A requirement for lipid rafts in B cell receptor induced Ca^{2+} flux

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Although the major biochemical events triggered by ligation of the B-cell receptor (BCR) have been well defined [1,2], little is known about the spatio-temporal organization of BCR signaling components within the cell membrane and the mechanisms by which signaling specificity is achieved. Partitioning of signaling complexes into specialized domains in the plasma membrane may provide a mechanism for channeling specific stimuli into distinct signaling pathways. Here, we report that multiple tyrosine-phosphorylated proteins accumulate transiently upon BCR activation in detergent-insoluble membrane microdomains known as lipid rafts. We found an activation-dependent translocation to the rafts of the BCR itself, as well as phospholipase Cγ2 (PLCγ2), an enzyme critical for BCR-induced Ca^{2+} flux in B cells. An intact raft structure was required for BCR-induced tyrosine phosphorylation of PLCγ2 and the induction of Ca^{2+} flux. Taken together, these data provide a functional role for lipid rafts in BCR signaling.

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Accepted: **7 February 2000**

Published: **24 March 2000**

Current Biology 2000, 10:393–396

0960-9822/00/$ – see front matter

Results and discussion

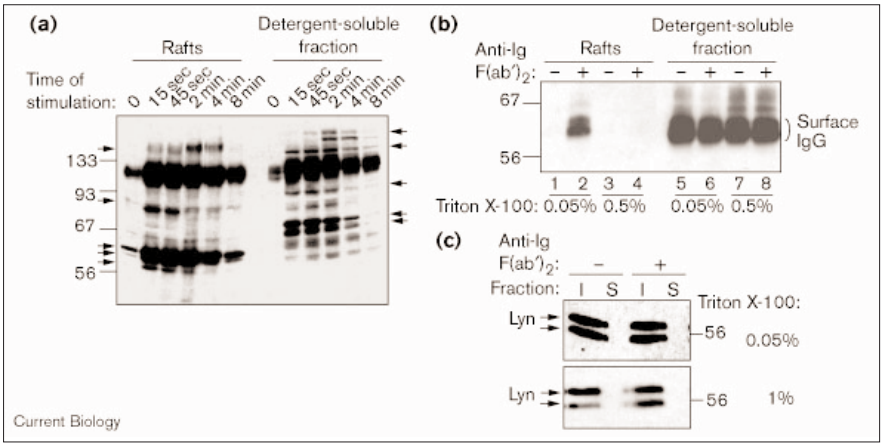
Studies of the apical sorting of lipids and proteins in epithelial cells have revealed the existence of highly ordered glycosphingolipid- and cholesterol-enriched microdomains within the plasma membrane [3]. These detergent-insoluble glycolipid-enriched complexes (DIGs) [3] or glycolipid-enriched membrane domains (GEMs) [4] can be isolated as low-density complexes by sucrose gradient ultracentrifugation. Although these microdomains were initially characterized biochemically, recent electron microscopy and immunofluorescence studies have demonstrated the existence of such structures as physical domains in living cells [5]. While glycosyl-phosphatidylinositol (GPI)-linked proteins and Src family kinases are tightly anchored into these microdomains, several signaling proteins can associate with GEMs in a regulated manner [3]. Thus, GEMs are believed to act as moving platforms or 'rafts', which selectively accommodate specific molecules involved in cell signaling and membrane trafficking [3].

On the basis of the detergent concentrations used for cell lysis, proteins that associate tightly or loosely with lipid rafts have been described [6–8]. To study the pattern of BCR-induced tyrosine phosphorylation in detergent-insoluble and detergent-soluble fractions, we initially used 0.05% Triton X-100 for cell lysis so that we could detect both low- and high-affinity interactions. A20 cells were stimulated by an $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin G (IgG) for different lengths of time. The detergent-soluble and insoluble (rafts) fractions were isolated by sucrose gradient ultracentrifugation and analyzed by immunoblotting with anti-phosphotyrosine antibody. BCR stimulation induced rapid tyrosine phosphorylation of multiple proteins both in the rafts and the soluble fraction (Figure 1a). Interestingly, while some phosphoproteins were detected in both fractions, others were either enriched or excluded from the raft fraction (indicated by arrows). We found that, typically, less than 0.5% of total proteins were found in the rafts (data not shown). Therefore, the distinct phosphorylated proteins readily detectable in this fraction suggested a selective enrichment of specific proximal mediators of BCR signaling.

The BCR consists of a transmembrane Ig molecule (IgG on the rafts within 15 seconds of BCR stimulation (Figure 1a) suggested that active receptor-mediated signaling might take place within these specialized domains. Therefore, we tested whether surface IgG, as the antigen-binding moiety of the receptor, was localized to the lipid rafts. Resting or activated A20 cells were lysed in either 0.05% or 0.5% Triton X-100, fractionated, and surface IgG immunoprecipitated using a rabbit anti-mouse Ig followed by immunoblotting with rabbit anti-mouse Ig that was labeled with horseradish peroxidase (HRP). Although surface IgG was not detectable in the raft fraction of unstimulated cells, significant enrichment of BCR in the rafts occurred after activation (Figure 1b, lanes 1,2 versus 5,6). BCR association with rafts was sensitive to detergent concentration and was hardly detectable at 0.5% Triton X-100 (Figure 1b). A similar sensitivity to detergents for association with lipid rafts has been reported for FcεRIγ and TCRζ [6,8]. These data demonstrated the activation-dependent association of BCR with lipid rafts and that this association is mediated by weak intermolecular interactions.

Src family kinases are major components of rafts, as shown for Fyn and Lck in T cells [9] and Lyn in monocytes [10]. Lyn is critically involved in the initial phase of BCR signaling [11]. We therefore tested the distribution of Lyn in B cells at different detergent concentrations. Lyn was

Figure 1



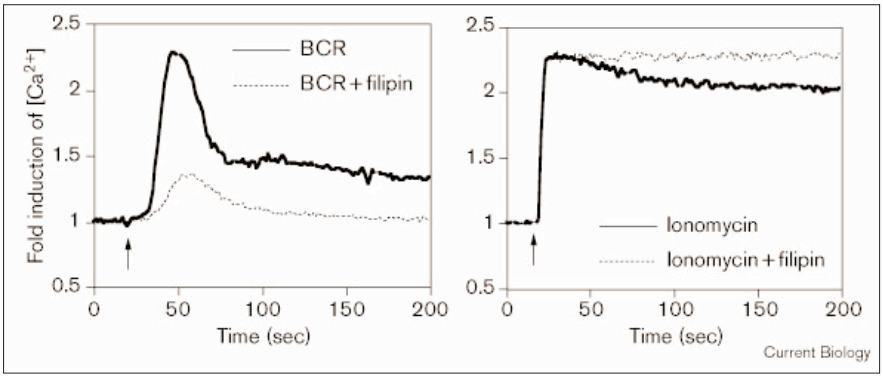
Detection of multiple tyrosine-phosphorylated proteins and the BCR itself in lipid rafts upon BCR activation. **(a)** A20 cells were stimulated with 15 μg/ml $F(ab')_2$ rabbit anti-mouse IgG for the indicated times, lysed in 0.05% Triton X-100 and fractionated. Rafts and detergent-soluble fractions were analyzed by immunoblotting with RC20 anti-phosphotyrosine antibody. **(b)** A20 cells were left unstimulated or activated as above for 45 sec, lysed in either 0.05% or 0.5% Triton X-100, and fractionated. The surface IgG was immunoprecipitated from each fraction using rabbit anti-mouse IgG and analyzed by immunoblotting with HRP-conjugated rabbit anti-mouse IgG. **(c)** Rafts (I) and soluble (S) fractions were prepared from resting or stimulated A20 cells, lysed in 0.05% or 1% Triton X-100, and analyzed for Lyn expression using rabbit anti-Lyn antibody. All molecular weights indicated here and in Figure 3 are in kDa.

almost exclusively found in the lipid rafts, independent of activation or detergent concentration (Figure 1c).

Activation of B cells through the BCR leads to increased intracellular Ca^{2+} concentration, and this Ca^{2+} flux has been shown to be critical for B-cell development and function [1,2]. We examined whether raft-dependent signaling is required for BCR-induced Ca^{2+} flux. The antifungal agent filipin has been shown to disrupt cholesterol-rich membrane microdomains [12]. We compared the pattern of BCR-induced Ca^{2+} flux in A20 cells pre-treated with filipin and untreated control cells. Treatment of cells with filipin potently inhibited BCR-induced Ca^{2+} flux (Figure 2, left panel). Filipin did not have any effect on ionomycin-mediated rise in intracellular Ca^{2+} levels (Figure 2, right panel), indicating that receptor-independent Ca^{2+} flux could still occur in filipin-treated cells and that the integrity of the cell membrane was intact. Filipin did not affect the viability (determined by trypan blue exclusion) or the BCR surface expression in the A20 cells (data not shown). These data strongly suggested that the BCR-mediated Ca^{2+} flux requires intact cholesterol-enriched membrane microdomains.

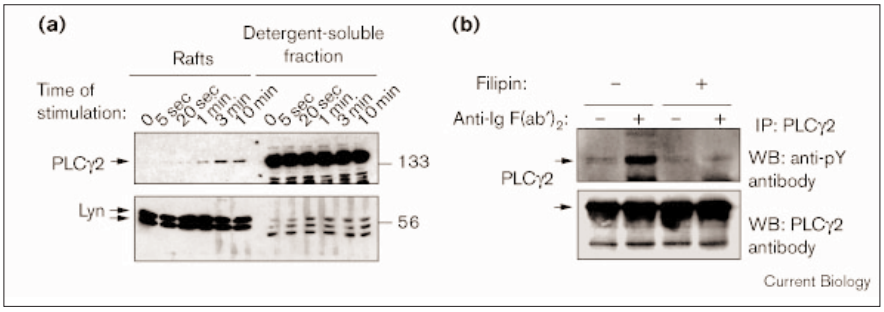
The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and the generation of soluble inositol 1,4,5-trisphosphate (IP_3) are critical for BCR-induced rise in intracellular Ca^{2+} levels [1]. Therefore, we examined whether PLCγ2 (the major isoform in A20 and other B cells) actively moves to lipid rafts upon BCR stimulation. A small but reproducible fraction of PLCγ2 was found to translocate into the lipid rafts after BCR stimulation as early as 5–20 seconds, with a peak between 1–3 minutes (Figure 3a). In some experiments we also observed some basal levels of PLCγ2 in the rafts which is further increased upon stimulation. As a control for the quality of raft preparation, the lower part

Figure 2



Disruption of rafts inhibits BCR-induced Ca^{2+} flux. A20 cells were loaded with indo-1. Where indicated, the cells were incubated with 1.6 μg/ml filipin for 5 min, washed extensively and analyzed for Ca^{2+} flux. Recording of fluorescence ratio was initiated before stimulation of cells with **(a)** 15 μg/ml $F(ab')_2$ rabbit anti-mouse IgG or **(b)** 1.0 μg/ml ionomycin. Arrows indicate the time point of addition of the antibody or ionomycin.

Figure 3



Translocation of PLCγ2 into rafts and the raft-dependent phosphorylation of PLCγ2. **(a)** A20 cells were stimulated with $F(ab')_2$ anti-mouse IgG for the indicated times, lysed in 0.5% Triton X-100, fractionated, and analyzed by immunoblotting with antibodies to PLCγ2 (top panel) or Lyn (bottom panel). **(b)** A20 cells were left untreated (lanes 1,2), or treated with 1.6 μg/ml filipin for 15 min at 37°C, and then either left unstimulated or activated for 3 min, and lysed. PLCγ2 was immunoprecipitated (IP) and analyzed by immunoblotting with HRP-conjugated anti-phosphotyrosine antibody RC20 (top panel). The nitrocellulose membrane was stripped and reprobed with anti-PLCγ2 antibody (bottom panel). WB, western blot.

of the same blot was analyzed for the presence of Lyn. As expected, Lyn was mainly localized in the detergent-insoluble fraction (Figure 3a). In initial experiments, we found that the association of PLCγ2 with rafts was detectable at both high (0.5%) and low (0.05%) concentrations of Triton X-100. The experiment shown in Figure 3a was performed with 0.5% Triton X-100.

Coordinated phosphorylation of PLCγ2 by the tyrosine kinases Syk and Btk is required for activation of PLCγ2 and the subsequent phosphoinositide turnover and BCR-induced Ca^{2+} flux [2]. We tested whether disruption of rafts would hamper the BCR-induced phosphorylation of PLCγ2 (Figure 3b). Treatment of cells with filipin almost completely inhibited the BCR-mediated tyrosine phosphorylation of PLCγ2. Comparable amounts of PLCγ2 were precipitated in filipin-treated and untreated conditions (Figure 3b).

These data suggest a critical role for lipid rafts in BCR-dependent tyrosine phosphorylation of PLCγ2, and provide a possible mechanism for the involvement of rafts in regulation of calcium flux. As we detected low levels of PLCγ2 in the rafts, it is possible that this translocation is very transient and serves solely the purpose of phosphorylation. Alternatively, rafts may be required for both activation of PLCγ2 and the subsequent hydrolysis of PIP_2 leading to initiation of Ca^{2+} flux. Interestingly, two recent reports showed the enrichment of PIP_2 along with phosphatidylinositol 4-phosphate in low-density membrane domains [13,14]. More detailed studies are needed to investigate whether PIP_2 hydrolysis takes place in the rafts.

The constitutive presence of Lyn in lipid rafts and its documented role in BCR signaling implies that at least part of the BCR-induced phosphorylation must take place in this compartment. In addition to the rapid phosphorylation of multiple substrates in the detergent-insoluble fraction, we also observed that disruption of raft structure decreased the overall tyrosine phosphorylation in response to BCR (data not shown). Thus, proteins may be trafficking into and out of rafts to become phosphorylated. Whether all of the phosphorylated proteins in rafts represent Lyn substrates remains to be clarified.

In addition to the proteins that are tightly anchored in the rafts through covalently bound fatty acyl chains (GPI-linked proteins, dually acylated Src-family kinases, and other acylated proteins such as LAT [3,4]), a second class of proteins associate with rafts through non-covalent interactions, typically in a transient and activation-dependent manner, the nature of which is not fully understood [3]. The second type of association with lipid rafts has been reported for TCRζ, Zap70, Shc, PLCγ1 and Syk in T cells [14], and for FcεRI in mast cells and basophils [6,15]. Our finding that the BCR moves into lipid rafts upon ligation suggests that active BCR-induced signaling takes place in the rafts. As the association of BCR with rafts is sensitive to high concentrations of detergent, such a weaker interaction with rafts may facilitate rapid and transient association and dissociation. During the submission of this work, Cheng *et al.* reported the activation-dependent translocation of sIgM and Igα into lipid rafts and showed a role for lipid rafts in BCR-mediated antigen internalization and processing [16]. Whereas we observed that association of surface IgG with rafts is sensitive to detergent concentration, Cheng *et al.* could detect sIgM in the rafts in 1% Triton X-100. Whether surface IgG and IgM have distinct affinities for raft microdomains remains to be determined.

The mechanism by which the BCR interacts with rafts is not clear. Field *et al.* reported that the association of FcεRIγ with lipid rafts is mediated by its transmembrane domain [15]. Interestingly, the transmembrane domain of TCRζ shows 90% homology with that of FcεRIγ [17]. We could not, however, detect a significant homology between

the transmembrane domains of surface IgG [18] and TCRζ or FcεRIγ. The precise contribution of the transmembrane domain, the short cytoplasmic tail and the extracellular regions of surface IgG remains to be determined. It would also be interesting to determine whether coreceptors that positively or negatively regulate B-cell functions influence the movement of the BCR or other signaling molecules to the rafts. Taken together, the data presented here and the recent work by Cheng *et al.* [16] provide a first step towards understanding the spatio-temporal regulation of BCR signaling and could have implications for B-cell development and function throughout ontogeny.

Materials and methods

Separation of rafts and detergent-soluble fractions

A20 cells (5×10^7) were lysed in 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 10 mM β-glycerophosphate, protease and phosphatase inhibitors, and Triton X-100 either at 0.05% or 0.5%. Lysates were then diluted 1:1 with 80% sucrose and transferred into a Beckman ultracentrifuge tube. The lysates were overlaid by 2 ml 30% sucrose followed by 1 ml 5% sucrose and centrifuged for 16–20 h at 200,000 × *g*. After the centrifugation, the lipid raft band visible at the interface of 30% and 5% sucrose (insoluble fraction) was removed and solubilized by adding octylglucoside (Sigma) at 50 mM. The lysate at the bottom of the tube represented the Triton-soluble fraction.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} levels were measured, using A20 cells loaded with indo-1 (1 μg/ml, 20 min, 37°C), in a Hitachi F-2500 fluorescence spectrophotometer, as previously described [19]. For experiments with filipin, the drug was added at 1.6 μg/ml during the last 5 min of incubation with indo-1 followed by extensive wash with PBS (5–6 times). After recording the background for 20 sec, cells were stimulated by injecting $F(ab')_2$ rabbit anti-mouse IgG antibody at 15 μg/ml or ionomycin at 1 μg/ml. Induced Ca^{2+} flux was calculated as fold-induction over the background.

Acknowledgements

This work was supported by a grant from the National Institutes of Health, USA (AI 43425-01) to K.S.R. M.J.A. was supported by an Immunology Training grant from the National Institutes of Health, USA.

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