Transient Translocation of the B Cell Receptor and Src Homology 2 Domain-Containing Inositol Phosphatase to Lipid Rafts: Evidence Toward a Role in Calcium Regulation

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Membrane microdomains (lipid rafts) are enriched in selected signaling molecules and may compartmentalize receptor-mediated signals. Here, we report that in primary human B lymphocytes and in Ramos B cells B cell receptor (BCR) stimulation induces rapid and transient redistribution of a subset of engaged BCRs to lipid rafts and phosphorylation of raft-associated tyrosine kinase substrates. Cholesterol sequestration disrupted the lipid rafts, preventing BCR redistribution, but did not inhibit tyrosine kinase activation or phosphorylation of mitogen-activated protein kinase/extracellular regulated kinase. However, raft disruption enhanced the release of calcium from intracellular stores, suggesting that rafts may sequester early signaling events that down-regulate calcium flux. Consistent with this, BCR stimulation induced rapid and transient translocation of the Src homology 2 domain-containing inositol phosphatase, SHIP, into lipid rafts.

The densely packed, liquid-ordered environment of rafts excludes most integral membrane proteins; however, a few transmembrane proteins translocate into lipid rafts with stimulation. These include the high-affinity IgE receptor in mast cells (18, 19), components of the TCR (14, 20), and CD20, a regulator of cell death. Unexpectedly, disruption of rafts by cholesterol sequestration enhanced BCR-mediated calcium mobilization, suggesting that these domains may play a role in down-regulating intracellular calcium release. Consistent with this, the Src homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP) transiently translocated to lipid rafts within the first minute of BCR stimulation.

Materials and Methods

Ramos, Raji, and Cess B cells were maintained by culture in RPMI 1640, 10% FBS. Primary B cells were isolated from tonsils obtained from the Alberta Children’s Hospital, essentially as described (23). Briefly, cells released from the tonsil tissue were depleted of T lymphocytes using SRBC pretreated with 2-aminoethylisothiouronium bromide (Sigma, St. Louis, MO), leaving >95% B lymphocytes. Dense (resting) B cells were then separated on Percoll density gradients.

Goat anti-human IgM F(ab')2 (Jackson ImmunoResearch, West Grove, PA) was used to stimulate the BCR. Monoclonal Abs 4G7 (CD19), 1F5 (CD20), G29.5 (CD21), HD39 (CD22), G28.1 (CD37), G28.5 (CD40), 9.4 (CD45), HIDE (MHC class I), and HB10a (MHC class II) were provided by Drs. J. Ledbetter and E. Clark (University of Washington, Seattle, WA). Anti-CD23 was purchased from Immunotech (Westbrook, ME), and anti-CD32 was obtained from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine mAb 4G10 and anti-mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) were obtained from Upstate Biotechnology (Lake Placid, NY), anti-phosphoMAPK was obtained from New England Biolabs (Beverly, MA), anti-PKCδ and anti-paxillin were obtained from Transduction Laboratories (Lexington, KY), anti-actin was obtained from Sigma, and anti-IgM was provided by Dr. Linda Matsuuchi, and SHIP Abs were provided by Drs. L. Rohrscheider (Seattle, WA) and A. Veillette.
FIGURE 1. Fractionation of lipid rafts. Ramos cells were lysed and fractionated on sucrose density gradients as described in Materials and Methods. Twelve 1-ml fractions were collected from the top to the bottom of the gradient. Fraction 6, which included the visible material at the 5%/30% interface, was recentrifuged as described in Materials and Methods, to obtain the insoluble low-density pellet (lane 6*). The low (lane 6*)- and the high (lane P)-density insoluble pellets were solubilized in SDS sample buffer. All lanes contain equivalent cell amounts except lane 6*, which contains 10-fold more cell equivalents. Samples were separated by SDS-PAGE and immunoblotted with Abs directed against the indicated proteins.

Sample preparation

F(ab') 2 anti-IgM (1 μg/10 6 cells) was added to cells at 37°C in an equal volume of warm RPMI 1640 to ensure efficient sample mixing. Cell stimulation was stopped by the addition of an equal volume of ice-cold 2× lysis buffer (2% Triton X-100 (Pierce) in 20 mM Tris, pH 7.5, 10 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, 10 mM EDTA, 2 mM Na3VO4), and samples were immediately placed on ice for 15 min. Sucrose density gradient centrifugation was performed essentially as described (21). Lysates from 10 7 cells were mixed with an equal volume of 80% sucrose in lysis buffer, overlayered with a discontinuous sucrose density gradient (4 ml 30% sucrose, then 6 ml 5% sucrose), and centrifuged at 37,000 rpm for 3 h. To confirm the location of lipid rafts on the gradients, 1-ml fractions were collected from the top to the bottom of the gradients, and the insoluble high-density pellets were washed four times and dissolved in 1 ml SDS sample buffer. Then, 10 μl of each fraction was mixed with an equal volume of SDS sample buffer and probed by immunoblot for the presence of Gαi, a dually acylated heterotrimeric G protein α subunit known to reside in rafts (13, 24). Gαi was exclusively detected in fraction 6, which included the 5%/30% interface where flocculent material was visible (Fig. 1). The majority of Gαi was pelleted as shown by dilution of fraction 6, recentrifugation, and dissolving the pellet material in 100 μl SDS sample buffer (Fig. 1, lane 6*). Tubulin and paxillin, predominantly cytosolic proteins, were detected primarily in fractions 11 and 12 (Fig. 1). Actin and ezrin, proteins associated with the cortical cytoskeleton, were detected in the high-density insoluble pellet, as well as in fractions 6, 11, and 12.

FIGURE 2. Translocation of the BCR to lipid rafts. A, Ramos cells were surface labeled with biotin, stimulated with F(ab') 2 anti-IgM for 15 min, lysed, and fractionated by sucrose density gradient centrifugation. Proteins in the fractions indicated were separated by SDS-PAGE and cell-surface proteins were detected by blotting with avidin-HRP. B, Dense tonsil cells were treated as in A. Biotin-labeled cell-surface proteins in the low-density insoluble fraction were detected by blotting with avidin-HRP. C, Ramos cells were stimulated with biotin-conjugated F(ab') 2 anti-IgM for 15 min, washed, lysed, and fractionated by sucrose density gradient centrifugation. The fractions indicated were separated by SDS-PAGE and probed by blotting with avidin-HRP. The bands detected are those of the receptor-bound stimulating Ab migrating at ~25 kDa.

Based on these data, for the remaining experiments, three fractions were collected. First, the visible, flocculent material at the 5%/30% interface, along with 1 ml of the gradient on either side of the interface, corresponded to fractions 5–7 and are hereafter termed low-density insoluble fraction or lipid rafts. These fractions were pooled, diluted with ~10 ml morpholinoethanesulfonic acid-buffered saline, and centrifuged at 37,000 rpm for 1 h to pellet and concentrate the raft-associated proteins. The pellets were solubilized in 100 μl SDS sample buffer. Second, a 1-ml sample of the soluble lysate was collected from the bottom of the tube (corresponding to fraction 12 in Fig. 1) and mixed with 0.33 ml of 4× SDS sample buffer. Third, the insoluble high-density pellet was washed four times and dissolved in 0.5 ml SDS sample buffer. Except where otherwise noted, 10 μl of each sample was loaded onto gels, thus representing 10 7, 7 x 10 7, and 2 x 10 7 cell equivalents from the low-density insoluble fraction, the soluble fraction, and the high-density insoluble pellet, respectively.

For experiments reported in Table I and Fig. 6, whole-cell lysates were cleared of insoluble material by centrifugation at 14,000 × g for 15 min at 4°C. For experiments in Table I, the insoluble pellet was washed and solubilized in SDS sample buffer as described (21) and contains both the high- and low-density insoluble fractions. Proteins were separated on 10% polyacrylamide gels and transferred to Immobilon P (Millipore, Bedford, MA) for immunoblotting.

Calcium measurements

Cells were incubated for 20 min with 20 μM fluo-3AM (Molecular Probes, Eugene, OR), washed, resuspended in 20 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, 1.5 mM CaCl2, 3 mM KCl, 10 mM glucose, and 250 μM sulfynilpyrazone (Sigma), and placed in a cuvette with magnetic stirrer in a SLM-Aminco series 2 luminescence spectrometer (SLM Instruments, Urbana, IL). Cells were excited at 480 nm (8 nm bandwidth), and emission was measured at 530 nm (8 nm bandwidth). A 495-nm long-pass filter was placed in front of the emission monochromator. Minimum and maximum fluorescence values for each experiment were obtained using fluo-3 (5 mM) and either A23187 (10 μM) or saponin (0.02%), respectively.

Results and Discussion

Partitioning of the activated BCR to lipid rafts

We initially sought to determine whether engagement of the BCR induces the redistribution of integral membrane surface proteins to lipid rafts. To that end, rafts were isolated by sucrose density gradient fractionation from Ramos B cells that had been surface labeled with biotin and stimulated with F(ab') 2 anti-IgM for 15 min before lysis (Fig. 2A). Detection of labeled proteins by avidin-HRP blotting revealed very few surface-labeled proteins in low-density fractions from unstimulated cells, even on overexposed films (Fig. 2A, lane 1). However, BCR stimulation induced the appearance in the low-density fraction of two prominent bands migrating at ~80 and ~25 kDa (Fig. 2A, lane 2) that were simultaneously lost from the detergent-soluble fraction (Fig. 2A, lanes 3 and 4). Similar results were obtained using dense tonsil B cells (Fig. 2B, and data not shown). Because the sizes of these proteins correspond to those

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of the heavy and light chains of the IgM BCR, biotin-labeled F(ab’)2 anti-IgM was used to stimulate Ramos B cells to determine whether receptor-bound Ab could be detected in the low-density insoluble fraction. The cells were stimulated with biotin-labeled F(ab’)2 anti-IgM for 15 min, washed, lysed, and fractionated on sucrose density gradients. A total of 105 cell equivalents from each fraction was analyzed by Western blotting with avidin-HRP (Fig. 2C). The intensity of the bands in each lane was measured by densitometry using a Fluor-S Max Multil Imaging system (Bio-Rad, Richmond, CA). The data indicated that only 16% of engaged receptors remained in the soluble fraction after 15 min stimulation, while 13% partitioned into the low-density insoluble fraction. The majority of receptors were found in the high-density insoluble pellet, consistent with previous reports of BCR translocation to the cytoskeleton (25–27).

The kinetics of BCR translocation to rafts was assessed in Ramos B cells by direct immunoblotting with anti-IgM (Fig. 3A). The mature IgM heavy chain (Fig. 3A, upper bands in all panels) was selectively and rapidly mobilized to lipid rafts (upper panels) and simultaneously lost from the detergent soluble fraction (middle panels). Receptor translocation to rafts was detected at the earliest time point tested (7 s) and peaked in the first 5 min of stimulation. Densitometry analysis of the upper bands in the top panels indicated that at 15 min there was 1.6-fold less BCR than at 5 min. Because we estimated 13% of engaged receptors in rafts at 15 min, this indicates that ~20% of BCRs are present in rafts 5 min after stimulation. Interestingly, translocation to the high-density insoluble pellet (Fig. 3A, bottom panels) involved both mature and immature (lower bands in all panels) forms of the receptor and, in contrast to raft-associated receptors, continued to increase over the 15-min period, accounting for the steady loss of receptors from the soluble fraction. Some irregularity in the protein bands in the pellets reflects the difficulty in solubilizing them for analysis.

The BCR is associated with Igα/Igβ components that facilitate signal transmission. Because it was recently reported that a proportion of BCRs dissociate from Igα/Igβ after stimulation (28), we sought to determine whether Igα/Igβ translocated to rafts with the BCR. Surface-labeled proteins corresponding to Igα and Igβ were not detected in lipid rafts after receptor stimulation (Fig. 2); however, we found that Igα and Igβ were only weakly labeled with biotin in comparison with the heavy and light chains of the BCR and may not have been detectable in lipid rafts by avidin blotting (data not shown). Immunoblotting with anti-Igα demonstrated that Igα was transiently recruited to lipid rafts with similar kinetics as the IgM component of the BCR (Fig. 3B). Similar data were obtained for Igβ (data not shown). Therefore, in agreement with the data of Cheng et al. (22), it is likely that the intact BCR/Igα/Igβ complex translocates to lipid rafts.

We have previously reported that Ab-mediated ligation of CD20, an integral membrane protein expressed on B cells, induces its redistribution to lipid rafts (21). To determine whether raft localization of surface proteins was a common consequence of Ab cross-linking, the detergent solubility of several other integral membrane proteins expressed in B cells, namely CD19, CD21, CD22, CD23, CD32, CD37, CD40, CD45, and MHC class I and II, was examined after Ab binding (Table I). Cells were exposed to specific Ab for 15 min at 37°C, unbound Ab was washed away, and receptor-bound Ab was tracked by immunoblotting with anti-mouse IgG-HRP. In addition to CD20 and the BCR, CD32 and CD45 were partly insoluble in Triton X-100, but all other proteins were completely soluble (Table I). To assess the subcellular localization of insoluble CD32 and CD45, low- and high-density detergent-insoluble proteins were isolated by sucrose density gradient centrifugation from cells pretreated with receptor-specific Abs. The insoluble components of both CD32 and CD45 were found exclusively in the high-density insoluble pellet, in contrast to CD20 and the BCR. This indicates that translocation to lipid rafts is not an inevitable outcome of Ab-mediated cross-linking of cell-surface proteins. Indeed, CD20 translocation to lipid rafts can be induced with highly purified Fab, indicating that cross-linking is not required (M. J. Polyak, H. Li, R. J. Petrie, and J. Deans, manuscript in preparation). It remains to be determined whether Fab of anti-IgM or monovalent Ag can induce BCR redistribution to lipid rafts.

Phosphorylation of tyrosine kinase substrates in lipid rafts

The earliest BCR-mediated signaling event known to occur is the activation of src family tyrosine kinases (1–3). Evidence of tyrosine kinase activation in lipid rafts was sought by phosphotyrosine immunoblotting of raft proteins after 15-min BCR stimulation of Ramos B cells (Fig. 4A). Substrates in the soluble fraction and in the high-density insoluble pellets were simultaneously assessed for comparison with the low-density fraction. Heavily phosphorylated proteins at ~55–60 kDa and 75–80 kDa were observed in lipid rafts from unstimulated samples and obscured any BCR-mediated signals that might otherwise be evident in that region of lipid rafts. 

FIGURE 3. Kinetics of BCR translocation to the detergent-insoluble compartments. A, Ramos cells were stimulated with F(ab’)2 anti-IgM for the times indicated, lysed, and fractionated. Two experiments are shown in which the stimulation times ranged from 7 to 120 s (Expt. 1) and 0.5 to 15 min (Expt. 2). Proteins in the low-density insoluble fraction (lipid rafts, upper panels), soluble fraction (middle panels), and the high-density insoluble pellet (bottom panels), were separated by SDS-PAGE and probed by anti-IgM immunoblotting. The upper and lower protein bands are the mature and immature forms, respectively, of the IgM heavy chain, migrating at ~80 kDa. B, Proteins in the raft fractions from Expt. 2 were immunoblotted with either anti-Igα or anti-Gαi.
the membrane. The 55- to 60-kDa proteins are likely to include src family kinases, but the identities of the 75- to 80-kDa proteins are not known. Increased tyrosine phosphorylation was observed on several substrates (arrows in Fig. 4A), indicating either the movement into rafts of phosphorylated proteins or the phosphorylation of resident proteins within rafts by activated kinases. A similar pattern of tyrosine-phosphorylated substrates was observed in lipid rafts isolated from dense tonsil B cells stimulated for 2 min with F(ab′)_2 anti-IgM (Fig. 4B). The difference in relative intensities of some of the bands observed in BCR-stimulated tonsil B cells compared with those in Ramos B cells can be attributed to the difference in stimulation time, because kinetic differences were noted in the phosphorylation of some of the raft-associated proteins. Peak detection of a protein migrating at 29 kDa, barely visible at the 15-min time point shown in Fig. 4A, occurred at 15 s, in contrast to the more delayed phosphorylation kinetics of substrates migrating between 35 and 50 kDa (data not shown). While the identity of the phosphorylated proteins has not yet been determined, the data suggest that a subset of BCR signals is relayed to lipid rafts after receptor engagement. It remains to be determined whether these signals are initiated from outside the rafts or are a direct consequence of BCR translocation into this compartment. It is established that dually acylated src family tyrosine kinases are highly enriched in low-density membranes, but because CD45 tyrosine phosphatase is excluded from rafts (29) (Table I), the mechanism of their activation is unknown. However, src family kinases can be hyperactivated in the absence of CD45 (30, 31), perhaps through accumulated phosphorylation on the positive regulatory tyrosine residue (32), or via high-affinity interactions with the SH3 domain (33).

**BCR-mediated activation of signaling pathways in the absence of lipid rafts**

The rapid redistribution of BCRs and the appearance of tyrosine-phosphorylated proteins in lipid rafts after receptor cross-linking, suggests a role for rafts in early signaling events. MBC, a small cyclic oligosaccharide with a hydrophobic core that selectively extracts cholesterol into its interior cavity (34, 35), was used to test the effect of raft disruption on BCR-mediated signaling. Unlike other compounds that extract cholesterol, MBC does so without binding or inserting into the cell membrane (36–38). Preliminary experiments indicated that pretreatment of Ramos B cells with 10 mM MBC for 10 min at 37°C would disrupt rafts without compromising cell viability as measured by trypan blue exclusion. Based on the work of others, these conditions are expected to extract 30–35% of membrane cholesterol (36). As shown in Fig. 5, pretreatment of Ramos cells for 10 min with 10 mM MBC prevented translocation of mature BCRs to the low-density insoluble

<table>
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<th>Cells Tested</th>
<th>Soluble</th>
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<th>High-Density Insoluble (cytoskeleton)</th>
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<tr>
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<td>Raji; tonsil B</td>
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*Methods described in the text.*
fraction, confirming the disruption of lipid rafts with these conditions. Fig. 5 also shows that translocation of BCRs to the high-density insoluble pellet occurs regardless of MBC treatment, suggesting that cytoskeletal attachment of BCRs may occur independently of rafts. Lower exposures did not reveal any difference in the representation of either upper or lower bands in the high-density insoluble fraction after receptor stimulation in the presence and absence of MBC pretreatment.

Because signaling from the BCR is initiated by src family tyrosine kinase activation, and because dually acylated src family kinases such as lyn and fyn, are enriched in lipid rafts (11, 12), it was possible that BCRs translocated into rafts to initiate downstream signals. To test this, we assessed the phosphorylation status of protein kinase Cδ (PKCδ) and MAPK/ERK as downstream indicators of activated signaling pathways. Recently, we reported that PKCδ is activated and tyrosine phosphorylated in response to BCR cross-linking (39). However, disruption of lipid rafts did not inhibit BCR-mediated tyrosine phosphorylation of PKCδ (Fig. 6A), indicating that raft integrity is not required for tyrosine kinase activation. Tyrosine phosphorylation of PKCδ is dependent not only on src family tyrosine kinase activity, but also on the activity of PI-3K and PLCγ (39). Therefore, tyrosine phosphorylation of PKCδ after raft disruption also suggests that activation of PI-3K and PLCγ can occur independent of raft integrity. One of the major signaling pathways downstream of BCR-mediated tyrosine kinase activation leads to activation of MAPK/ERK. ERK activation, as measured by detection of its phosphorylated form, was also not inhibited by raft disruption (Fig. 6B), even when suboptimal doses of anti-IgM were used to stimulate the BCR (data not shown). Others have shown that ERK is hyperactivated by the epidermal growth factor receptor in cholesterol-depleted fibroblasts (40). Although we did not measure ERK activity per se, the detection of similar levels of phosphorylated ERK in MBC-treated and untreated BCR-stimulated B cells indicates that MAPK can occur independently of lipid rafts.

**Raft disruption enhances intracellular Ca2+ concentration [Ca2+]i, mobilization**

To investigate a potential role for lipid rafts in signaling pathways leading to intracellular calcium elevation, Ramos cells were loaded with fluo-3 and pretreated with MBC before BCR stimulation. MBC pretreatment caused a marked enhancement in the initial calcium burst induced by BCR stimulation (Fig. 7A, left panel). The enhanced calcium response in MBC-treated cells was resistant to chelation of extracellular calcium (Fig. 7A, right panel), suggesting that it was due to greater release from internal stores. MBC-treated and untreated Ramos B cells had identical levels of basal calcium and internal calcium stores as shown in Fig. 7A (right panel inset). The higher levels of [Ca2+]i, observed in BCR-stimulated MBC-treated Ramos cells was confirmed to be due to enhanced release from intracellular stores, because, compared with untreated BCR-stimulated cells, there was less calcium available for subsequent release by A23187 (data not shown).

Enhanced calcium flux was also observed in MBC-treated, anti-BCR-stimulated dense tonsil B cells (Fig. 7B, left panel). Compared with Ramos cells, tonsil B cells showed a more sustained elevation in [Ca2+]i, presumably due to calcium influx. It is interesting that in MBC-treated tonsil B cells there was a more rapid decline in [Ca2+]i, following the initial peak, perhaps indicating some dependence of the calcium channels on lipid microdomains. In both untreated and MBC-treated primary B cells, the height of the calcium peak was lower in the absence of extracellular calcium because of the loss of the component of the peak that is due to calcium influx (Fig. 7B, right panel). In primary B cells, as in Ramos, MBC enhanced calcium release even in the absence of extracellular calcium. However, whereas in Ramos cells the enhancing effect of cholesterol depletion appears to be completely independent of extracellular calcium, in primary B cells the enhancing effect is not as great as in the presence of calcium, again consistent with the possibility that calcium influx is partially dependent on lipid microdomains.

Others have reported that MBC inhibits the TCR-mediated calcium flux in T lymphocytes (14). This suggests that T and B lymphocytes may differ with respect to the role of lipid microdomains in Ag receptor signaling. The requirement for lipid rafts in TCR-mediated calcium mobilization can be understood in light of the fact that the adaptor protein LAT, which couples the TCR to PLCγ activation (41, 42), is palmitoylated and selectively localized to
of the response, in which SHIP is recruited to FcRγIIB and terminates calcium influx (53). It is important to note that in the studies reported here FcRγIIB is not involved because F(ab’2) anti-IgM was always used for BCR stimulation. However, SHIP is also involved in regulating BCR signals in the absence of FcRγIIB engagement (54, 55). Evidence indicates that it is the early, inositol 1,4,5-triphosphate-dependent phase of calcium flux that is primarily regulated by SHIP (55). Therefore, we examined low-density insoluble fractions of Ramos lysates for the presence of SHIP before and after BCR stimulation and found that SHIP was detectable at very low levels in rafts before stimulation, but rapidly accumulated there upon BCR cross-linking (Fig. 8A). Phosphotyrosine blots of raft fractions revealed a phosphorylated protein that comigrated with SHIP (Fig. 8B), suggesting that raft-associated SHIP is tyrosine phosphorylated. The mechanism of SHIP recruitment to rafts is not yet known but could conceivably involve phosphotyrosyl-based docking interactions.

The primary substrate for SHIP, phosphatidylinositol-3,4,5-trisphosphate, is generated by phosphorylation of phosphatidylinositol 4,5-bisphosphate by PI-3K. Thus, if SHIP is functional in lipid rafts one would expect to also find PI-3K in that compartment. Indeed, like SHIP, PI-3K was detectable at very low levels in rafts before stimulation, but rapidly increased transiently after BCR engagement (Fig. 8B). The amount of SHIP and PI-3K detected in rafts was small relative to that of total cellular SHIP and PI-3K, as indicated by the relative intensities of the bands in lanes 1–6 (Fig. 8C). The amount of SHIP and PI-3K detected in rafts was a small proportion of the total cellular SHIP and PI-3K, as indicated by the relative intensities of the bands in lanes 1–6 with that in lane 7; however, compared with PI-3K, a higher proportion of total cellular SHIP was detected in rafts. Recruitment of signaling molecules to rafts is selective because, as mentioned earlier, we did not...
detect BLNK. Similarly, PKCδ, which is rapidly activated and tyrosine phosphorylated in Ramos cells upon BCR stimulation (39), was not detected in lipid rafts before or after receptor stimulation (data not shown).

Our preliminary data indicate that 42–43% of total phosphatidylinositol phosphates in unstimulated cells are found in the low-density insoluble fraction. When lipids were extracted from either soluble or low-density insoluble fractions and analyzed by TLC, we found that phosphatidylinositol trisphosphate accumulated predominantly in the low-density insoluble fraction before BCR stimulation and was rapidly dephosphorylated (data not shown). Because TLC of extracted lipids does not discriminate between different isoforms of phosphatidylinositol phosphates, the complexity of the phosphatidylinositol bisphosphate content of lipid rafts after receptor stimulation was not revealed by these preliminary experiments, and further detailed work needs to be done to determine the activity of SHIP in lipid microdomains.

Lipid rafts isolated from Ramos B cells exclude >99% of total surface-labeled proteins (data not shown). In this report, we have demonstrated that, in both Ramos cells and in primary human B cells, a subset of engaged BCRs translocates into lipid rafts. This is an unusual property among B cell integral membrane proteins that has previously been ascribed only to CD20. In contrast to CD20, which translocates into lipid rafts but is never detected in the high-density insoluble pellet, the BCR is rapidly translocated to both compartments. Although rafts contain both actin and ezrin, whether and how lipid rafts are tethered to the cytoskeleton is presently unknown. Cytoskeletal attachment of the BCR did not appear to be dependent on prior residency in lipid rafts (Fig. 5), and inhibition of actin polymerization with cytochalasin D did not prevent translocation of receptors into rafts (data not shown).

Phosphorylation of tyrosine kinase substrates in rafts after BCR engagement, as shown in both Ramos and primary B cells, indicates that selected early signaling events are targeted to lipid rafts. Although the integrity of lipid rafts does not appear to be essential for the activation of signaling pathways leading to phosphorylation of PKCδ or ERK, it is nevertheless possible that signaling events involved in these pathways normally occur in rafts. Further, signaling pathways other than those tested here may have a requirement for intact raft microdomains. Whether or not rafts provide an essential conduit for receptor-mediated signaling pathways, it seems likely that signaling events occurring in rafts play a role in modulating the outcome of BCR signaling. Our preliminary data indicates that turnover of inositol phosphates occurs primarily in lipid rafts after receptor stimulation was not revealed by these preliminary experiments, and further detailed work needs to be done to determine the activity of SHIP in lipid microdomains.

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