Colocalization of the B Cell Receptor and CD20 Followed by Activation-Dependent Dissociation in Distinct Lipid Rafts

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The B cell Ag receptor (BCR) and CD20, a putative calcium channel, inducibly associate with cholesterol-dependent membrane microdomains known as lipid rafts. A functional association between the BCR and CD20 is suggested by the effects of CD20-specific mAbs, which can modulate cell cycle transitions elicited by BCR signaling. Using immunofluorescence microscopy we show here that the BCR and CD20 colocalize after receptor ligation and then rapidly dissociate at the cell surface before endocytosis of the BCR. After separation, surface BCR and CD20 were detected in distinct lipid rafts isolated as low density, detergent-resistant membrane fragments. Pretreatment with methyl-β-cyclodextrin, which we have previously shown to enhance receptor-mediated calcium mobilization, did not prevent colocalization of the BCR and CD20, but slowed their dissociation. The data demonstrate rapid dynamics of the BCR in relation to CD20 at the cell surface. Activation-dependent dissociation of the BCR from CD20 occurs before receptor endocytosis and appears to require in part the integrity of lipid rafts. The Journal of Immunology, 2002, 169: 2886–2891.

The plasma membrane is a highly organized and dynamic structure. Preferential clustering of sphingolipids and cholesterol forms ordered domains, lipid rafts, that have been implicated in receptor-mediated signal transduction through their ability to concentrate or exclude protein and lipid mediators. Signaling molecules found in lipid rafts include dually acylated Src family tyrosine kinases, heterotrimERIC G protein subunits, adapter proteins, phosphatidylinositol-3,4-bisphosphate, and lipid kinases and phosphatases (1, 2). Rafts are also abundant in proteins associated with the cytoskeleton and are involved in membrane trafficking and receptor internalization (1, 3).

Engagement of the B cell receptor (BCR) by Ag triggers accelerated endocytosis of the receptor along with a cascade of signaling events that can result in diverse cellular responses (4, 5). In immature and tolerized B cells, where BCR signaling may lead to apoptosis and anergy, respectively, the BCR is excluded from lipid rafts (6, 7). In pre-B and mature B cells, where BCR signaling is associated with cell activation, the BCR and many of the molecular components of the BCR signaling cascade associate with lipid rafts following stimulation (8–10). Coligation of the BCR with the CD19/CD21 complex prolongs BCR association with lipid rafts, while CD40 costimulation accelerates movement of the BCR out of rafts (11, 12). Expression of the EBV latent membrane protein LMP2A blocks both entry of the BCR into rafts and BCR signaling (13). The evidence thus favors a role for lipid rafts in BCR signal transduction. However, raft disruption using methyl-β-cyclodextrin (MβCD) does not inhibit BCR-mediated tyrosine kinase activation, ERK activation, or calcium release from intracellular stores (9, 14). Taken together, the data suggest that rafts function to organize and limit selected signaling events, rather than serving as essential conduits for all BCR signals.

CD20 is a B cell integral membrane protein that shares with the BCR the ability to associate with lipid rafts (15). CD20 belongs to a newly discovered, largely uncharacterized, family of proteins expressed in a variety of tissues (16, 17) and is involved in the regulation of cell cycle progression in B lymphocytes as either a component or a regulator of a calcium channel (18–20). In human peripheral B lymphocytes, signals delivered through CD20 can increase levels of c-Myc and synergize with the BCR to drive cellular proliferation (18). Lipid rafts could represent the compartment of the cell membrane where CD20 regulates BCR signaling, possibly through modulating levels of intracellular calcium.

Evidence of a functional association between the BCR and CD20 and their mutual association with lipid rafts prompted us to examine their relative distribution in B cells. In this report we show that the BCR and CD20 colocalize on the surface of stimulated B lymphocytes, then rapidly dissociate before BCR internalization. Dissociation was retarded in cholesterol-depleted cells. Surface-labeled BCR and CD20 were visualized in isolated rafts using a novel strategy involving tracking of fluorochrome-conjugated Abs bound to live cells before lysis. CD20 and the BCR were partially colocalized in isolated rafts under conditions where cell surface colocalization was observed. When the BCR and CD20 were dissociated at the cell surface, they were correspondingly segregated in isolated rafts. The data suggest that the BCR, upon stimulation, redistributes to lipid rafts with CD20 and subsequently traffics away in a distinct population of rafts before undergoing endocytosis.

Materials and Methods

Cells, Abs, and reagents

Ramos B cells were maintained in culture in RPMI 1640 and 7.5% FBS. Goat F(ab')2 anti-human IgM, either unconjugated or conjugated to Cy3 or biotin (Jackson ImmunoResearch Laboratories, West Grove, PA), was used.
to stimulate and label the BCR. Streptavidin-Cy3 (Jackson Immuno-
Research Laboratories) or streptavidin-PE (BD Biosciences, San Jose, CA) was used to detect biotin-labeled BCR. The CD20-specific mAb 2H7 and anti-CD45 mAb 9.4 were provided by Dr. J. Ledbetter (University of Washington, Seattle, WA). 2H7 conjugated to Alexa 488 was generated using the Alexa Fluor 488 protein labeling kit (Molecular Probes, Eugene, OR). Rabbit antiserum to CD20, used for immunoblotting, was generated using a peptide corresponding to the intracellular C-terminal (aa 280–297) of human CD20 conjugated to GST. Cholera toxin B (CTB) and rabbit anti-CTB were purchased from Sigma-Aldrich (St. Louis, MO). Alexa 488-conjugated transferrin (TI) was purchased from Molecular Probes.

Detergent-resistant membrane isolation
Lipid rafts were isolated as low density, detergent-resistant membranes (DRMs) by sucrose density centrifugation after lysis in Triton X-100 essentially as previously described (15). Cells (10⁶) were lysed in ice-cold lysis buffer (25 mM morpholineethane-sulfonic acid, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, 10 mM EDTA, 2 mM Na₂VO₃, and 1% Triton X-100). Samples were immediately placed on ice for 20 min. Lysates were mixed with an equal volume of 80% sucrose in MBS (25 mM MES and 150 mM NaCl), overlaid with 5 ml of 30% sucrose and 5 ml of 5% sucrose in MBS, and centrifuged at 37,000 rpm for 3 h in a Sw41TI swinging bucket rotor in a Beckman XL-70 ultracentrifuge (Fullerton, CA). The layer of opaque material at the 5/30% interface, previously shown to contain lipid rafts (9), was collected in 1 ml, diluted in 11 ml cold 1× MBS, and resuspended at 37,000 rpm for 1 h. The pelletted material was solubilized in 100 μl of 2× SDS sample buffer.

Immunoblots
Samples were heated at 95°C for 5 min. separated on 10% SDS-polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA). The membranes were blocked in 5% BSA and incubated with anti-CD20 antiserum or biotin-conjugated goat F(ab')₂, anti-human IgM. Following washing, protein A-HRP (Bio-Rad, Richmond, CA) or NeutrAvidin-HRP (Southern Biotechnology Associates, Birmingham, AL) was used to detect anti-CD20 or biotin-conjugated goat F(ab')₂, anti-human IgM, respectively. Immunoblots were developed using Super Signal chemiluminescence (Pierce, Rockford, IL). The bands were visualized using the Fluor S-Max (Bio-Rad) imaging system.

Immunofluorescence microscopy and digital deconvolution
CD20 and the BCR were stimulated and labeled at 37°C or on ice (0°C) by addition of 2H7-Alexa 488 and/or F(ab')₂, anti-IgM-Cy3 (1 μg/10⁶ cells). For detection of surface BCR, cells were stimulated with F(ab')₂, anti-IgM-biotin for the specified times and rapidly pelleted at 0°C, and the supernatants were aspirated. Cell pellets were placed at 0°C for 30 s and mixed with cold streptavidin-Cy3 (Jackson Immunoresearch Laboratories) for an additional 30 s. GM1 was labeled at 0°C by sequential 15-min incubations with CTB, rabbit anti-CTB, and goat anti-rabbit IgG-Cy3 (Jackson ImmunoResearch Laboratories). The negative control for GM1 staining replaced anti-CTX with normal rabbit serum (not shown). For CD45 labeling, cells were incubated at 0°C with 9.4 mAb or IgG2b as a control, then with anti-mouse IgG-FITC. Tf receptor was labeled by incubating cells at 37°C with Tf-Alexa 488 for 30 min. Cells were fixed by the addition of 1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 1× PBS. The fixed and stained cells were washed and placed on microscope slides coated with poly-n-lysine (Sigma-Aldrich). For imaging DRMs, cells were stimulated and labeled as described above before lysis and sucrose density gradient centrifugation; 0.5 ml was removed from the 5/30% sucrose interface, and 12 μl was placed on a poly-n-lysine-coated slide. The cells of DRMs were visualized using a Leica DMRXA microscope (Rockleigh, NJ) attached to a 14-bit cooled CCD camera (Princeton Instruments, Monmouth Junction, NJ). Digital deconvolution was performed using a nearest neighbor algorithm, Microtome for Windows (VayTek, Fairfield, IA).

Image quantitation
Deconvolved images were converted to binary images in ImageJ (National Institutes of Health, Bethesda, MD). The mean fluorescence across one entire image was determined; a threshold for positive staining was set and maintained for all images within each experiment. The percent colocalization from each image was derived from the number of positive pixels common to both images (BCR/CD20), divided by the number of positive pixels within the CD20 image. Paired Student’s t test was performed to determine significance.

Electron microscopy
For visualization by transmission electron microscopy, 400 μl DRMs were removed from the sucrose gradient, fixed, and stained in 0.8% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) with rotation at room temperature for 30 min. The DRMs were washed and re-suspended in 400 μl of 1× PBS. Four microfilters were placed on a carbon-coated copper grid, air-dried, and visualized on an H-7000 transmission electron microscope (Hitachi, Schaumburg, IL).

Results
CD20 and the BCR transiently colocalize on intact cells
The membrane distribution of CD20 and the BCR in unstimulated Ramos B cells was assessed by labeling fixed cells with 2H7-Alexa 488 and F(ab')₂, anti-IgM-Cy3 (Fig. 1, top panel). Both were uniformly distributed around the plasma membrane, with areas of coincident brighter staining. Cells were then exposed to the fluorochrome-labeled Abs before fixation at either 37 or 0°C, for times ranging from 1–60 min. At 0°C, CD20 and the BCR were clustered and colocalized at all times; the results at 60 min shown in Fig. 1, second panel from the top, were also typical of data obtained at 1, 5, and 15 min. The amount of colocalization was estimated at 46 ± 2.7% (see Table I). At 37°C, CD20 and the BCR had the highest degree of colocalization at 1 min, with the extent of colocalization decreasing over time (Fig. 1 and Table I). CD20,

FIGURE 1. Colocalization and rapid dissociation of BCR and CD20. For the zero time point Ramos cells were fixed, then labeled with 2H7-A488 and F(ab')₂, anti-IgM-Cy3. For all other samples cells were treated with 2H7-A488 and F(ab')₂, anti-IgM-Cy3 for the indicated times and temperatures, then fixed. The results shown are representative of five independent experiments. The percent colocalization was estimated and analyzed as described in Materials and Methods (see Table I).
BCR/CD20 dissociation requires BCR stimulation

In immunofluorescence experiments represented in all but the top panel of Fig. 1, CD20 was visualized by incubating viable cells with fluorochrome-conjugated Abs before fixation. This was because we found that extracellular CD20 epitopes were sensitive to fixation and Ab binding after fixation was weak. Since we have shown previously that CD20 mAbs induce biochemical alterations in CD20 consistent with its redistribution to lipid rafts, it was important to determine whether Ab binding to CD20 contributed to its dissociation from the BCR. Ramos cells were incubated with anti-CD20 at 37°C for 5 min in the absence of anti-IgM, fixed, and stained for BCR. The results show that the BCR remained colocalized with CD20 (Fig. 2, top panel; percent colocalization estimated at 72.3 ± 2.9). In contrast, when cells were incubated with anti-IgM, fixed, and stained for CD20, the BCR was predominantly segregated from CD20 (Fig. 2, middle panel; percent colocalization, 15.8 ± 2.6), with relative staining patterns similar to those observed when the BCR and CD20 were both engaged before fixation (Fig. 2, bottom panel; percent colocalization, 22.5 ± 1.0). Thus, dissociation of CD20 and the BCR is a consequence of BCR stimulation.

CD20 and the BCR dissociate at the cell surface

Dissociation of the BCR and CD20 appeared to have occurred at the cell surface. However, it was possible that endocytosis was already underway by 5 min. If this were the case, labeled BCR that was just under the surface might not be distinguishable from surface BCR. To determine whether the BCR was still on the surface at times when it was separated from CD20, biotin-conjugated F(ab’)_2 anti-IgM was used to stimulate the BCR for 5, 15, and 60 min. After washing away unbound Ab, the cells were rapidly chilled and briefly incubated at 0°C with streptavidin-Cy3 to label only surface BCR. As expected, the amount of labeled BCR decreased over time (Fig. 3). However, the BCR could still be detected on the cell surface at times when it was no longer colocalized with CD20, indicating that separation occurred after BCR cross-linking, but before internalization.

Surface-labeled BCR and CD20 in distinct DRM

Translocation of the BCR to lipid rafts has previously been shown to occur in cells held at 0°C (7, 8, 22). At 37°C, the association of the BCR with rafts is transient, peaking at 5–15 min and declining thereafter (7–9), whereas CD20 translocation at 37°C does not decline over the course of 1 h (15). The ability of CD20 to translocate at 0°C has not been reported. To compare BCR and CD20 cell surface distribution with their localization to lipid rafts, DRM were isolated from Ramos cells pretreated at either 37 or 0°C with F(ab’)_2 anti-IgM and 2H7 Abs. DRM isolated from Ramos cells have previously been shown to be enriched in Src family kinase Lyn, Grx, actin, and ezrin, but not tubulin or paxillin (9, 14). Redistribution of the BCR and CD20 to DRM at various times after Ab addition was determined by immunoblotting. The results obtained for the BCR at both temperatures and for CD20 at 37°C were consistent with those previously reported. Translocation of CD20 to DRM also occurred at 0°C (Fig. 4A).

We have previously estimated that the amount of CD20 localized to lipid rafts after incubation with the 2H7 mAb is 95% (15). We and others (8, 9) estimated the maximal amount of BCR associated with lipid rafts to be ~20% 5–15 min after stimulation. Within this time frame, it was possible that surface BCR could be in non-raft domains, while raft-associated BCR was internalized.

### Table 1. BCR/CD20 colocalization in the presence or absence of MJCD

| Stimulation Conditions | −MJCD | +MJCD |
|------------------------|--------|-------|
| 0°C                    | 46.4 ± 2.7 | 46.0 ± 6.1 |
| 37°C, 1 min            | 47.0 ± 5.2 | 55.8 ± 4.9 |
| 37°C, 5 min            | 21.4 ± 3.6 | 56.3 ± 5.4 |
| 37°C, 15 min           | 15.2 ± 3.4 | 49.3 ± 3.8 |
| 37°C, 60 min           | 4.2 ± 1.9  | 35.7 ± 6.4 |

*Values are the percent colocalization ± SE measured as described in Materials and Methods.

*Conditions significantly different from 0°C (p < 0.05).
FIGURE 4. BCR/CD20 dissociation in distinct lipid rafts at the cell surface. A, Ramos cells were treated for the indicated times at either 37 or 0°C with F(ab')2 anti-IgM and 2H7 and lysed in Triton X-100. DRMs were collected from sucrose gradients and probed by immunoblotting for the presence of CD20 or BCR as indicated. The intensity of the bands was analyzed by densitometry and expressed as a ratio of the intensity of the darkest band on each membrane. The results shown are representative of two independent experiments. B, GM1, CD45, Tf receptor, and CD20 were labeled as described in Materials and Methods. An aliquot of cells was removed for imaging (upper panel), and the remaining cells were lysed in 1% Triton X-100. DRMs were isolated by sucrose gradient centrifugation and visualized by immunofluorescence microscopy. Staining was observed as isolated particles of fluorescence. Fluorescent particles were counted in multiple fields for each sample using Image J (National Institutes of Health) analysis software, and the number of green particles (i.e., IgG2b, CD45, TIR, or CD20) was expressed as a percentage of the number of red (GM1) particles (0.04, 0.03, 0.08, and 164%, respectively). C, Ramos cells were stimulated and surface-labeled for BCR and CD20, as described in Fig. 3, for the indicated times and temperatures before lysis and isolation of DRMs. In the top panel there are four red and three green distinct isolated particles of fluorescence; in the bottom panel there are seven red and eight green, of which six are colocalized. Sixteen images from two independent experiments were analyzed, and the percent colocalization was estimated to be 12.6 ± 4.2 and 42.0 ± 4.3 at 37 and 0°C, respectively (p < 0.0003; bar = 1 μm). D, DRMs from unlabeled Ramos cells were processed for transmission electron microscopy (TEM; bar = 260 nm).

Alternatively, a proportion of surface BCR may be associated with lipid rafts, either colocalized with CD20 or in distinct rafts. To test this, DRMs were isolated from Ramos cells that, before lysis, had been stimulated and surface-labeled with fluorochrome-conjugated Abs as described for Fig. 3, and then observed and analyzed by immunofluorescence microscopy. To confirm that this method would detect known DRM-associated molecules, but not membrane proteins thought to be excluded from rafts, cells were pre-labeled at 0°C for sphingolipid GM1 and for CD45. As an additional negative control, the Tf receptor was labeled with Tf-Alexa 488 at 37°C. Tf and its receptor associate with early recycling endosomes, through which the internalized BCR trafficks on its way to the class II MHC loading compartment (23). An aliquot of cells was removed from each sample to confirm appropriate labeling (Fig. 4B, upper panel). GM1 and CD45 were brightly stained at the cell surface, and Tf was internalized as expected. The remaining cells were lysed and processed for DRM isolation. Isolated DRMs were found to be brightly stained for GM1, but not for CD45 or Tf receptor (Fig. 4B, lower panel). CD20 mAb 2H7 (IgG2b, as is CD45 mAb 9.4) was used as a positive control for dual staining with GM1. Interestingly, GM1 and CD20 were not colocalized either at the cell surface or in isolated DRMs, indicating their localization to distinct lipid rafts.

Using this technique, surface-labeled BCR was indeed detected in DRMs (Fig. 4C). When isolated from cells that were incubated and labeled at 0°C, there was an incidence of BCR/CD20 colocalization in DRMs similar to that observed at the surface of intact cells under these conditions. In contrast, when cells were stimulated for 5 min at 37°C, surface BCR was largely segregated from CD20 in DRMs, as it is at the cell surface. Transmission electron microscopy performed on DRMs that were similarly isolated demonstrated that they consisted of a heterogeneous mixture of large and small particles ranging from 50 to 250 nm in size (Fig. 4D). This is consistent with several reports of lipid raft size measured in vivo (24–26). The size of the particles of fluorescence in Fig. 4, B and C, was estimated and corresponded to the largest particles observed by electron microscopy.

MβCD slows the kinetics of BCR/CD20 dissociation

The effect of lipid raft disruption on the dynamics of BCR/CD20 association was examined using MβCD under conditions that we have previously demonstrated to efficiently prevent the association of both BCR and CD20 with DRMs in Ramos cells (9, 14). Interestingly, MβCD did not prevent the colocalization of BCR and CD20 observed at early time points of stimulation, but delayed their dissociation (Fig. 5 and Table I). At 5 and 15 min of BCR stimulation there was still coincident staining with CD20 (compare with Fig. 1; see Table I). Internalization of the BCR was also reduced, but not completely prevented, as confirmed using flow cytometry (data not shown).

Discussion

The novel findings presented in this paper include 1) the transient colocalization and rapid dissociation of the BCR and CD20 after receptor stimulation, and 2) the presence of the BCR and CD20 in distinct plasma membrane DRMs, representing the first report of heterogeneity among lipid rafts at the cell surface in B lymphocytes.

In Ramos B cells that were fixed before staining, BCR and CD20 were uniformly distributed around the plasma membrane, with areas of coincident brighter staining that hinted at colocalization in resting cells. The distribution of CD20 in fixed cells was difficult to determine with confidence because extracellular CD20 epitopes are sensitive to fixation, and staining was weak. In unfixed cells CD20 cross-linking coclustered the BCR in the absence
FIGURE 5. Effect of MβCD on BCR/CD20 distribution on the cell surface. Ramos cells were treated with 10 mM MβCD for 10 min at 37°C, washed, then treated for the indicated times with 2H7-A488 and F(ab’)$_2$ anti-IgM-Cy3 and fixed. The results are representative of two independent experiments. The percent colocalization was estimated and analyzed (see Table I).

of BCR stimulation (Fig. 2), strongly suggesting that the BCR and CD20 are indeed colocalized before receptor stimulation.

Coclustering of BCR and CD20 was also observed when both were Ab-ligated at 0°C. It is known that stimulation at low temperatures prevents capping and internalization, but not clustering, of the BCR (27, 28). Consistent with this, colocalization of BCR and CD20 was stable at 0°C. Following stimulation at 37°C, however, colocalization was transient, and the BCR and CD20 dissociated while both were still on the cell surface. Separation occurred only after BCR stimulation; CD20 ligation was neither sufficient nor required. These receptor dynamics were also observed in another human B cell line, BJAB, and in tonsil B cells, although the kinetics were variable (data not shown). In tonsil cells BCR and CD20 were already dissociated at 1 min after stimulation at 37°C, and the BCR was completely internalized by 5 min. In BJAB cells colocalization and dissociation were observed at 1 and 5 min, respectively, but BCR internalization was faster than in Ramos, essentially complete by 15 min.

In parallel with BCR/CD20 colocalization at the cell surface was the finding of surface BCR and CD20 in the same or closely apposed DRMs. When DRMs were isolated from cells preincubated with fluorochrome-conjugated Abs at 0°C, the estimated colocalization was similar to that found in intact cells (42.0 ± 4.3 and 46.4 ± 2.7%, respectively). Similarly, dissociation of BCR and CD20 at the cell surface corresponded to the finding of surface BCR and CD20 in predominantly distinct DRMs (down from 42 ± 4.3 to 12.6 ± 4.2% colocalization). The most straightforward interpretation of these data is that the BCR and CD20 transiently colocalize in the same lipid rafts and then dissociate into distinct lipid rafts before BCR internalization. Unclustering of proteins within lipid rafts has, to our knowledge, not previously been described. Alternatively, it is possible that the BCR and CD20 are never colocalized in the same lipid rafts, but are present in closely apposed rafts that separate during BCR stimulation. New strategies for detection and/or isolation of rafts will be required to distinguish these possibilities.

The inhibitory effects of MβCD on BCR/CD20 dissociation indicate a requirement for the integrity of lipid rafts in this process. Lipid rafts are rich in actin and actin-associated proteins, and it is well known that engaged BCRs become attached to the cytoskeleton (29). It would not be surprising to find that BCR dissociation from CD20 is mediated via the actin cytoskeleton, but this remains to be tested. As well, the consequences of BCR/CD20 colocalization and their rapid dissociation are not yet known. CD20 is a putative calcium channel, and lipid rafts are likely sites of calcium regulation (30). It is possible that calcium-dependent signaling events are initiated within BCR/CD20-positive lipid rafts immediately after stimulation. These signaling events could be limited in part by dissociation of the BCR from these sites of calcium entry. This would be consistent with the increased BCR-mediated intracellular calcium flux and the slower rate of BCR/CD20 dissociation observed in MβCD-treated B cells (9, 14).

Evidence for heterogeneity among plasma membrane microdomains is accumulating rapidly. Specialized non-raft microdomains in Ag-presenting B cells contain a subset of MHC-peptide complexes in association with tetraspanins (31). Glycosphingolipids GM1 and GD3 segregate to distinct lipid rafts in neuronal cells (32), and GM3 and GM1 differentially localize to the leading and trailing edges, respectively, of migrating T cells (33). CD20 was shown here to localize to rafts distinct from those containing GM1 (Fig. 4B). Since this experiment was performed under conditions where CD20 and the BCR colocalize, i.e., at 0°C, it is likely that BCR and GM1 are also in distinct rafts, at least during the earliest stage of BCR stimulation. Our preliminary data indicate that this is indeed the case, and it will be interesting to determine whether there is differential segregation of GM1 and BCR/CD20 during B cell migration. Using immunogold electron microscopy of plasma membrane sheets from resting peripheral blood T cells, Schade and Levine (34) observed Lck and linker for activation of T cells (LAT) in separate raft microdomains that apparently converge after TCR activation. Interestingly, raft association of LAT was found by these investigators to be resistant to cholesterol depletion. Although a contradictory result for LAT was previously reported by another group (35), this finding is similar to our recent report (14) that the related adaptor protein Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains (Cbp/PAG) is resistant to cholesterol depletion. Unlike Cbp/PAG, BCR and CD20 raft associations are both sensitive to the effects of MβCD, suggesting that Cbp/PAG could be present in a third population of lipid raft microdomains in B cells.

Lipid rafts may have multiple roles in coordinating the organization of membrane molecules in response to the extracellular environment. In addition to the potential role in signaling regulation mentioned above, it is possible that distinct lipid rafts are involved in organizing molecular events leading to B cell migration. Additionally, a recent report described the formation of a synapse between B-T cell conjugates involving the redistribution of BCRs to the contact site along with actin and signaling effectors (36). Localization of functionally related molecules in separate lipid rafts may be involved in organization of the B cell synapse. Finally, unclustering of proteins within rafts may be a sorting process that promotes selective internalization of the Ag receptor while leaving other raft-associated proteins, such as CD20, on the cell surface. The rapid activation-dependent membrane trafficking events described here may reflect a mechanism integrating the early phases
of more than one of these components of B cell activation. Elucidation of the composition and function of the distinct rafts in which BCR and CD20 reside after activation should help to address these important questions.

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References

1. Brown, D. A., and E. London. 2000. Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J. Biol. Chem. 275:17221.
2. Langlet, C., A. M. Bernard, P. Drevot, and H. T. He. 2000. Membrane rafts and signaling by the multichain immune recognition receptors. Curr. Opin. Immunol. 12:250.
3. von Haller, P. D., S. Donohoe, D. R. Goodlett, R. Aebersold, and J. D. Watts. 2000. Cutting edge: B cell antigen presentation to class II-restricted T lymphocytes. J. Immunol. 31:3789.
4. Cherukuri, A., P. C. Cheng, H. W. Sohn, and S. K. Pierce. 2001. The CD19/CD21 signaling by the multichain immune recognition receptors. EMBO J. 19:1092.
5. Yanke, T. M., and E. A. Clark. 2000. Signaling through the B cell antigen receptor with lipid rafts in mature and immature B cells. J. Immunol. 165:1220.
6. Weintraub, B. C., J. E. Jun, A. C. Bishop, K. M. Shokat, M. L. Thomas, and C. C. Goodnow. 2000. Entry of B cell receptor into signaling domains is inhibited in tolerant B cells. J. Exp. Med. 191:1443.
7. Sproul, T. W., S. Malapati, J. Kim, and S. K. Pierce. 2000. Cutting edge: B cell antigen receptor signaling occurs outside lipid rafts in immature B cells. J. Immunol. 165:6020.
8. Cheng, P. C., M. L. Dykstra, R. N. Mitchell, and S. K. Pierce. 1999. A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. J. Exp. Med. 190:1549.
9. Petrie, R. J., P. P. Schnetkamp, K. D. Patel, M. Awasthi-Kalia, and J. P. Deans. 2000. Transient translocation of the B cell receptor and Src homology domain-containing inositol phosphatase to lipid rafts: evidence toward a role in calcium regulation. J. Immunol. 165:1220.
10. Guo, B., R. M. Kato, M. Garcia-Lloret, M. I. Wahl, and D. J. Rawlings. 2000. Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex. Immunity 13:243.
11. Cherukuri, A., P. C. Cheng, H. W. Sohn, and S. K. Pierce. 2001. The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. Immunity 14:169.
12. Malapati, S., and S. K. Pierce. 2001. The influence of CD40 on the association of the B cell antigen receptor with lipid rafts in mature and immature cells. Eur. J. Immunol. 31:3789.
13. Dykstra, M. L., R. Longnecker, and S. K. Pierce. 2001. Epstein-Barr virus coopts lipid rafts to block the signaling and antigen transport functions of the BCR. Immunity 14:57.
14. Awasthi-Kalia, M., P. P. Schnetkamp, and J. P. Deans. 2001. Differential effects of filipin and methyl-β-cyclodextrin on B cell receptor signaling. Biochim. Biophys. Acta 287:77.
15. Deans, J. F., S. M. Robbins, M. J. Polylak, and J. A. Savage. 1998. Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. J. Biol. Chem. 273:344.
16. Ishibashi, K., M. Suzuki, S. Sasaki, and M. Imai. 2001. Identification of a new multigene four-transmembrane family (MS4A) related to CD20, HTM4 and β subunit of the high-affinity IgE receptor. Gene 264:87.
17. Liang, Y., and T. F. Tedder. 2001. Identification of a CD20-, FcerIβ-, and HTM4-related gene family: sixteen new MS4A family members expressed in human and mouse. Genomics 72:119.
18. Tedder, T. F., and P. Engel. 1994. CD20: a regulator of cell-cycle progression of B lymphocytes. Immunol. Today 15:450.
19. Bubien, J. K., L. J. Zhou, P. D. Bell, R. A. Frizzell, and T. F. Tedder. 1993. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca++ conductance found constitutively in B lymphocytes. J. Cell Biol. 121:1121.
20. Kanzaki, M., H. Shibata, H. Mogami, and I. Kojima. 1995. Expression of calcium-permeable cation channel CD20 accelerates progression through the G phase in BALB/c 3T3 cells. J. Biol. Chem. 270:13099.
21. Press, O. W., J. Howell-Clark, S. Anderson, and I. Bernstein. 1994. Retention of B-cell-specific monoclonal antibodies by human lymphoma cells. Blood 83:1359.
22. Cheng, P. C., B. K. Brown, W. Song, and S. K. Pierce. 2001. Translocation of the B cell antigen receptor into lipid rafts reveals a novel step in signaling. J. Immunol. 166:3693.
23. Brown, B. K., and W. Song. 2001. The actin cytoskeleton is required for the trafficking of the B cell antigen receptor to the late endosomes. Traffic 2:414.
24. Varma, R., and S. Mayor. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. Nature 394:798.
25. Schuetz, G. J., G. Kada, V. P. Pastushenko, and H. Schindler. 2000. Properties of lipid microdomains in a muscle cell membrane visualized by single molecule microscopy. Annu. Rev. Phys. Chem. 51:73.
26. Pralle, A., P. Keller, E. L. Florin, K. Simons, and J. K. Horber. 2000. Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of malignant cells. J. Cell Biol. 148:997.
27. de Petris, S., and M. C. Raff. 1973. Normal distribution, patching and capping of the B cell antigen receptor with lipid rafts in mature and immature B cells. J. Cell Biol. 59:132.
28. Schreiner, G. F., and E. R. Unanue. 1976. Membrane and cytoplasmic changes in B lymphocytes induced by ligand-surface immunoglobulin interaction. Adv. Immunol. 24:37.
29. Caplan, S., and M. Banyash. 1995. Multisubunit receptors in the immune system and their association with the cytoketin: in search of functional significance. Adv. Immunol. 59:29.
30. Ishikii, M., and R. G. Anderson. 1999. Calcium signal transduction from caveolae. Cell Calcium 26:201.
31. Kropshofer, H., S. Spinadelrehreger, T. A. Rohn, N. Platania, C. Grygar, N. Daniel, A. Wolfg, H. Langen, V. Horejsi, and A. B. Vogl. 2002. Tetraspan microdomains distinct from lipid rafts enrich select peptide-MHC class II complexes. Nat. Immunol. 3:61.
32. Vyse, K. A., H. V. Patel, A. A. Vyus, and R. L. Schmaas. 2001. Segregation of gangliosides GM1 and GD3 on cell membranes, isolated membrane rafts, and defined supported lipid monolayers. Biol. Chem. 382:241.
33. Gomez-Mouton, C., J. L. Abad, E. Mira, R. A. Lacalle, E. Gallardo, S. Jimenez-Baranda, I. Illa, A. Bernad, S. Manes, and A. C. Martinez. 2001. Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. Proc. Natl. Acad. Sci. USA 98:9642.
34. Schade, A. E., and A. D. Levine. 2002. Lipid raft heterogeneity in human peripheral blood T lymphoblasts: a mechanism for regulating the initiation of TCR signal transduction. J. Immunol. 168:2231.
35. Kabouridis, P. S., J. Janzen, A. L. Magee, and S. C. Ley. 2000. Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes. Eur. J. Immunol. 30:954.
36. Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. Nature 411:489.