J. Biochem. 129, 861–868 (2001)

Detergent-Resistant Membrane Domains Are Required for Mast Cell Activation but Dispensable for Tyrosine Phosphorylation upon Aggregation of the High Affinity Receptor for IgE

Toshiyuki Yamashita,[1] Takayuki Yamaguchi, Kiichi Murakami, and Shigeharu Nagasawa

Division of Hygienic Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12 Nishi-6, Kita-ku, Sapporo 060-0812

Received February 5, 2001; accepted March 5, 2001

Aggregation of the high affinity receptor for IgE (FcεRI) on mast cells results in the rapid phosphorylation of tyrosines on the β and γ chains of the receptor by the Src family kinase Lyn, which initiates the signaling cascades leading to secretion of inflammatory mediators. The detergent-resistant membranes (DRMs) have been implicated in FcεRI signaling because aggregated receptors emigrate to DRMs that are enriched in certain signaling components. We evaluated the role of DRMs in FcεRI signaling by disruption of DRMs using a cholesterol-binding agent, methyl-β-cyclodextrin (MBCD). While treatment of rat basophilic leukemia cells with MBCD inhibits degranulation and Ca^{2+} mobilization upon aggregation of FcεRI, MBCD hardly affects the aggregation-induced tyrosine phosphorylation of FcεRI as well as other signaling molecules such as phospholipase C-γ1 (PLC-γ1). MBCD delocalizes phosphatidylinositol 4,5-bisphosphate from DRMs, which may prevent MBCD-treated cells from producing inositol 1,4,5-trisphosphate by means of activated PLC-γ1. These data suggest an indispensable role for DRMs in the Ca^{2+} response rather than tyrosine phosphorylation, and support a model of receptor phosphorylation in which aggregated FcεRI is tyrosine phosphorylated outside DRMs by constitutively associated Src family kinase Lyn *via* a transphosphorylation mechanism.

Key words: DRM, FcεRI, Lyn, methyl-β-cyclodextrin, RBL-2H3.

Aggregation of the high affinity receptor for IgE (FcεRI) on mast cells and basophils initiates a signaling cascade that results in degranulation and the release of mediators of the allergic reaction (*1*, *2*). The cytoplasmic domains of the β and γ chains contain a sequence termed the immunoreceptor tyrosine-based activation motif (ITAM) (*3*). The phosphorylation of two conserved tyrosine residues within the ITAM consensus sequence plays a critical role in receptor-mediated signal transduction. Previous studies have revealed that the Src family kinase Lyn is responsible for the tyrosine phosphorylation of FcεRI subunits (*4–8*). Tyrosine phosphorylation of γ chain ITAM recruits another non-receptor PTK, Syk, a member of the Syk/ZAP-70 family, *via* its tandem Src homology 2 (SH2) domain (*9*). This binding and consequent activation of Syk result in increased tyrosine phosphorylation, and activation of a number of cellular proteins such as phospholipase C (PLC)-γ1, Vav, and Shc (*10–12*).

Although the tyrosine phosphorylation of FcεRI by Lyn is a critical event leading to mast cell activation, the mechanism by which receptor aggregation initiates this event is not well understood. Substantial evidence suggests that Lyn is associated with the β chain of FcεRI prior to receptor aggregation (*6*, *13*). This preassociated Lyn is hypothesized to mediate the tyrosine phosphorylation of β- and γ-chains of the receptors through a process of transphosphorylation when the receptors are brought into proximity upon aggregation (*14*). On the other hand, specialized membrane domains have been proposed to be involved in the FcεRI signaling (*15–18*). These detergent-resistant membrane rafts (DRMs), which can be separated as a low density membrane fraction on density gradient centrifugation, are enriched in sphingolipids, cholesterol, and saturated glycerophospholipids (*19*, *20*). Furthermore, a number of proteins that are involved in signal transduction, including dually acylated Src family kinases, low molecular weight and heterotrimeric G proteins, are partitioned into these microdomains. Previous biochemical studies have revealed that FcεRI is excluded from or only weakly associated with DRMs in resting cells, while the aggregation of receptors triggers its recruitment to DRMs (*16*, *17*). The association of aggregated FcεRI with DRMs has also been demonstrated in living cells (*21*). The high concentration of active Lyn in DRMs may then allow the tyrosine phosphorylation of the DRM-associated FcεRI. Thus, in this scenario, DRMs

[1] To whom correspondence should be addressed. Tel: +81-11-706-3245, Fax: +81-11-706-4989, E-mail: yamashit@pharm.hokudai.ac.jp

Abbreviations: Ag, antigen; $[Ca^{2+}]_i$, intracellular free calcium concentration; DNP, dinitrophenyl; DRM, detergent-resistant membrane; FcεRI, high affinity receptor for IgE; IP_3, inositol 1,4,5-trisphosphate; ITAM, immunoreceptor tyrosine-based activation motif; MBCD, methyl-β-cyclodextrin; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; RBL, rat basophilic leukemia; SH2, Src homology 2; TCR, T cell receptor.

function to facilitate the initial coupling of aggregated FcεRI and Lyn.

Accumulating evidence suggests the possibility that DRM microdomains play a pivotal role in signal transduction through many types of membrane receptors including the T cell receptor (TCR). Recent data indicate that DRMs are necessary for TCR-mediated Ca^{2+} mobilization and tyrosine phosphorylation of signaling molecules such as PLC-γ1 as well as the ζ subunits of the TCR complex itself (*22–24*). DRMs have also been proposed to be important in inducing TCR-mediated cytoskeletal reorganization (*23*). In addition, phosphatidylinositol 4,5-bisphosphate (PIP_2), the primary PLC substrate in the plasma membrane, appears to be concentrated in DRMs (*25–27*). Thus, DRMs may function as platforms for signal transduction.

In the present study, we investigated the functional role of DRMs in RBL-2H3 mast cells by means of disruption of DRMs. Here we used the cholesterol-binding agent methyl-β-cyclodextrin (MBCD), which selectively extracts cholesterol from the plasma membrane and thereby disrupts DRMs (*28–31*). We show that intact DRMs are necessary for FcεRI-mediated Ca^{2+} mobilization as a reservoir of PIP_2. However, DRM integrity is not required for tyrosine phosphorylation of FcεRI. The relevance of these data as to current ideas on aggregation-induced tyrosine phosphorylation of FcεRI by Lyn is discussed.

MATERIALS AND METHODS

Materials—Monoclonal anti-dinitrophenyl (DNP) IgE was prepared from the ascites of mice bearing an H1 DNP-ε-26.82 hybridoma (*32*). [^{125}I]IgE was prepared by the chloramine-T method (*33*). Some IgE and [^{125}I]IgE was biotinylated with D-biotinoyl-ε-amidocaproic acid–*N*-hydroxysuccinimide ester (Boehringer Mannheim) (*15*). MBCD was purchased from Sigma. Immunoprecipitating anti-PLCγ1 antibodies (immunizing antigen amino acids 1249–1262 of bovine PLCγ1) were obtained from Santa Cruz Biotechnology. The other reagents we used have already been described (*34, 35*).

Treatment Cells with MBCD—RBL-2H3 cells were grown as adherent monolayers, and harvested following exposure to trypsin and EDTA as previously described (*35*). The cells (10^7/ml) were incubated with 10 μg/ml mouse anti-DNP IgE for 1 h at room temperature. The IgE-sensitized cells were washed twice, and then resuspended in assay buffer containing 25 mM PIPES (pH 7.2), 119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgSO_4$, and 5.4 mM glucose. The cells (10^7/ml) were treated with the indicated concentrations of MBCD in 1 ml of the assay buffer at 37°C for 20 min and then washed twice with the assay buffer containing 0.1% BSA. In some experiments, MBCD-treated cells were further incubated with cholesterol-MBCD complex (0.2 mM cholesterol, Sigma) at 37°C for 10 min. Except where otherwise indicated, the IgE-sensitized cells (10^7/ml) were stimulated with 1 μg/ml DNP-BSA for 2 min at 37°C. In some experiments, cells were sensitized with biotinylated IgE and then stimulated with 0.5 μg/ml streptavidin for 2 min at 37°C.

Isolation of DRMs—Cells (5×10^6) were lysed in 1 ml of lysis buffer containing 0.05% Triton X-100, 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin. Lysates were incubated for 1 h on ice with frequent agitation. Each lysate was mixed with an equal volume of 85% sucrose in STE (50 mM NaCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 0.05% Triton X-100, and 1 mM Na_3VO_4) and then placed at the bottom of a centrifuge tube. Two milliliters of 35% sucrose in STE was layered on top, followed by 1 ml of 5% sucrose in STE. The discontinuous gradients were then centrifuged for 16 h at 4°C at 200,000 ×*g* in an SW50.1 Ti rotor. Fractions of 0.5 ml were collected from the top of the gradients.

Immunoprecipitation and Immunoblotting—The conditions for immunoprecipitation and immunoblotting were described previously (*35*). In brief, cells were solubilized with lysis buffer containing 0.5% Triton X-100, and precleared lysates were incubated with appropriate antibodies followed by incubation with protein A- or protein G-agarose. To assay the association of FcεRI with Lyn, IgE-sensitized cells (5×10^6/ml) were solubilized in lysis buffer containing 0.05% Triton X-100 as in the case of DRM isolation, and IgE-bound FcεRI was directly immunoprecipitated with bead-bound anti–mouse IgE. Lysates or immunoprecipitates were resolved by SDS/PAGE, transferred to nitrocellulose membranes, and then visualized with proper antibodies and an enhanced chemiluminescence system (Amersham).

Degranulation and Ca^{2+} Mobilization Assay—The assay conditions for degranulation were described previously (*35*). Measurement of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed using Fura-PE3 as a Ca^{2+} indicator (*34, 36*).

Analysis of IP_3 Production—The assays were performed using a D-*myo*-inositol 1,4,5-trisphosphate [^{3}H] assay system (Amersham). To prepare samples for the assays, 5×10^6 cells sensitized with IgE were incubated without a stimulus or stimulated with DNP-BSA (1 μg/ml) in 800 μl of assay buffer at 37°C for the times indicated. IP_3 was extracted with perchloric acid according to the manufacturer's recommendations. Each sample was assayed in duplicate.

Immune Complex Phospholipase Assay—Lysates of 2×10^7 cells were immunoprecipitated with anti-PLC-γ1 antibodies and 30 μl of rProtein A Sepharose Fast Flow (Amersham Pharmacia Biotech AB). The immunoprecipitates were washed with reaction buffer containing 35 mM NaH_2PO_4, pH 6.8, 70 mM KCl, 0.8 mM EGTA, and 0.8 mM $CaCl_2$, and then suspended in the same buffer (50 μl) supplemented with 0.2 mM PIP_2 (Boehringer Mannheim), 0.15% octyl glucoside, and 0.05% Triton-X-100 (*37, 38*). After incubation for 30 min at 37°C, IP_3 produced was quantitated by means of the radioreceptor assay described above.

Analysis of [^{3}H]Inositol-Labeled Lipid—Cells (2.5×10^7) were cultured with 30 ml of inositol-free DMEM (GIBCO BRL) containing 10% dialyzed fetal bovine serum (GIBCO BRL) and 1 μCi/ml *myo*-[^{3}H]inositol (Amersham Pharmacia Biotech AB) for 48 h. After centrifugation to isolate DRMs as described above, each fraction was extracted in chloroform:methanol:HCl as described (*25*). For the analysis of inositol phospholipids, potassium oxalate-impregnated silica gel plates (Silica gel 60, MERCK) were used and developed in a solvent comprising $CHCl_3/CH_3OH$/4 M NH_3 (45:35:10). The TLC plates were sprayed with EN^{3}HANCE (NEN) and then subjected to autoradiography.

RESULTS

Effect of MBCD Treatment on Partitioning of FcεRI and Lyn into DRMs—To determine the effects of cholesterol removal on the integrity of DRMs, IgE-sensitized RBL-2H3 cells were treated in the absence or presence of 25 mM MBCD for 20 min. Cells treated in this manner remained viable, as assessed by trypan blue staining (data not shown). It should be noted that approximately 60% of FcεRI was lost from cells on MBCD-treatment, as reported by Sheets *et al.* (*18*). When lysates of untreated cells were analyzed on sucrose density gradients, a considerable fraction of aggregated FcεRI migrated to the low density sucrose region, whereas unaggregated FcεRI was found almost entirely in the high density fraction containing solubilized proteins (Fig. 1A). However, aggregated FcεRI was nearly absent in the low density fractions of MBCD-treated cells, similar to the gradient distributions of control unaggregated cells (Fig. 1B). As shown in Fig. 1C, treatment of RBL-2H3 cells with MBCD also led to the loss of compartmentalization of Lyn, which was found almost exclusively in the high density regions in MBCD-treated cells. These data suggest that the removal of cholesterol disrupts the integrity of the low density DRMs, which results in the delocalization of aggregated FcεRI and Lyn.

Disruption of DRMs Inhibits the Mast Cell Activation Following Aggregation of FcεRI—When RBL-2H3 cells were treated with increasing concentrations of MBCD, we observed dose-dependent inhibition of antigen-induced degranulation, as assessed as the release of β-hexosaminidase (Fig. 2A). In contrast, MBCD did not affect the degranulation induced by PMA or calcium ionophore A23187, indicating that cholesterol depletion under these conditions does not significantly compromise cell integrity. We also found that Ca^{2+} mobilization induced by FcεRI aggregation is impaired in MBCD-treated RBL-2H3 cells (Fig. 2B). MBCD treatment failed to affect Ca^{2+} mobilization induced by ionomycin (data not shown). Whereas MBCD extracts cholesterol from plasma membranes, water soluble complexes of cholesterol and MBCD are capable of incorporating cholesterol into membranes (*28*). To determine whether or not the impaired Ca^{2+} mobilization was due to the depletion of cholesterol, MBCD-treated RBL-2H3 cells were subsequently incubated with either the buffer alone or cholesterol-MBCD complexes for 10 min. As shown in Fig. 2C, treatment of MBCD-treated cells with cholesterol-MBCD complexes (*cholesterol*) reversed the diminished Ca^{2+} mobilization, whereas incubation in buffer alone (*MBCD*) did not lead to restoration of the response, indicating that the effect of MBCD results from its ability to remove cholesterol from membranes rather than its possible other activity. Taken together, these results provide strong evidence that DRMs are important for mast cell activation initiated by aggregation of FcεRI.

Cholesterol Lowering Hardly Affects Tyrosine Phosphorylation of FcεRI and Receptor Association with Lyn—To dissect the requirement of DRM integrity for mast cell activation, we investigated whether or not treatment of cells with MBCD affects the tyrosine phosphorylation of FcεRI. For this purpose, RBL-2H3 cells were sensitized with [^{125}I]IgE, untreated or treated with MBCD, and left unstimulated or stimulated with the optimum concentration of antigen for 2 min. The cells were then solubilized, and FcεRI was immunoprecipitated from these cells. Based on the precipitated radioactivity, equal amounts of IgE-bound FcεRI were analyzed by immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10. Interestingly, cholesterol depletion by MBCD only partially inhibited the aggregation-induced tyrosine phosphorylation of both the β- and γ-subunits of FcεRI even at the concentration of 25 mM, with which antigen-induced degranulation and Ca^{2+} mobilization is almost completely inhibited (Fig. 3A). When the blots were scanned, and the absorbance of the β and γ bands was measured, we found 12.1 ± 5.4% (n = 6) and

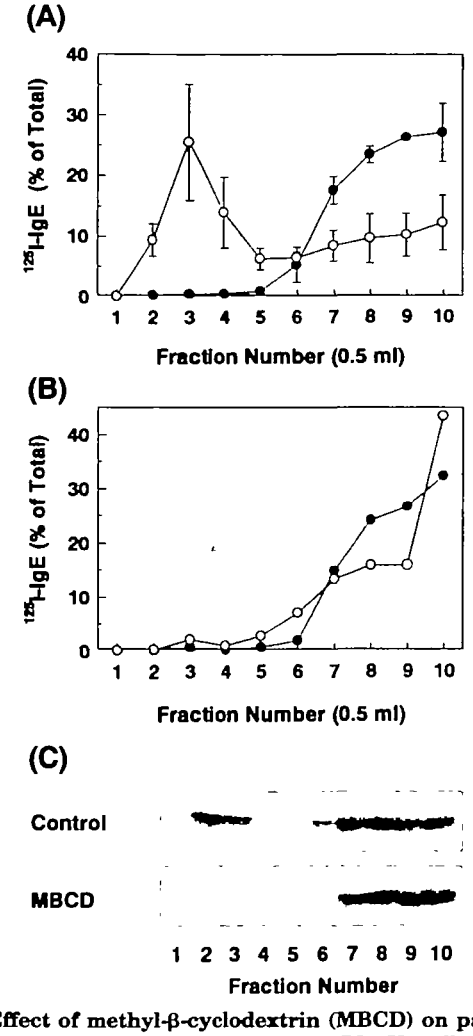

Fig. 1. **Effect of methyl-β-cyclodextrin (MBCD) on partitioning of Lyn and FcεRI into DRMs.** (A) RBL-2H3 cells that had been sensitized with [^{125}I]- and biotin-conjugated mouse IgE were unstimulated (closed circles) or stimulated with streptavidin (open circles). The cells were lysed and fractionated by sucrose density gradient centrifugation. The [^{125}I] present in each fraction is shown as a percentage of the total [^{125}I]IgE in the entire gradient. Data shown are the means ± SD of three separate experiments. (B) The distribution of FcεRI from unstimulated (closed circles) or stimulated RBL-2H3 cells (open circles) that had been treated with 25 mM MBCD was analyzed. (C) RBL-2H3 cells were incubated with or without 25 mM MBCD for 20 min at 37°C. The cells were then lysed and fractionated by sucrose density gradient centrifugation. Equal aliquots of each fraction were analyzed by SDS-PAGE and Western blotting for the presence of Lyn.

17.7 ± 9.6% (n = 6) less tyrosine phosphorylation of β and γ, respectively. This partial inhibition of tyrosine phosphorylation of FcεRI is likely due to a reduction in the amount of FcεRI in the MBCD-treated cells, since a proportional relationship between receptor number and β phosphorylation has been reported (*18*).

We also determined whether or not MBCD affects the extent of association of FcεRI with Lyn. In agreement with previously published data (*8*), FcεRI was constitutively associated with Lyn, and this association was significantly enhanced after receptor aggregation (Fig. 3B). Cholesterol lowering by MBCD again failed to inhibit either the constitutive or inducible association of FcεRI with Lyn. Thus, the influence of cholesterol depletion on signal transduction *via* FcεRI seems not to be due to destruction of the initial coupling of FcεRI with Lyn.

PLC-γ1 Is Activated upon FcεRI Aggregation, while IP_3 Is Not Generated in MBCD-Treated Cells—Aggregation of FcεRI leads to the tyrosine phosphorylation and activation of Syk, and the absolute requirement for Syk in Ca^{2+} signaling and many downstream functions has been demonstrated (*39, 40*). We found that Syk in MBCD-treated cells was normally tyrosine phosphorylated upon FcεRI aggregation (data not shown). Moreover, PLC-γ1, which is one of the multiple protein substrates of Syk and responsible for the aggregation-stimulated IP_3 production (*41*), was also tyrosine phosphorylated even in the MBCD-treated cells (Fig. 4A). When we directly measured the enzymatic activity of PLC-γ1 through immune complex phospholipase assays, we found that FcεRI aggregation caused a substantial increase in PLC-γ1 activity, and that there was no significant difference in the level between untreated and MBCD-treated cells (Fig. 4B).

We also studied *in vivo* IP_3 production by extracting IP_3 from Ag-stimulated cells and quantitating it by means of a radio receptor binding assay. Aggregation of FcεRI on control RBL-2H3 cells induced IP_3 production, which became a maximum at around 15 s and persisted for at least 2 min (Fig. 4C). However, in contrast to the results of *in vitro* phospholipase assays, MBCD-treated cells did not produce IP_3 in response to FcεRI aggregation. Thus, disruption of DRMs inhibits *in vivo* IP_3 production induced by aggregation of FcεRI without affecting the enzymatic activity of PLC-γ1.

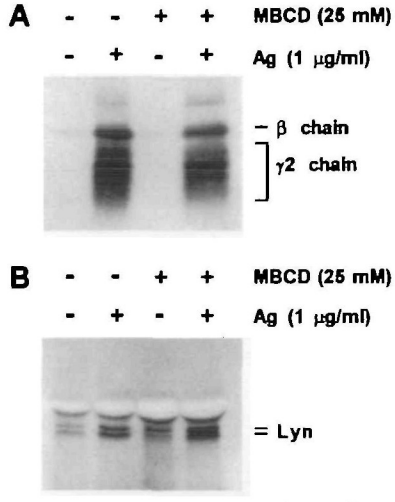

Fig. 3. **Cholesterol depletion hardly affects the aggregation-induced tyrosine phosphorylation of FcεRI and the level of FcεRI-Lyn association.** (A) RBL-2H3 cells that had been sensitized with [^{125}I]IgE anti-DNP were untreated or treated with 25 mM MBCD. Cells were then unstimulated or stimulated with DNP-BSA (Ag), and IgE-bound FcεRI was immunoprecipitated with anti-mouse IgE-bound Sepharose beads. Immunoprecipitates equivalent to 1 × 10^6 cells (based on the precipitated [^{125}I]) were subjected to Western blotting with an anti-phosphotyrosine monoclonal antibody (4G10). (B) MBCD-treated or untreated RBL-2H3 cells were unstimulated or stimulated with Ag, and then anti-IgE immunoprecipitates were analyzed by Western blotting with anti-Lyn serum.

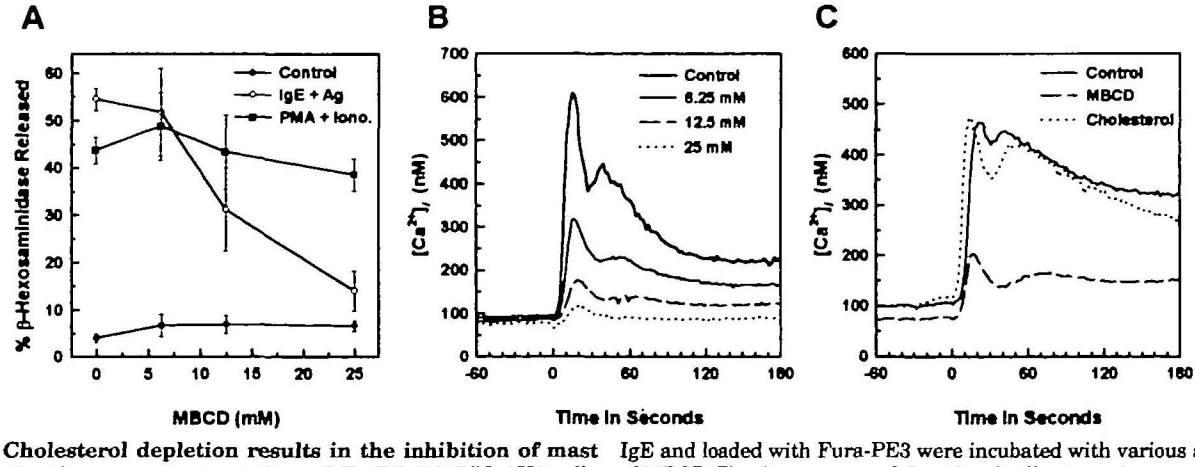

Fig. 2. **Cholesterol depletion results in the inhibition of mast cell activation upon aggregation of FcεRI.** (A) RBL-2H3 cells that had been sensitized with anti-DNP IgE were incubated with various amounts of MBCD. Cells were incubated with buffer alone or 1 μg/ml DNP-BSA for 40 min at 37°C. Cells were also incubated with 50 nM PMA and 5 μM calcium ionophore A23187. β-Hexosaminidase in the supernatant was measured as a percentage of total β-hexosaminidase. Each point represents the mean ± SD of three independent experiments. (B) RBL-2H3 cells that had been sensitized with anti-DNP IgE and loaded with Fura-PE3 were incubated with various amounts of MBCD. The fluorescence of the stirred cell suspension was continuously monitored with a fluorescence spectrophotometer. Cells were stimulated with 1 μg/ml Ag at time 0. (C) IgE-sensitized and Fura-PE3–loaded RBL-2H3 cells were untreated (control) or treated with 12.5 mM MBCD. The MBCD-treated cells were then incubated with buffer alone (MBCD) or MBCD-cholesterol complex (0.2 mM cholesterol) (Cholesterol) for 10 min at 37°C. Ca^{2+} mobilization was determined as described above.

Effect of Cholesterol Depletion on Localization of PIP_2—Studies involving A431 and other cells have indicated that as much as half of the cellular PIP_2 is present in low density, detergent-resistant domains enriched in caveolin (*25, 26, 42*). Therefore, we hypothesized that in RBL-2H3 cells also PIP_2 may be concentrated in cholesterol-rich DRMs, and that cholesterol depletion may delocalize PIP_2 and thereby inhibit IP_3 production, even though PLC-γ1 is activated following FcεRI aggregation. To determine the compartmentalization of PIP_2, RBL-2H3 cells were labeled with [^{3}H]-inositol, and cell lysates were fractionated on sucrose density gradients as described in Fig. 1. From each fraction, inositol phospholipids were then extracted and separated by TLC. As shown in Fig. 5A, we found the localization of PIP_2 in low density DRMs (Fractions 1–3) on RBL-2H3 cells. We also found that cholesterol depletion resulted in the loss of compartmentalization of PIP_2 (Fig. 5B). Furthermore, the MBCD-induced delocalization of PIP_2 was reversed after treatment with cholesterol-MBCD complexes (Fig. 5C).

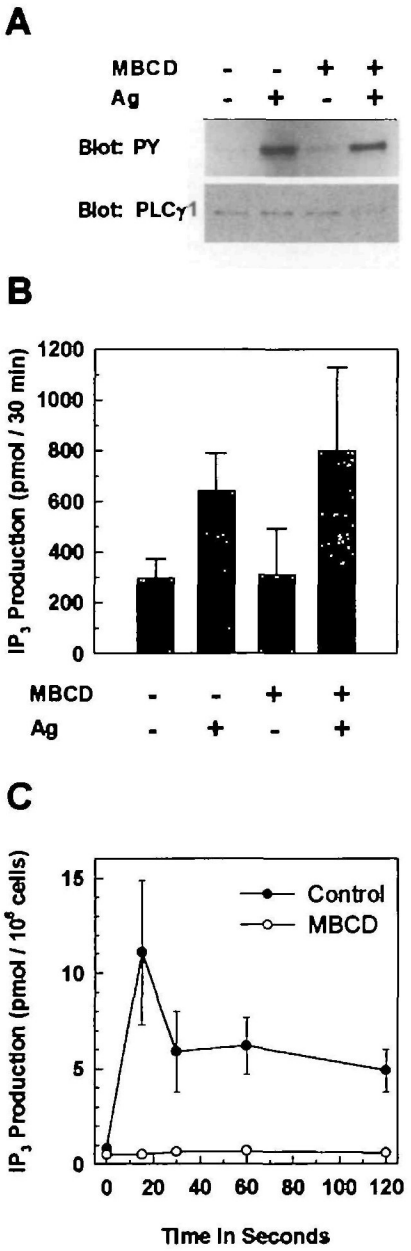

Fig. 4. **Effects of MBCD treatment on tyrosine phosphorylation of PLC-γ1, *in vitro* PLC activity, and *in vivo* IP_3 production following FcεRI aggregation.** (A) RBL-2H3 cells sensitized with anti-DNP IgE were untreated or treated with 25 mM MBCD. Cells were then unstimulated or stimulated with Ag, immunoprecipitated with anti–PLC-γ1, and subjected to Western blotting with anti-phosphotyrosine. The same blot was stripped and reprobed with anti–PLC-γ1. (B) PLC-γ1 was immunoprecipitated and incubated with 0.2 mM PIP_2. IP_3 generated in 30 min was quantitated by means of a radioreceptor binding assay. The results shown represent the mean values ± SD of three separate experiments. (C) RBL-2H3 cells were treated with or without 25 mM MBCD. At the indicated times after stimulation with 1 μg/ml Ag at 37°C, IP_3 was extracted using perchloric acid and quantitated as described above. Each point represents the mean ± SD of three independent experiments.

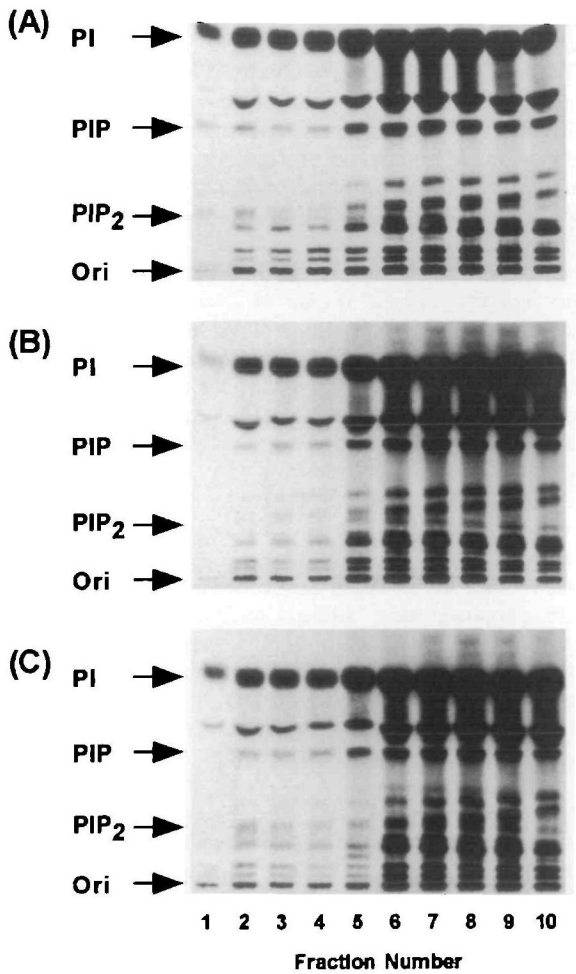

Fig. 5. **Effect of MBCD treatment on the partitioning of inositol phospholipids into DRMs.** RBL-2H3 cells were labeled for 48 h with [^{3}H]inositol. Cells were untreated (A) or treated with 12.5 mM MBCD. MBCD-treated cells were then incubated with buffer alone (B) or buffer containing MBCD-cholesterol complex (0.2 mM cholesterol) (C) for 10 min at 37°C. After centrifugation to isolate DRMs as described in Fig. 1, each fraction was extracted with chloroform:methanol:HCl to isolate inositol phospholipids. Phosphoinositides were separated by TLC and visualized by autoradiography. The bands corresponding to PI, PIP, and PIP_2 were identified by comigration with standards.

DISCUSSION

In T cells, dispersion of DRMs with various cholesterol-binding reagents including MBCD inhibits Ca^{2+} mobilization following TCR cross-linking (*24*). The association of TCR with the cytoskeleton is also decreased in cells treated in this manner (*23*). The TCR-mediated tyrosine phosphorylation of cellular proteins including the TCR ζ chain itself is also inhibited in these cells (*22–24*). Thus, DRMs have been proposed to function as platforms for signal transduction and cytoskeletal reorganization. In this study, we established an essential role for DRMs in FcεRI signaling by demonstrating that treatment of RBL-2H3 cells with MBCD results in potent inhibition of degranulation and Ca^{2+} mobilization in response to aggregation of FcεRI. However, the most striking finding in our studies is that aggregation-induced tyrosine phosphorylation of FcεRI is hardly affected by pretreatment of cells with MBCD, indicating that DRMs are dispensable for tyrosine phosphorylation of FcεRI upon aggregation.

The results of our study have important implications as to understanding of the molecular mechanism by which FcεRI is tyrosine phosphorylated upon aggregation. Many studies have demonstrated that FcεRI is preassociated with Lyn, and this constitutively associated kinase mediates the trans-phosphorylation of adjacent receptors upon aggregation (*5, 6, 8, 14, 43*). Experimental data obtained in several laboratories have shown that this interaction occurs *via* the unique region of Lyn with nonphosphorylated ITAM of the receptor β chain (*6, 13*). However, the structural basis of the FcεRI-Lyn interaction is not yet fully understood. For example, Lyn is able to phosphorylate aggregated FcεRI when the receptor is expressed without the β chain, and aggregation of chimeric receptors containing only the intracellular domain of the γ chain serves to initiate the signaling cascade leading to mast cell activation, but a much lower level than in the case of the $\alpha\beta\gamma_2$ complex (*4, 44, 45*). Based on the observation that aggregation of FcεRI results in its rapid association with DRMs, Field *et al.* (*16*) proposed an additional mechanism for the tyrosine phosphorylation of FcεRI upon aggregation. Their model shows that unaggregated FcεRI may weakly associate with certain components of DRMs, probably Lyn or a ganglioside such as GD_{1b}. The aggregation of receptors rapidly stabilizes the receptor-DRM associations, thereby facilitating the phosphorylation of the receptors by a heightened concentration of Lyn in DRMs. Consistent with their results, we also demonstrate the recruitment of aggregated FcεRI to DRMs (Fig. 1A). However, our findings that FcεRI is normally tyrosine phosphorylated in the absence of intact DRM structures and that unaggregated receptors maintain their association with Lyn kinase even in such conditions argue against a model in which DRMs would be the structural basis for the initial coupling of FcεRI and Lyn. We speculate that FcεRI is associated with Lyn through protein:protein interactions rather in a DRM-dependent manner, and this receptor-associated kinase phosphorylates aggregated receptors outside DRMs through a transphosphorylation mechanism. The phosphorylated receptors may then rapidly be translocated to DRMs, where additional Lyn, Syk, and other signaling molecules with SH2 domains are recruited and activated. Thus, we propose that intact DRMs are not important for the initial tyrosine phosphorylation of FcεRI following aggregation. However, our results do not exclude the possibility that MBCD-resistant DRMs, if present, are involved in the phosphorylation of FcεRI. In addition, a recent study has indicated that the cholesterol extracted by MBCD is not required for the organization of GPI-anchored proteins and sphingolipids in DRMs (*31*).

It should be noted that, while this work was being completed, Sheets *et al.* (*18*) reported the effects of depletion of cellular cholesterol on aggregation-induced tyrosine phosphorylation of FcεRI on RBL-2H3 cells. In contrast to our results, they found that cholesterol depletion with MBCD almost completely inhibits the stimulated tyrosine phosphorylation of FcεRI. Nonetheless they failed to observe any inhibition of degranulation upon FcεRI aggregation and rather they showed an enhancing effect of MBCD on degranulation in response to stimulation by the Ca^{2+} ionophore, A23187. Therefore, their results indicate that DRMs are indispensable for initial tyrosine phosphorylation of FcεRI but dispensable for mast cell activation. The reason for the observed difference is not clear. The differences between our results and those of Sheets *et al.* are likely to be technical in nature. For example, our conditions for treatment cells with MBCD are different from theirs. However, we still found that tyrosine phosphorylation of FcεRI was not affected and that Ag-induced degranulation was inhibited by MBCD even when we mimicked their conditions (data not shown). In support of our conclusions, experiments in which chimeric constructs were used to explore the interaction between Lyn and the receptor subunits also suggested that these associations can occur outside the DRMs (*46*). Recently, in Ramos B cells, cholesterol sequestration was found not to inhibit tyrosine kinase activation induced by B cell receptor stimulation (*47*).

In addition to the fact that aggregation-induced tyrosine phosphorylation of FcεRI is not affected by pretreatment of cells with MBCD, we demonstrated that PLCγ1 was tyrosine phosphorylated following FcεRI aggregation even in MBCD-treated cells. More importantly, an *in vitro* phospholipase assay revealed that PLC-γ1 can be inducibly activated without DRMs. Despite activation of PLCγ1, cholesterol depletion by MBCD inhibits FcεRI-stimulated IP_3 production. This is associated with the loss of compartmentalization of PIP_2, the primary PLC substrate in the plasma membrane, in DRM microdomains. This suggests that localization of PIP_2 to DRMs is necessary for efficient IP_3 generation leading to intracellular Ca^{2+} mobilization and degranulation, though the mechanism of this localization is unknown. Thus, the localization of both protein and lipid components to DRMs may be required for the proper function of signal transduction through FcεRI. Our finding is consistent with those with A431 and other cells indicating that a fraction of PIP_2 is present in low density, detergent-insoluble domains enriched in caveolin (*25, 26*). In addition, cholesterol depletion by MBCD inhibits EGF-stimulated PI turnover in A431 cells (*42*). However, it is not clear whether or not this inhibition is due to the loss of localization of PIP_2, because EGF is also localized to DRMs and MBCD treatment induces the delocalization of EGF receptor itself (*42*). In contrast, our study clearly demonstrates the absolute requirement of PIP_2 localization to DRMs for efficient IP_3 production following FcεRI aggregation.

In summary, our findings indicate that the primary role

for DRM in signaling through mast cell FcεRI may be to co-localize signaling proteins and lipids for an efficient Ca^{2+} response rather than to serve as a site of initial tyrosine phosphorylation of receptors. Future studies will be directed toward the molecular mechanisms for the aggregation-dependent association of FcεRI with DRMs. A recent study suggested that the transmembrane domains of FcεRI play the major role in this aggregation-induced association (*17*). In addition, further investigations will be required to answer the important question about the regulation of FcεRI-associated Lyn activity, probably by CD45 and Csk, within DRMs.

The authors are grateful to Drs. Yasuyuki Igarashi and Jin-ichi Inokuchi for the many helpful discussions and the PIP_2 analysis.

REFERENCES

1. Ravetch, J.V. and Kinet, J.P. (1991) Fc receptors. *Annu. Rev. Immunol.* **9**, 457–492
2. Kinet, J.P. (1999) The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* **17**, 931–972
3. Cambier, J.C. (1995) Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J. Immunol.* **155**, 3281–3285
4. Eiseman, E. and Bolen, J.B. (1992) Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* **355**, 78–80
5. Hutchcroft, J.E., Geahlen, R.L., Deanin, G.G., and Oliver, J.M. (1992) FcεRI-mediated tyrosine phosphorylation and activation of the 72-kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc. Natl. Acad. Sci. USA* **89**, 9107–9111
6. Jouvin, M.H., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A., and Kinet, J.P. (1994) Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem.* **269**, 5918–5925
7. Oliver, J.M., Burg, D.L., Wilson, B.S., McLaughlin, J.L., and Geahlen, R.L. (1994) Inhibition of mast cell FcεRI-mediated signaling and effector function by the Syk-selective inhibitor, piceatannol. *J. Biol. Chem.* **269**, 29697–29703
8. Yamashita, T., Mao, S.Y., and Metzger, H. (1994) Aggregation of the high-affinity IgE receptor and enhanced activity of $p53/56^{lyn}$ protein-tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **91**, 11251–11255
9. Benhamou, M., Ryba, N.J., Kihara, H., Nishikata, H., and Siraganian, R.P. (1993) Protein-tyrosine kinase $p72^{syk}$ in high affinity IgE receptor signaling. Identification as a component of pp72 and association with the receptor γ chain after receptor aggregation. *J. Biol. Chem.* **268**, 23318–23324
10. Deckert, M., Tartare-Deckert, S., Couture, C., Mustelin, T., and Altman, A. (1996) Functional and physical interactions of Syk family kinases with the Vav proto-oncogene product. *Immunity* **5**, 591–604
11. Jabril-Cuenod, B., Zhang, C., Scharenberg, A.M., Paolini, R., Numerof, R., Beaven, M.A., and Kinet, J.P. (1996) Syk-dependent phosphorylation of Shc. A potential link between FcεRI and the Ras/mitogen-activated protein kinase signaling pathway through SOS and Grb2. *J. Biol. Chem.* **271**, 16268–16272
12. Law, C.L., Chandran, K.A., Sidorenko, S.P., and Clark, E.A. (1996) Phospholipase C-γ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Mol. Cell. Biol.* **16**, 1305–1315
13. Vonakis, B.M., Chen, H., Haleem-Smith, H., and Metzger, H. (1997) The unique domain as the site on Lyn kinase for its constitutive association with the high affinity receptor for IgE. *J. Biol. Chem.* **272**, 24072–24080
14. Pribluda, V.S., Pribluda, C., and Metzger, H. (1994) Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation. *Proc. Natl. Acad. Sci. USA* **91**, 11246–11250
15. Field, K.A., Holowka, D., and Baird, B. (1995) FcεRI-mediated recruitment of $p53/56^{lyn}$ to detergent-resistant membrane domains accompanies cellular signaling. *Proc. Natl. Acad. Sci. USA* **92**, 9201–9205
16. Field, K.A., Holowka, D., and Baird, B. (1997) Compartmentalized activation of the high affinity immunoglobulin E receptor within membrane domains. *J. Biol. Chem.* **272**, 4276–4280
17. Field, K.A., Holowka, D., and Baird, B. (1999) Structural aspects of the association of FcεRI with detergent-resistant membranes. *J. Biol. Chem.* **274**, 1753–1758
18. Sheets, E.D., Holowka, D., and Baird, B. (1999) Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcεRI and their association with detergent-resistant membranes. *J. Cell Biol.* **145**, 877–887
19. Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569–572
20. Brown, D.A. and London, E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136
21. Stauffer, T.P. and Meyer, T. (1997) Compartmentalized IgE receptor-mediated signal transduction in living cells. *J. Cell Biol.* **139**, 1447–1454
22. Montixi, C. *et al.* (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* **17**, 5334–5348
23. Moran, M. and Miceli, M.C. (1998) Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. *Immunity* **9**, 787–796
24. Xavier, R., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998) Membrane compartmentation is required for efficient T cell activation. *Immunity* **8**, 723–732
25. Hope, H.R. and Pike, L.J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. *Mol. Biol. Cell* **7**, 843–851
26. Pike, L.J. and Casey, L. (1996) Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains. *J. Biol. Chem.* **271**, 26453–26456
27. Liu, Y., Casey, L., and Pike, L.J. (1998) Compartmentalization of phosphatidylinositol 4,5-bisphosphate in low-density membrane domains in the absence of caveolin. *Biochem. Biophys. Res. Commun.* **245**, 684–690
28. Klein, U., Gimpl, G., and Fahrenholz, F. (1995) Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry* **34**, 13784–13793
29. Yancey, P.G., Rodrigueza, W.V., Kilsdonk, E.P.C., Stoudt, G.W., Johnson, W.J., Phillips, M.C., and Rothblat, G.H. (1996) Cellular cholesterol efflux mediated by cyclodextrins. Demonstration of kinetic pools and mechanism of efflux. *J. Biol. Chem.* **271**, 16026–16034
30. Scheiffele, P., Roth, M.G., and Simons, K. (1997) Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. *EMBO J.* **16**, 5501–5508
31. Ilangumaran, S. and Hoessli, D.C. (1998) Effects of cholesterol depletion by cyclodextrin on the sphingolipid microdomains of the plasma membrane. *Biochem. J.* **335**, 433–440
32. Liu, F.T., Bohn, J.W., Ferry, E.L., Yamamoto, H., Molinaro, C.A., Sherman, L.A., Klinman, N.R., and Katz, D.H. (1980) Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation, and characterization. *J. Immunol.* **124**, 2728–2737
33. McConahey, P.J. and Dixon, F.J. (1966) A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**, 185–189
34. Murakami, K., Sato, S., Nagasawa, S., and Yamashita, T. (2000) Regulation of mast cell signaling through high-affinity IgE receptor by CD45 protein tyrosine phosphatase. *Int. Immunol.* **12**, 169–176
35. Yamashita, T., Kairiyama, L., Araki, M., and Nagasawa, S. (1998) Evidence for involvement of two isoforms of Syk protein-tyrosine kinase in signal transduction through the high affinity

IgE receptor on rat basophilic leukemia cells. *J. Biochem.* **123**, 1199–1207
36. Vorndran, C., Minta, A., and Poenie, M. (1995) New fluorescent calcium indicators designed for cytosolic retention or measuring calcium near membranes. *Biophys. J.* **69**, 2112–2124
37. Wahl, M.I., Daniel, T.O., and Carpenter, G. (1988) Antiphosphotyrosine recovery of phospholipase C activity after EGF treatment of A-431 cells. *Science* **241**, 968–970
38. Wahl, M.I., Jones, G.A., Nishibe, S., Rhee, S.G., and Carpenter, G. (1992) Growth factor stimulation of phospholipase C-γ1 activity. Comparative properties of control and activated enzymes. *J. Biol. Chem.* **267**, 10447–10456
39. Costello, P.S., Turner, M., Walters, A.E., Cunningham, C.N., Bauer, P.H., Downward, J., and Tybulewicz, V.L. (1996) Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. *Oncogene* **13**, 2595–2605
40. Zhang, J., Berenstein, E.H., Evans, R.L., and Siraganian, R.P. (1996) Transfection of Syk protein tyrosine kinase reconstitutes high affinity IgE receptor-mediated degranulation in a Syk-negative variant of rat basophilic leukemia RBL-2H3 cells. *J. Exp. Med.* **184**, 71–79
41. Barker, S.A., Caldwell, K.K., Pfeiffer, J.R., and Wilson, B.S. (1998) Wortmannin-sensitive phosphorylation, translocation, and activation of PLC-γ1, but not PLC-γ2, in antigen-stimulated RBL-2H3 mast cells. *Mol. Biol. Cell* **9**, 483–496
42. Pike, L.J. and Miller, J.M. (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J. Biol. Chem.* **273**, 22298–22304
43. Eiseman, E. and Bolen, J.B. (1992) Signal transduction by the cytoplasmic domains of FcεRI-γ and TCR-ζ in rat basophilic leukemia cells. *J. Biol. Chem.* **267**, 21027–21032
44. Alber, G., Miller, L., Jelsema, C.L., Varin-Blank, N., and Metzger, H. (1991) Structure-function relationships in the mast cell high affinity receptor for IgE. Role of the cytoplasmic domains and of the β subunit. *J. Biol. Chem.* **266**, 22613–22620
45. Lin, S., Cicala, C., Scharenberg, A.M., and Kinet, J.P. (1996) The FcεRIβ subunit functions as an amplifier of FcεRIγ-mediated cell activation signals. *Cell* **85**, 985–995
46. Vonakis, B.M., Haleem-Smith, H., Benjamin, P., and Metzger, H. (2001) Interaction between the unphosphorylated receptor with high affinity for IgE and Lyn kinase. *J. Biol. Chem.* **276**, 1041–1050
47. Petrie, R.J., Schnetkamp, P.P., Patel, K.D., Awasthi-Kalia, M., and Deans, J.P. (2000) 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. *J. Immunol.* **165**, 1220–1227