Biochemical and Biophysical Research Communications **287,** 77–82 (2001)
doi:10.1006/bbrc.2001.5536, available online at http://www.idealibrary.com on IDEAL®

Differential Effects of Filipin and Methyl-β-cyclodextrin on B Cell Receptor Signaling[1]

Manjula Awasthi-Kalia,* Paul P. M. Schnetkamp,† and Julie P. Deans*,[2]
**Department of Biochemistry and Molecular Biology and †Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada*

Received August 9, 2001

Methyl-β-cyclodextrin and filipin are cholesterol-binding reagents often used interchangeably to investigate functional requirements for lipid rafts in receptor-mediated signal transduction. Recently, contradictory results were reported by two groups using these reagents in different model systems to investigate the role of lipid rafts in BCR signaling. We confirm here that BCR-mediated calcium release is inhibited by filipin and enhanced by cyclodextrin. The inhibitory effect of filipin could not be attributed to raft disruption, however, because its ability to release raft-associated proteins into the detergent-soluble phase of cell lysates was less than that of cyclodextrin. In contrast, we found that filipin profoundly inhibited phosphorylation of the raft-associated adaptor protein Cbp/PAG, whereas the effect of cyclodextrin was minor. Thus, filipin and cyclodextrin modify cholesterol-rich microdomains through different mechanisms with different consequences on receptor signaling. In addition, the enhanced calcium release observed under conditions of maximum raft disruption suggests that rafts have a role in negatively regulating BCR signals.

Key Words: **B cell receptor; rafts; signal transduction; biochemistry; cholesterol; filipin; cyclodextrin; Cbp/PAG; calcium.**

Lipid rafts and caveolae, specialized membrane microdomains in which cholesterol and glycosphingolipids are the major components, are enriched in acylated signaling molecules and appear to compartmentalize receptor-mediated signals (1–4). Cholesterol is the key component in maintaining the integrity of membrane microdomains, and various studies have demonstrated the inhibitory effects of cholesterol-reducing agents on signaling. Two such reagents that have been extensively used are methyl-β-cyclodextrin (MβCD) and filipin.

MβDC is a membrane impermeable, small cyclic oligosaccharide with a hydrophobic core that selectively and rapidly extracts cholesterol from the plasma membrane (5, 6). Pretreatment of cells with MβCD prevents the recruitment of the high affinity IgE receptor (7) and the B cell antigen receptor (BCR) to lipid rafts (8), and causes loss of compartmentalization of the Src family kinases Lyn and Lck (7, 9), influenza hemagglutinin (10, 11), LAT and Gβ (9), caveolin, Gq, EGF receptor, and PtdIns 4,5-P2 (12). Some GPI-linked proteins are unclustered by MβCD (13) although others are not (7, 14, 15). Consistent with the idea that cholesterol-rich microdomains organize signaling platforms in the plasma membrane, MβCD inhibits antigen receptor-mediated intracellular calcium release in Jurkat T cells (9, 16), and hormone-stimulated PtdIns turnover (12). On the other hand, MβCD activates extracellular receptor kinase (ERK) in some cells (9, 17).

Filipin, a polyene antibiotic with antifungal properties, binds selectively to cholesterol, forming complexes in the plasma membrane that sequester cholesterol and induce structural disorder (18–20). Filipin disperses caveolar domains, unclusters GPI-linked and other receptors, induces significant loss of compartmentalization of PtdIns 4,5-P2, and inhibits PDGF receptor-mediated signaling events (21–24). Like MβCD, filipin inhibits TCR-mediated calcium release and activates ERK in T cells (9). Thus, despite using different mechanisms to sequester cholesterol in membranes, MβCD and filipin have similar effects in a variety of cell types.

Several groups have reported that the BCR, upon stimulation in mature B lymphocytes, translocates into

Abbreviations used: BCR, B cell receptor; ERK, extracellular signal-related kinase; Cbp/PAG, Csk binding protein/Phosphoprotein associated with glycosphingolipid-enriched microdomains; MAPK, mitogen-activated protein kinase.

[1] This work was supported by an operating grant from The Arthritis Society and by salary awards from the Alberta Heritage Foundation for Medical Research to J.P.D. (AHFMR Senior Scholar) and to P.P.M.S. (AHFMR Senior Scientist).

[2] To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, University of Calgary, Health Sciences Center, 3330 Hospital Drive NW, Calgary, Alberta, T2N 4N1, Canada. Fax: (403) 283-1267. E-mail: ideans@ucalgary.ca.

0006-291X/01 $35.00

lipid rafts and induces signaling events detectable in that compartment (8, 25, 26). Aman and Ravichandran showed that filipin inhibited BCR-induced calcium flux, suggesting that the integrity of lipid rafts is required for BCR signaling (25). However, work from this laboratory demonstrated that MβCD enhanced calcium release from intracellular stores after BCR stimulation (8). These apparently contradicatory observations prompted us to compare the effects of MβCD and filipin on lipid rafts and BCR signaling in Ramos B cells. This is the first report of differential effects of these reagents in the same model system.

MATERIALS AND METHODS

Cells, antibodies, and reagents. Ramos B cells were maintained by culture in RPMI 1640, 7.5% FBS. Anti-Cbp was a gift from Dr. Masato Okada (Osaka University, Osaka, Japan). All other antibodies used in the study were previously described (8). MβCD, filipin, and laurylmaltoside were purchased from Sigma.

Cell stimulation and sample preparation. Cells (1×10^8/sample) were either untreated or treated with 10 mM MβCD for 10′ or 2 μg/ml filipin for 10′; these conditions were selected after time- and dose-response studies established that higher doses of MβCD and filipin led to significant cell death as measured by trypan blue exclusion and propidium iodide staining. To induce translocation of CD20 to lipid rafts, the cells were treated with 2H7 mAb (0.5 μg/10^6 cells) at 37°C for 15 min. For BCR stimulation, cells were incubated with $F(ab')_2$ anti-IgM (1 μg/10^6 cells) for 5′ at 37°C. Cells were lysed in 1% Triton X-100. Sucrose density gradient centrifugation was performed as described (8). A total of either 1.5-ml fractions were collected from the top of the gradient. Fractions 3 and 4 correspond to lipid rafts and fractions 7 and 8 contain the soluble proteins.

For tyrosine phosphorylation and MAPK studies, 2×10^6 cells were given appropriate drug treatment prior to BCR stimulation and lysed in 0.5% Triton X-100. For Cbp/PAG localization analysis, fractions from sucrose density gradients were pooled in pairs and concentrated as follows: fractions 3 and 4 containing membrane rafts were centrifuged and the pellet dissolved in a 100-μl SDS sample buffer; fractions 1 and 2, 5 and 6, and 7 and 8 were TCA precipitated and also dissolved in a 100-μl SDS sample buffer; equal volumes were loaded for SDS–PAGE and immunoblotting. For Cbp/PAG immunoprecipitation the cells were lysed in 1% laurylmaltoside to solubilize lipid rafts. Protein separation and immunoblotting was performed as described (8). Silver staining was performed on pooled raft fractions using a silver stain kit purchased from Bio-Rad.

Calcium measurements. Cells were incubated for 20 min with 20 μM fluo-3AM (Molecular Probes, Eugene, OR), washed and treated with either 10 mM MβCD or 2 μg/ml filipin for 10′. The experimental procedure and instrumentation were previously described (8).

RESULTS

Opposite Effects of MβCD and Filipin on Intracellular Ca^{2+} Mobilization in B Cells

Possible explanations for the conflicting reports on the effects of cholesterol depletion on BCR-mediated intracellular calcium release include the use of different cell lines, i.e., human Ramos B cells vs murine A20 B cells, as well as the use of different cholesterol depleting reagents (8, 25). We show here (Fig. 1) that treatment of Ramos B cells with filipin inhibited the BCR-induced rise in cytosolic free calcium, whereas, as reported previously, MβCD enhanced calcium release. Neither reagent compromised the internal calcium stores of the cells (data not shown). These data rule out the possibility that the apparently contradictory results were due to the use of different cell lines, and indicate that the effects are due to differences in the actions of filipin and cyclodextrin.

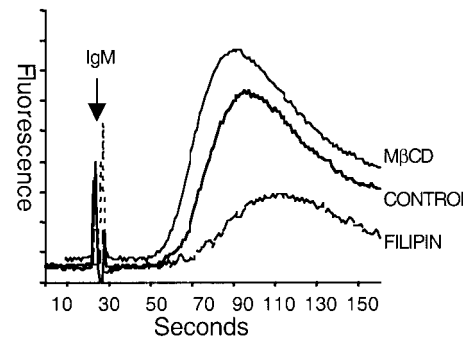

FIG. 1. Effects of MβCD and filipin on BCR-stimulated intracellular Ca^{2+} release. Basal calcium levels in fluo-3 loaded Ramos cells, either untreated (control) or pretreated with MβCD or filipin, were recorded for ~30 s before stimulation with $F(ab')_2$ anti-IgM.

Differential Effects of MβCD and Filipin on the Integrity of Lipid Rafts

In order to try and determine why filipin and cyclodextrin have different effects on BCR signaling, we first compared the ability of these reagents to disrupt lipid rafts. We selected two dually acylated proteins, the Src family kinase Lyn and the heterotrimeric G protein α subunit, $G\alpha_i$, as markers of lipid raft integrity. Lipid rafts can be isolated using sucrose density gradients on the basis of their insolubility in Triton X-100 and buoyant density. In untreated cells, both Lyn and $G\alpha_i$ were present predominantly in the low density lipid raft fraction (fraction 4, Fig. 2). Treatment with MβCD prevented the association of these proteins with the low density fraction, as seen by the appearance of these proteins in the soluble fractions 7 and 8. Treatment with filipin also caused these proteins to appear in the soluble cellular fractions, indicating that tight compartmentalization of these proteins in lipid rafts was lost. However, densitometry analysis estimated that approximately 50% of Lyn and $G\alpha_i$ remained in the low density fractions following treatment with filipin.

We also examined the effects of these drugs on two integral membrane proteins that inducibly associate with lipid rafts following stimulation, CD20 and the BCR. Treatment of Ramos B cells with 2H7 anti-CD20 mAb induced translocation of the majority of CD20 from the soluble cellular compartment to the low density insoluble fraction (Fig. 2), as previously described

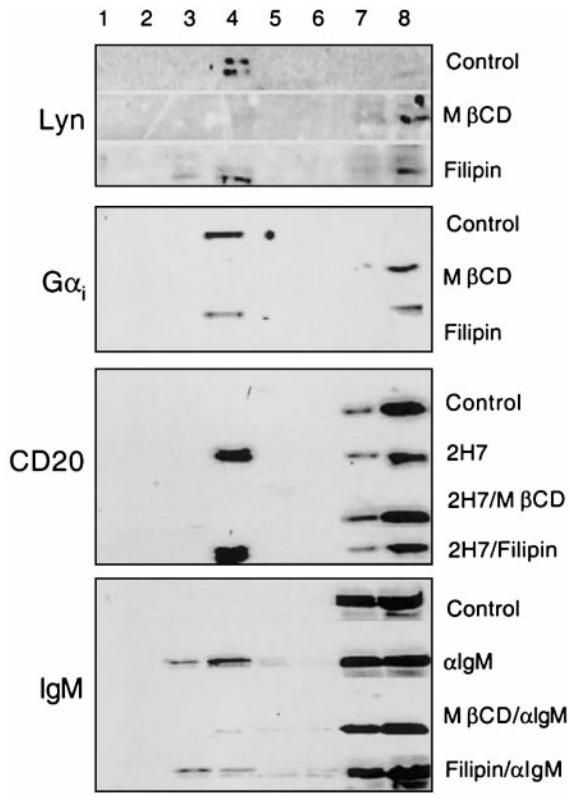

FIG. 2. Effects of MβCD and filipin on the integrity of lipid rafts. Ramos cells were either untreated (control) or treated with MβCD or filipin before lysis in Triton X-100. Lysates were subjected to sucrose density ultracentrifugation and fractions 1–8 were collected from the top of the gradients. Samples from each fraction were blotted for Lyn and Gαi, as indicated. To test effects on raft-associated CD20, cells were untreated (control) or treated with 2H7 mAb for 15′ followed by MβCD or filipin. To test for effects on translocation of the BCR to rafts, cells were pretreated with MβCD or filipin before addition of $F(ab')_2$ anti IgM for 5′.

(27). After mAb-induced CD20 translocation into rafts, cells were treated with MβCD causing complete loss of CD20 from the low density fraction and its reappearance in the soluble fraction (Fig. 2). In contrast, filipin had no effect on CD20-raft association (Fig. 2). Similar results were seen when the cells were treated first with MβCD or with filipin and then with 2H7 mAb (data not shown). Pretreatment with MβCD also prevented translocation of the mature form of the BCR (upper band) into the low density insoluble fraction (Fig. 2), as previously reported (8). Pretreatment with filipin, however, only partially inhibited BCR translocation into the low density fraction. Together, these data indicate that MβCD disrupts lipid rafts more effectively than does filipin. This conclusion is also supported by the protein profile of lipid raft fractions observed by silver staining. Treatment with MβCD led to significant loss of proteins compared to untreated and filipin treated lipid raft fractions (Fig. 4C).

Effects of MβCD and Filipin on Tyrosine Phosphorylation of Cellular Proteins and ERK Phosphorylation

Since the inhibitory effect of filipin on BCR-induced calcium release could not be explained by raft disruption, we next examined the ability of both reagents to modulate tyrosine kinase-dependent signaling events. Treatment of Ramos cells with either filipin or MβCD enhanced tyrosine phosphorylation of selected cellular proteins in the absence of BCR crosslinking (Figs. 3A and 3B). The heavily tyrosine phosphorylated bands at ~55 kDa may correspond to Lyn, since Lyn was released from lipid rafts following MβCD or filipin treatment (Figs. 2, 3A, and 3B). Cyclodextrin did not lead to MAPK (ERK) phosphorylation in B cells (Fig. 3A), as it does in T cells (9). Filipin, however, activated ERK as also observed in T cells (Fig. 3B). Pretreatment of Ramos cells with MβCD or with filipin did not significantly affect BCR-induced phosphorylation of ERK (Figs. 3C and 3D), indicating that BCR induced signaling events leading to ERK can occur independently of rafts.

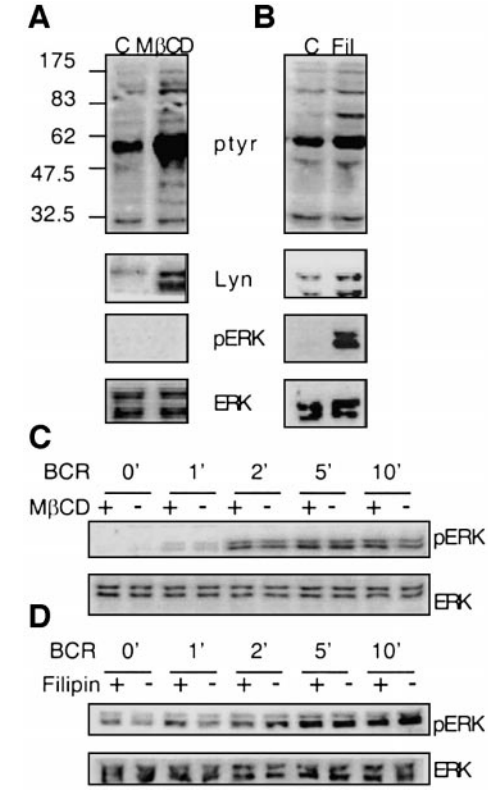

FIG. 3. Effects of MβCD and filipin on tyrosine phosphorylation of cellular proteins, solubility of lyn, and MAPK activation. Ramos cells were treated with (A) MβCD or (B) filipin and lysed in Triton X-100. Lysates were immunoblotted for phosphotyrosine, lyn, pERK, and ERK, as indicated. (C) Ramos cells, untreated or treated with MβCD or (D) filipin, were stimulated with $F(ab')_2$ anti IgM for the times indicated and lysed in Triton X-100. Lysates were immunoblotted for pERK and ERK.

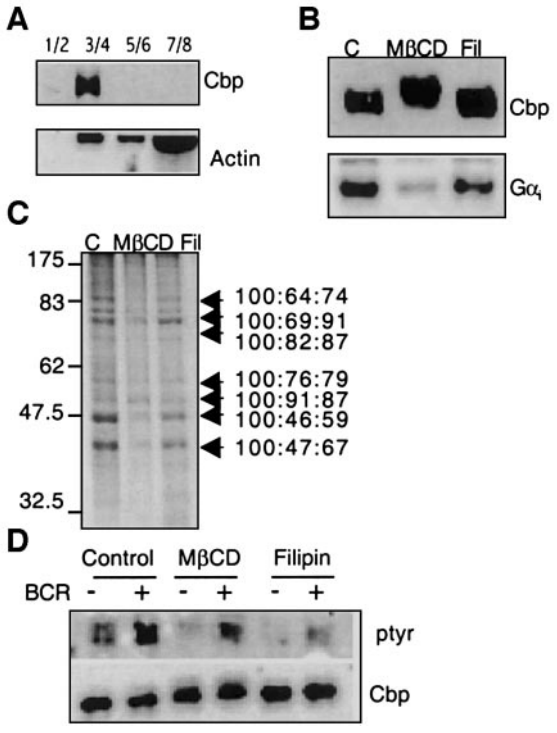

FIG. 4. Effects of MβCD and filipin on Csk-binding protein. (A) Untreated Ramos cells were lysed in 1% Triton X-100 and subjected to sucrose density gradient centrifugation. Fractions were pooled in pairs, as indicated, and concentrated as described under Materials and Methods. Equal cell equivalents were probed for Cbp/PAG and actin by immunoblotting. (B) Lipid raft fractions from untreated, MβCD- or filipin-treated Ramos cells were blotted for Cbp/PAG and Gαi. (C) Silver-stained proteins from samples in B. The intensity of individual bands was measured by densitometry (arrows). The relative intensity of each band in the MβCD- and filipin-treated samples was expressed as a percentage of the control. (D) Ramos cells, untreated (control) or treated with MβCD or filipin, were stimulated with anti-IgM for 1′ and lysed in laurylmaltoside. Cbp/PAG was immunoprecipitated from the lysates and the samples were immunoblotted for phosphotyrosine and Cbp/PAG.

Filipin Inhibits Phosphorylation of Cbp/PAG

Since inhibitory effects of filipin were not detected in the detergent soluble phase of cellular lysates, we next examined tyrosine kinase-dependent signaling events in lipid rafts. Cbp/PAG is a broadly expressed transmembrane adaptor protein localized to membrane rafts and is the major tyrosine phosphorylated protein in that compartment (28, 29). As expected, Cbp/PAG was found exclusively in the lipid raft fractions isolated from Ramos B cells (Fig. 4A) and was constitutively phosphorylated on tyrosine residues (Fig. 4D, lane 1). Surprisingly, the subcellular localization of Cbp/PAG was not affected by treatment with either MβCD or filipin, even though loss of Gα_i could be demonstrated in the same samples (Fig. 4B). Silver staining after SDS–PAGE showed loss of protein from raft-containing gradient fractions that was more profound with MβCD than with filipin pretreatment (Fig. 4C), although densitometry analysis of several bands on the gel indicated that there was unequal loss of individual proteins (Fig. 4C). To assess the phosphorylation status of Cbp/PAG, cells were lysed in 1% laurylmaltoside in order to solubilize raft proteins for immunoprecipitation. Phosphotyrosine on Cbp/PAG increased after 1 min of BCR stimulation (Fig. 4D), an unexpected finding since receptor stimulation in T cells inhibits phosphorylation of Cbp/PAG at the same time point (28, 29). Pretreatment with filipin profoundly inhibited both constitutive and BCR-induced tyrosine phosphorylation of Cbp/PAG (Fig. 4D), in contrast with its enhancing effects on tyrosine phosphorylation of proteins excluded from rafts (Fig. 3).

DISCUSSION

Differential effects of MβCD and filipin on specific receptor signaling events occurring under identical conditions has not been previously reported. In other studies where both reagents were used, similar effects were found; for example, both reagents inhibit TCR-induced calcium release (16). At the outset of this study, Ramos cells were exposed for varying times to the titrated reagents, and conditions were selected based on minimal effects on cell viability and maximum effects on protein association with rafts. The doses and exposures selected were similar to those used by others in various reports. However, it remains possible that a subtle technical difference in the experimental conditions may alter the cellular response to either reagent. A more provocative explanation is that a fundamental difference in receptor signaling and/or cholesterol effects in B cells underlies the differences observed. Activation of ERK by MβCD in T cells, but not in B cells, lends some support to the latter possibility.

Differential effects of MβCD and filipin on receptor-mediated calcium release in B cells can be attributed to differences in the mode of action of the reagents. By extracting cholesterol without binding to the membrane (5), MβCD effectively disperses a large proportion of most of the constituents of lipid rafts, and prevents BCR translocation into rafts. These data clearly demonstrate that in B cells, antigen receptor-mediated signaling events leading to calcium release are not compromised by raft disruption, suggesting that rafts have a negative regulatory role. Filipin does not extract cholesterol but rather inserts into the membrane and sequesters cholesterol *in situ* (18, 20, 30). Filipin caused partial dispersal of raft constituents, but this cannot account for its inhibitory effects on calcium mobilization because more complete raft disruption by MβCD enhanced calcium mobilization. Inhibition by filipin must be derived from effects other than raft disruption.

No inhibitory effects of filipin were detected by examining proteins in the detergent-soluble phase of cell lysates. However, profound inhibition of Cbp/PAG phosphorylation indicates that filipin exerts inhibitory effects in the raft environment. The cytoplasmic region of Cbp/PAG contains multiple tyrosine phosphorylation sites and is likely to recruit signaling effectors in addition to Csk (28, 29). It is conceivable, therefore, that filipin-mediated inhibition of calcium release may be linked to reduced Cbp/PAG phosphorylation. Interestingly, raft localization of Cbp/PAG was remarkably resistant to both MβCD and filipin. Cbp/PAG is the only transmembrane protein reported so far to have this property.

In conclusion, inhibition of BCR-mediated calcium release by filipin cannot be attributed to the inability of the BCR to gain access to lipid rafts, or to raft disruption, but may be a result of inhibitory effects on selected raft proteins occurring as consequence of filipin binding to membranes. Enhanced calcium release caused by MβCD attests to the ability of signaling effectors in the phospholipase C pathway to make efficient connections in the absence of organized rafts. Therefore, compartmentalization of the BCR and signaling effectors in lipid rafts likely serves a regulatory role. Filipin and MβCD are useful reagents for investigating the role of rafts in receptor signaling. However, they may exert different effects depending on the types of cells and receptor signaling systems being investigated, and must be used with caution.

ACKNOWLEDGMENTS

We thank Dr. Masato Okada (Osaka University, Osaka, Japan) for providing us with anti-Cbp, and Cathlin Mutch and Dr. Stephen Robbins for critical comments on the manuscript.

REFERENCES

1. Simons, K., and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387,** 589.
2. Brown, D. A., and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J. Biol. Chem.* **275,** 17221.
3. Fielding, C. J., and Fielding, P. E. (2000) Cholesterol and caveolae: Structural and functional relationships. *Biochim. Biophys. Acta* **1529,** 210.
4. Langlet, C., Bernard, A. M., Drevot, P., and He, H. T. (2000) Membrane rafts and signaling by the multichain immune recognition receptors. *Curr. Opin. Immunol.* **12,** 250.
5. Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K., and Pitha, J. (1989) Differential effects of alpha-, beta-, and gamma-cyclodextrins on human erythrocytes. *Eur. J. Biochem.* **186,** 17.
6. Kilsdonk, E. P., Yancey, P. G., Stoudt, G. W., Bangerter, F. W., Johnson, W. J., Phillips, M. C., and Rothblat, G. H. (1995) Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270,** 17250.
7. Sheets, E. D., Holowka, D., and Baird, B. (1999) Critical role for cholesterol in Lyn-mediated tyrosine phosphorylation of FcepsilonRI and their association with detergent-resistant membranes. *J. Cell Biol.* **145,** 877.
8. 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.
9. Kabouridis, P. S., Janzen, J., Magee, A. L., and Ley, S. C. (2000) Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes [in process citation]. *Eur. J. Immunol.* **30,** 954.
10. Keller, P., and Simons, K. (1998) Cholesterol is required for surface transport of influenza virus hemagglutinin. *J. Cell Biol.* **140,** 1357.
11. 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.
12. Pike, L. J., and Miller, J. M. (1998) Cholesterol depletion delocalizes phosphatidylinositol bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J. Biol. Chem.* **273,** 22298.
13. Friedrichson, T., and Kurzchalia, T. V. (1998) Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. *Nature* **394,** 802.
14. Hanada, K., Nishijima, M., Akamatsu, Y., and Pagano, R. E. (1995) Both sphingolipids and cholesterol participate in the detergent insolubility of alkaline phosphatase, a glycosylphosphatidylinositol-anchored protein, in mammalian membranes. *J. Biol. Chem.* **270,** 6254.
15. Ostermeyer, A. G., Beckrich, B. T., Ivarson, K. A., Grove, K. E., and Brown, D. A. (1999) Glycosphingolipids are not essential for formation of detergent-resistant membrane rafts in melanoma cells. methyl-beta-cyclodextrin does not affect cell surface transport of a GPI-anchored protein. *J. Biol. Chem.* **274,** 34459.
16. Xavier, B., Brennan, T., Li, Q., McCormack, C., and Seed, B. (1998) Membrane compartmentation is required for efficient T cell activation. *Immunity* **8,** 723.
17. Furuchi, T., and Anderson, R. G. (1998) Cholesterol depletion of caveolae causes hyperactivation of extracellular signal-related kinase (ERK). *J. Biol. Chem.* **273,** 21099.
18. McGookey, D. J., Fagerberg, K., and Anderson, R. G. (1983) Filipin-cholesterol complexes form in uncoated vesicle membrane derived from coated vesicles during receptor-mediated endocytosis of low density lipoprotein. *J. Cell Biol.* **96,** 1273.
19. Bolard, J. (1986) How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim. Biophys. Acta* **864,** 257.
20. Robinson, J. M., and Karnovsky, M. J. (1980) Evaluation of the polyene antibiotic filipin as a cytochemical probe for membrane cholesterol. *J. Histochem. Cytochem.* **28,** 161.
21. Hope, H. R., and Pike, L. J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. *Mol. Biol. Cell* **7,** 843.
22. Liu, J., Oh, P., Horner, T., Rogers, R. A., and Schnitzer, J. E. (1997) Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphospatidylinositol-anchored protein microdomains. *J. Biol. Chem.* **272,** 7211.
23. Rothberg, K. G., Ying, Y. S., Kamen, B. A., and Anderson, R. G. (1990) Cholesterol controls are clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell Biol.* **111,** 2931.
24. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994) Filipin-sensitive caveolae-mediated transport in endothelium: Reduced

transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **127,** 1217.

25. Aman, M. J., and Ravichandran, K. S. (2000) A requirement for lipid rafts in B cell receptor induced Ca(2+) flux. *Curr. Biol.* **10,** 393.
26. Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999) A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med.* **190,** 1549.
27. Deans, J. P., Robbins, S. R., Polyak, M. J., and Savage, J. A. (1998) Rapid redistribution of CD20 to a low-density detergent-insoluble membrane compartment. *J. Biol. Chem.* **273,** 344.
28. Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korinek, V., Angelisova, P., Scherer, J., Shevchenko, A., Hilgert, I., Cerny, J., Drbal, K., Kuramitsu, Y., Kornacker, B., Horejsi, V., and Schraven, B. (2000) Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. *J. Exp. Med.* **191,** 1591.
29. Kawabuchi, M., Satommi, Y., Takao, T., Shimonishi, Y., Nada, S., Nagai, K., Tarakhovsky, A., and Okada, M. (2000) Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. *Nature* **404,** 999.
30. Elias, P. M., Goerke, J., Friend, D. S., and Brown, B. E. (1978) Freeze-fracture identification of sterol-digitonin complexes in cell and liposome membranes. *J. Cell Biol.* **78,** 577.