II MHC molecules might be transported along cytonemes, and carried to the tips of the cytonemes where they might be presented at the surface. This may provide an antigen-presenting B cell with a more efficient mechanism to search and find the rare T cells with the right TCR specificity. In this regard, it is interesting to note that actin-containing cytoneme-like filopodia were recently reported to be present on dendritic cells, which are the antigen-presenting cells thought to initiate T-cell activation in a primary immune response (Raghunathan, 2001).

In summary, our microscopic imaging experiments have confirmed previous biochemical reports that engagement of the BCR induces it to localize to the lipid raft subdomains of the plasma membrane. We further observed that early signaling events associated with BCR signaling were primarily localized to lipid rafts. The observations indicate that the lipid raft compartment of the plasma membrane acts as the "business district" of a mature B cell where the BCR assembles upon ligation by an antigen and subsequently recruits other positive effectors in a concentrated space so as to bring about efficient and sustained signal transduction, resulting in B-cell activation.

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