Critical Role for Cholesterol in Lyn-mediated Tyrosine Phosphorylation of FcεRI and Their Association with Detergent-resistant Membranes

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Abstract. Tyrosine phosphorylation of the high affinity immunoglobulin (Ig)E receptor (FcεRI) by the Src family kinase Lyn is the first known biochemical step that occurs during activation of mast cells and basophils after cross-linking of FcεRI by antigen. The hypothesis that specialized regions in the plasma membrane, enriched in sphingolipids and cholesterol, facilitate the coupling of Lyn and FcεRI was tested by investigating functional and structural effects of cholesterol depletion on Lyn/FcεRI interactions. We find that cholesterol depletion with methyl-β-cyclodextrin substantially reduces stimulated tyrosine phosphorylation of FcεRI and other proteins while enhancing more downstream events that lead to stimulated exocytosis. In parallel to its inhibition of tyrosine phosphorylation, cholesterol depletion disrupts the interactions of aggregated FcεRI and Lyn on intact cells and also disrupts those interactions with detergent-resistant membranes that are isolated by sucrose gradient ultracentrifugation of lysed cells. Importantly, cholesterol repletion restores receptor phosphorylation together with the structural interactions. These results provide strong evidence that membrane structure, maintained by cholesterol, plays a critical role in the initiation of FcεRI signaling.

Key words: methyl-β-cyclodextrin • immunoglobulin E receptor • plasma membrane structure • lipid domains • signal transduction

The first known biochemical event in antigen stimulation of mast cells and basophils is tyrosine phosphorylation of the β and γ subunits of the high affinity IgE receptor (FcεRI) by the Src family kinase Lyn; however, the mechanism by which these proteins interact is not fully understood. A common view is that this receptor-kinase coupling occurs strictly via protein–protein interactions. For example, in the transphosphorylation model, antigen-induced cross-linking causes Lyn, which is bound weakly to one receptor, to phosphorylate immune tyrosine activation motifs on a juxtaposed receptor, thereby initiating signal transduction (Jouvin et al., 1994; Pribluda et al., 1994). We recently presented a different view that the plasma membrane structure plays an integral role in facilitating coupling between Lyn and FcεRI. In particular, we proposed that FcεRI tyrosine phosphorylation occurs within specialized regions in the plasma membrane, enriched in sphingolipids and cholesterol (Field et al., 1997; Sheets et al., 1999). Membrane structures of this composition have been isolated from many cell types and primarily characterized on the basis of their resistance to solubilization by nonionic detergents such as Triton X-100 (TX-100) and their consequent buoyancy in sucrose density gradients. These isolated detergent-resistant membranes (DRMs) (also referred to in the literature as DIGs [detergent insoluble glycolipid domains], GEMs [glycolipid-enriched membranes], and membrane rafts) are enriched in cholesterol, sphingomyelin, glycosphingolipids, and saturated glycerophospholipids, as well as dually acylated Src family kinases (e.g., Lyn, Fyn, Ys) and glycosylphosphatidylinositol (GPI)-anchored proteins (for review see Brown and London, 1998a; Simons and Ikonen, 1997). They are postulated to represent plasma membrane domains that may function as centers for signal transduction and membrane trafficking, although their nature on the cell surface is controversial (Brown and London, 1998a; E didin, 1997).

In initial studies we found that cross-linking of FcεRI increases the percentage of cellular Lyn recovered in DRMs from TX-100-lysed RBL-2H3 mast cells, suggesting that FcεRI aggregation causes an alteration of DRMs that may...
be involved in FcεRI-mediated signaling (Field et al., 1995). With sufficiently low concentrations of T X-100 to lyse the cells, aggregated (but not monomeric) FcεRI also associate with DRM vesicles, and only this population of receptors is phosphorylated upon cross-linking (Field et al., 1997). Furthermore, fluorescence microscopy on intact cells revealed that cross-linking of FcεRI induces co-redistribution with DRM components, including a GD\textsubscript{1b} ganglioside (Pieri et al., 1996), as well as Ly and the GPI-anchored protein Thy-1 (Holowka, D., E.D. Sheets, and B. Baid, manuscript in preparation), and with saturated phospholipid analogues (Thomas et al., 1994). Together, these results are consistent with the view that specialized membrane domains function in cells to facilitate coupling between aggregated FcεRI and Ly.

The detergent resistance of these membrane structures has been hypothesized to depend upon their lipid phase (Brown and London, 1998b). Cholesterol, which profoundly affects phase behavior of lipids, was found to be a major lipid component of isolated DRM vesicles derived from M DCK cells (Brown and Rose, 1992). In subsequent studies, Brown and colleagues found that model membranes with compositions similar to DRM vesicles are not solubilized by T X-100, and this detergent resistance was observed to correlate with cholesterol concentrations that induce formation of the liquid-ordered (L\textsubscript{o}) phase (Schroeder et al., 1994; Ahmed et al., 1997; Schroeder et al., 1998). This phase results from cholesterol having a gel phase–like ordering effect on the saturated and near-saturated acyl chains of glycerophospholipids and sphingolipids; yet the lipids retain a high degree of lateral and rotational mobility, similar to lipids in the fluid liquid crystalline phase (Brown and London, 1998a,b). The relevance of this structure for biological membranes is supported by recent electron spin resonance measurements that showed parameters characteristic of the L\textsubscript{o} phase for DRM vesicles isolated from R BL cells (Ge et al., 1999). Overall, these results support the hypothesis that ordered lipid domains coalesce on the plasma membrane after FcεRI aggregation serve to co-localize FcεRI and Ly and thereby initiate receptor phosphorylation and signaling.

As reported here, we tested this hypothesis by using methyl-\(\beta\)-cyclohextrin (M\textbeta CD) to selectively deplete cholesterol from RBL-2H3 cells, and we investigated the functional and structural effects of the M\textbeta CD treatment on FcεRI/Ly interactions. This reagent has been used recently for efficient removal of cholesterol from a variety of cell types (Klsdonk et al., 1995; Yancey et al., 1996; Christian et al., 1997; Gimpl et al., 1997; Scheiffele et al., 1997; Friedrichson and Kurzchalia, 1998; Keller and Simons, 1998; Varma and Mayor, 1998). We find that cholesterol depletion substantially reduces stimulated tyrosine phosphorylation of FcεRI and other substrates in RBL cells. Furthermore, we find that cholesterol depletion selectively disrupts the structural interactions between aggregated FcεRI and Ly both for DRM vesicles from lysed cells that are isolated on sucrose gradients and for intact cells as assessed by confocal fluorescence microscopy. When cholesterol levels are repleted, these functional effects and molecular associations are restored. These results provide strong evidence that cholesterol is required for effective functional coupling between aggregated FcεRI and Ly, and they are consistent with an important structural role for liquid-ordered membrane domains in this coupling.

### Materials and Methods

#### Cholesterol Depletion and Repletion

RBL-2H3 cells were maintained and harvested as previously described (Pieri et al., 1996). Mouse monoclonal IgE specific for 2,4-dinitrophenyl (DNP) (Liu et al., 1980) was purified as previously described (Subramanian et al., 1996b; biotinylated and iodinated (Field et al., 1995) or FITC-labeled (Pieri et al., 1996) IgE was used to sensitize cells in some experiments. Mouse monoclonal anti-1,5-dansyl IgE was affinity purified as previously described (Weetall et al., 1993). Other RBL cell membrane components were labeled with A4A mAb (a gift from Dr. Reuben Siraganian, National Institutes of Health, Bethesda, MD), specific for the α-galactosyl GD\textsubscript{1b} ganglioside derivative; OX-7 (PharMingen), specific for the GPI-anchored protein Thy-1; and anti-Lyn (Upstate Biotechnology, Inc., and Santa Cruz Biotechnology) as previously described (Field et al., 1995; Pierini et al., 1996). Transferrin receptors (TIR; CD 71) were labeled with a monoclonal antibody from PharMingen, followed by Cy3-goat anti-mouse γ chain (Southern Biotechnology Associates).

To remove cholesterol, suspended cells (2-4×10\(^6\) cells/ml) were incubated for 1 h at 37°C in the presence or absence of 10 mM M \textbeta CD (Sigma Chemical Co.) in BSA-containing buffered saline solution (BSA/BSS; 20 mM Hepes, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5.6 mM glucose, and 1 mg/ml BSA), then washed with BSA/BSS before stimulation. For some experiments, cholesterol (A vanti Polar Lipids) was added back to cholesterol-depleted cells (2×10\(^6\) cells/ml) in BSA/BSS by incubation for 2 h at 37°C with indicated concentrations of M\textbeta CD (cholesterol 8:1, mol/mol) complexes. These complexes were prepared similarly to a previously described procedure (Racchi et al., 1997). In brief, cholesterol in a chloroform solution was dried under nitrogen in a glass culture tube precleaned with ethanolic KOH. A n appropriate volume of sterile-filtered 300 mM M \textbeta CD in BSA/BSS was added to the tube, and the resulting suspension was vortexed and bath sonicated until the suspension clarified. The complex was then incubated in a rocking water bath overnight at 37°C to maximize formation of soluble complexes.

#### In Vivo Tyrosine Phosphorylation Assays

Suspended RBL-2H3 cells that had been sensitized with anti-DNP IgE (Chang et al., 1995) and cholesterol depleted/repleted or not were stimulated at a density of 10\(^6\) cells/ml with multivalent DNP-BSA (1 mg/ml; X u et al., 1996) at 37°C for indicated times, lyzed by addition of 5× SDS sample buffer (50% glycerol, 0.25 M Tris, pH 6.8, 5% SDS, 0.5% bromophenol blue) and boiled for 5 min and centrifuged for 5 min at 13,000 g. Equal numbers of cell equivalents of lysates (typically, 5×10\(^6\) cells equivalents) were electrophoresed on 12% SDS polyacrylamide gels, transferred to Immobilon-P membranes (Millipore Corp.), and probed with horseradish peroxidase-conjugated antiporphophorytyrosine (4G10-HRP; Upstate Biotechnology, Inc.). Enhanced chemiluminescence (Pierce) was used for detection. Phosphorylation as a function of stimulation time was quantified after scanning blots and analyzing with U n-Scan-It (Silk Scientific) and Igor Pro (Wavemetrics).
showed no evidence for internalization of FITC-IgE after MβCD treatment.

To examine the relationship between the density of anti-DNP IgE on the cells and antigen-stimulated tyrosine phosphorylation, FcεRI were saturated with mixtures of anti-DNP IgE and antidual IgE in percentage mixtures of 30:70, 50:50, and 100:0. Washed cells were stimulated with 1 µg/ml DNP-BSA at 37°C for various times and tyrosine phosphorylation was analyzed. Competition binding between each of these unlabeled antibodies and FITC-labeled anti-DNP IgE was carried out under identical conditions. With steady-state fluorimetry to quantify the amount of cell-bound FITC-IgE, we confirmed that the percentages of anti-DNP IgE and antidual IgE bound to FcεRI in the tyrosine phosphorylation experiments were identical to the percentages added with an uncertainty of ± 8%. Furthermore, fluorimetry experiments with mixtures of FITC anti-DNP IgE and unlabeled antidual IgE showed that DNP-BSA binding and cross-linking induced internalization of FITC-IgE-FcεRI proportionally to the amount of FITC-IgE bound in the range of 30–100% occupancy by this IgE (data not shown; Xu et al., 1998a,b).

Sucrose Gradients
Cholesterol-depleted/repleted or untreated cells were fractionated on sucrose step gradients as previously described (Field et al., 1997), except that the concentration of TX-100 during lysis was 0.04% instead of 0.05%. A liquids (200 µl) were removed from the top of the gradient, and γ-radiation of biotinylated 125I-IgE was counted. The gradient fractions were then pooled as indicated, boiled with SDS sample buffer, and boiled as described above, except that the primary antibody was rabbit anti-Lyn antibody (Upstate Biotechnology, Inc.) and the secondary antibody was HRP-conjugated donkey anti-rabbit Ig (Amersham Pharmacia Biotech). To determine the location of CD 8β in the gradients, cells labeled with 125I- A 4 and biotinylated IgE were analyzed as previously described (Field et al., 1995). In some experiments, the pooled gradient fractions were electrophoresed on 12% nonreduced SDS-acrylamide gels and subsequently transblotted to nitrocellulose. The blots were incubated with rabbit anti-rabbit IgG (HRP) (Jackson ImmunoResearch Lab, West Grove, PA), and bound HRP was visualized using a chemiluminescent detection kit (Pierce, Rockford, IL). Reactivity of the bands was quantified with a Molecular Imager Chemidoc System (Bio-Rad, Hercules, CA). In experiments designed to test the hypothesis that the degree of co-redistribution of Lyn or TfR with antigen–cross-linked IgE showed that DNP-BSA binding and cross-linking induced internalization of FITC-IgE-FcεRI proportionally to the amount of FITC-IgE bound in the range of 30–100% occupancy by this IgE (data not shown; Xu et al., 1998a,b).

Immunodepletion of FcεRI
An anti-DNP IgE-sensitized cells were stimulated for the indicated times with DNP-BSA (1 µg/ml) and lysed on ice with TX-100 lysis buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM NaVO₄, 30 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 0.02 U/ml aprotinin, 0.01% NaN₃, 1 mM 4-[(2-aminoethyl)-benzenesulfonyl] fluoride, and 0.2% TX-100) followed by addition of 10 µM DNP-aminoacaproyl-L-tyrosine, as previously described (Harris et al., 1997). A fraction centrifugation at 13,000 g for 5 min to remove insoluble material, lysates were incubated with or without 20 µg/ml rabbit anti-IgE (M enon et al., 1984). A fter incubation with protein A–agarose beads (Pierce), samples were centrifuged to pellet the beads and supernatants were removed, boiled in SDS sample buffer, electrophoresed, and blotted with 4G10-HRP as described above. Rabbit anti-IgE–IgG–FcεRI complexes are bound to the protein A–agarose, which result in selective depletion of the FcεRI β and γ bands.

Degranulation Assays
The degranulation response that occurs after stimulation with the antigen DNP-BSA or the calcium ionophore A 23187 (Calbiochem-Novabiochem) for 1 h at 37°C was carried out as described previously (Harris et al., 1997), except that cells were treated with or without MβCD immediately before stimulation.

Confocal Immunofluorescence Microscopy
Cells sensitized with FITC-IgE were treated with or without MβCD, then washed and incubated with cytochalasin D (1 µg/ml) for 5 min while temperature to prevent antigen-stimulated IgE–FcεRI internalization and to sustain Lyn co-redistribution with patch IgE–FcεRI at the cell surface. The cells were then stimulated with 1.7 µg/ml DNP-BSA at room temperature for 20 min and subsequently fixed with cold methanol for Lyn labeling or with formaldehyde for Gd 8 or TRβ labeling as previously described (Pierini et al., 1996). Confocal fluorescence microscopy was performed as previously described (Pierini et al., 1996). Cross correlation analysis of the co-redistribution of Lyn or TRβ with antigen–cross-linked FITC-IgE–FcεRI were carried out on equatorial images of individual cells using a computational procedure similar to that previously described (Stauffer and M eyer, 1997). Peak values can be calculated from this analysis.
Figure 1. Effects of cholesterol depletion on antigen-stimulated tyrosine phosphorylation. (a) Tyrosine phosphorylation of whole cell lysates. IgE-sensitized RBL-2H3 cells treated with (+) or without (−) 10 mM MβCD for 1 h at 37°C were washed and stimulated with 1 μg/ml DNP-BSA at 37°C for the indicated times, then lysed with SDS sample buffer. Equal numbers of cell equivalents (8,000/lane) were Western blot analyzed and probed with antiphosphotyrosine. (b) Quantitation of FceRI β tyrosine phosphorylation in the MβCD-treated cells was confirmed by specific immunodepletion of these bands with anti-IgE (Fig. 1 c).

To further characterize the cells under conditions of MβCD treatment, we determined that untreated cells contain 6.7 ± 0.7 nmol free cholesterol/10^6 cells (n = 5), and, after incubation with 10 mM MβCD at 37°C for 1 h, the amount of free cholesterol was determined to be 2.7 ± 1.0 nmol/10^6 cells (n = 5). Thus, the fraction of free cholesterol remaining after treatment is 0.40 ± 0.15 as compared with untreated cells, consistent with previously described levels of cholesterol depletion for a variety of cell types under similar conditions (Kilsdonk et al., 1995; Yancey et al., 1996; Christian et al., 1997; Gimpl et al., 1997; Scheiffele et al., 1997; Friedrichson and Kurzchalia, 1998; Keller and Simmons, 1998; Varma and Mayor, 1998). TLC analysis of cellular lipid extracts showed that there was no detectable difference in phospholipid composition before and after MβCD treatment, and this is also consistent with previous reports from other laboratories for other cell types (Kilsdonk et al., 1995; Yancey et al., 1996; Christian et al., 1997; Gimpl et al., 1997). In particular, we found that the amounts of phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin present in the total lipid extracts from MβCD-treated cells were not obviously different from untreated cell lipids (data not shown). The amounts of two other unidentified lipid species (one polar lipid species and one neutral lipid species) observed with TLC also were unaffected by MβCD treatment.

To further investigate the basis for the dramatic reduction in tyrosine phosphorylation of FceRI β, we measured the amount of FceRI β before and after MβCD treatment using 125I-IgE. Under our optimal conditions for inhibition of tyrosine phosphorylation (10 mM MβCD for 1 h at 37°C), we observed a 70 ± 6% (n = 6) loss of receptor-bound IgE from the cells that could be recovered in the supernatants of the cell washes after MβCD treatment. Similarly, 64 ± 7% (n = 2) of the ganglioside GD1b was lost from the cells due to MβCD treatment as detected using 125I-A A4 mAb. The loss of FceRI β is probably due to vesicle shedding caused by the MβCD treatment because a substantial fraction of 125I-IgE in the post-wash supernatant after MβCD treatment can be pelleted by high speed centrifugation as membrane vesicles that are detectable by phase contrast microscopy (data not shown).

In steady-state fluorescence measurements of FITC-IgE bound to MβCD-treated cells, 65 ± 10% (n = 3) IgE–FceRI loss was observed. Qualitatively consistent results were visualized with FITC-IgE and Cy3-A A4 in fluorescence microscopy of labeled cells, and a similar reduction in the cell surface expression of the GPI-anchored protein Thy-1 was also observed (data not shown). Despite these substantial reductions in FceRI β and the outer leaflet markers for DRM's in these cells after MβCD treatment, the plasma membrane expression of Lyn was only modestly reduced as assessed from fluorescence microscopy and Western blot analysis of sucrose gradient fractions (see below). Silver-stained polyacrylamide gels of RBL cell lysates showed no significant alterations in the amounts or composition of proteins detected in this manner (data not shown).

These results suggest that the inhibition of antigen-stimulated tyrosine phosphorylation is due in part to a reduction in the amount of FceRI β available for phosphorylation in the MβCD-treated cells. To assess the magnitude of this effect, we determined the relationship between FceRI β tyrosine phosphorylation and the effective concentration of...
this receptor in the plasma membrane by comparing the amount of FcεRI β tyrosine phosphorylation for cells in which 30% of the receptors were occupied by anti-DNP IgE, and 70% were occupied by anti-1,5-dansyl IgE, which does not bind DNP ligands (Weetall et al., 1993; see Material and Methods). Using the same conditions for stimulation and analysis as was used for the MβCD-treated cells, we determined that at the time point for maximal stimulation DNP-BSA caused 66% (n = 2) less tyrosine phosphorylation of FcεRI β for the cells occupied with 30% anti-DNP IgE compared with those occupied with 100% anti-DNP IgE. Likewise, when FcεRI on cells were occupied by 50% anti-DNP IgE, DNP-BSA caused 41% (n = 2) less tyrosine phosphorylation of FcεRI β than those occupied with 100% anti-DNP IgE. Assuming that this approximately proportional relationship between receptor number and β phosphorylation is valid for the MβCD-treated cells over this range of anti-DNP IgE densities, then the expected reduction in tyrosine phosphorylation of FcεRI β due to 70% loss of FcεRI should be ~70%. Thus, the actual reduction in this value (95 ± 4%) represents an 83% inhibition of the stimulated FcεRI β tyrosine phosphorylation expected for the amount of cross-linked receptors present.

Because of the nearly complete inhibition of stimulated FcεRI tyrosine phosphorylation caused by MβCD-mediated cholesterol depletion, we examined the effects of this treatment on cellular degranulation as measured by release of β-hexosaminidase. Fig. 2 summarizes the results from three separate experiments and shows that cholesterol depletion does not significantly inhibit degranulation stimulated by an optimal dose of antigen. Furthermore, cholesterol depletion actually enhances the amount of degranulation observed in response to stimulation by the Ca2+ ionophore, A23187, without altering the amount of β-hexosaminidase released in unstimulated cells. These results indicate that reduction in cellular cholesterol enhances one or more of the downstream events that follow Ca2+ elevation and lead to degranulation. They also demonstrate that cholesterol depletion under these conditions is not cytotoxic. As seen in Fig. 1, tyrosine phosphorylation of FcεRI is usually inhibited more strongly than stimulated phosphorylation of some other substrates (e.g., the stimulated bands in the range of 70–100 kD) that are known to be dependent on activation of the tyrosine kinase Syk (Zhang et al., 1996). These results indicate that relatively small amounts of stimulated tyrosine phosphorylation can result in substantial degranulation responses in the MβCD-treated cells. Consistent with this, stimulation of maximal degranulation requires effective cross-linking of only ~10% of the IgE receptors on untreated RBL-2H3 cells (Fewtrell, 1985).

**Cholesterol Depletion Disrupts the Interactions of Cross-linked IgE–FcεRI with DRM Vesicles**

To determine the importance of cholesterol in DRM interactions, we examined the distributions of IgE–FcεRI and Lyn across sucrose gradients of cholesterol-depleted or untreated RBL cells. As previously demonstrated for untreated cells (Field et al., 1997), monomeric IgE–FcεRI (Fig. 3 a, □) is found predominantly in the 40% sucrose region of the gradient where cytoplasmic and detergent-solubilized membrane proteins are characteristically observed, and a large percentage of cross-linked IgE–FcεRI (Fig. 3 a, ○) is located in the low density region of the gradient where DRM vesicles are found (Field et al., 1997; Scheiffele et al., 1997; Wolf et al., 1998). A flter MβCD treatment, monomeric IgE–FcεRI (Fig. 3 a, ■) is located in the 40% sucrose region, similar to those in the untreated cells. However, cross-linked IgE–FcεRI (Fig. 3 a, □) no longer float to the DRM region, but rather appear in the 50–60% sucrose region where aggregates of IgE–FcεRI characteristically locate in the absence of interactions with DRM vesicles (Field et al., 1997). These results are representative of four experiments, and they show that ~60% reduction in cholesterol almost completely prevents the association of cross-linked IgE–FcεRI with DRM vesicles.

A as shown in Fig. 3 b (top), the distribution of Lyn in the gradient fractions of stimulated (+ sa v) and unstimulated (– sa v) cells that were not treated with MβCD is qualitatively similar to that observed by Field et al. (1995), who used higher concentrations of TX-100. In the present experiments, it is notable that the p56 isoform of Lyn is selectively enriched in the low density, DRM region of the gradient (fractions 4–9), whereas the p53 isoform is located predominately in the 40% sucrose region (fractions 10–18). A flter cholesterol depletion, Lyn no longer localizes in the low density region of the gradient for both stimulated and unstimulated cells, whereas the distribution of p53 Lyn in the 40% sucrose region remains essentially unchanged from the untreated cells (Fig. 3 b, middle). In the cholesterol-depleted cells, it appears that p56 Lyn is relatively enriched in the gradient pellet (bottom fraction), both for the stimulated and the unstimulated cells. Overall, the total amount of Lyn detected in the gradients is only moderately reduced in the MβCD-treated cells compared with untreated control cells.

The loss of both aggregated FcεRI and Lyn from the low density region of the gradient raises the question of whether DRM vesicles are disrupted entirely in the cholesterol-depleted cells. To address this question, we investigated the distribution of other DRM markers in sucrose gradients after lysis of cholesterol-depleted cells. As shown in Fig. 3 c, AA4-labeled GD1b from cholesterol-depleted cells is found almost completely in the low density region.
of the gradients, indicating that DRMs still exist in some form after ~60% cholesterol depletion. The shift in GDb distribution to a slightly higher density after cholesterol depletion suggests an increase in the protein/lipid ratio, consistent with the substantial loss of cholesterol, a major lipid component of the DRM (Brown and Rose, 1992). These same trends are observed for both stimulated (Fig. 3 c, open symbols) and unstimulated (Fig. 3 c, closed symbols) cells. Cells were then lysed with cold TX-100 lysis buffer containing 0.040% TX-100, loaded onto sucrose step gradients, and analyzed. Before cross-linking with sAv (or not), cells were incubated: (1) without MβCD for 1 h at 37°C, then subsequently for 2 h at 37°C in the absence of MβCD or cholesterol–MβCD (untreated; circles); (2) with 10 mM MβCD for 1 h at 37°C, followed by a subsequent treatment with 3 mM MβCD for 2 h at 37°C (depleted; squares); or (3) with 10 mM MβCD for 1 h at 37°C, followed by cholesterol/MβCD (3 mM MβCD; MβCD/cholesterol, 8:1, mol/mol) for 2 h at 37°C (repleted; triangles). (b) Lyn distributions. Equal amounts of sucrose gradient fractions pooled as indicated were loaded onto nonreducing SDS polyacrylamide gels and immunoblotted for Lyn. Top, untreated cells; middle, cholesterol-depleted cells; and bottom, cholesterol-repleted cells as described in a. Note that the fraction denoted bottom includes the pellet. (c) GDb distributions. RBL cells sensitized with biotinylated IgE were labeled with 125I-AA4 (anti-GDb), cross-linked or not with sAv, lysed, and subjected to sucrose density gradient analysis as in a. Distributions of GDb from cells stimulated with sAv as in a are denoted by open symbols and those from unstimulated cells are denoted by closed symbols. Circles indicate untreated cells, and squares indicate cells that had been treated with 10 mM MβCD for 1 h.

**Cholesterol Depletion Prevents the Redistribution of Lyn with Cross-linked IgE–FceRI on Intact Cells**

To evaluate how cholesterol depletion affects FceRI on intact cells, we used confocal microscopy to examine the redistributions of Lyn and GDb with cross-linked IgE–FceRI. Representative images in Fig. 4, a and b, show that both monomeric IgE–FceRI (left panels) and Lyn (right panels) are uniformly distributed in the plasma membrane in the absence and presence of MβCD, respectively. When IgE–FceRI is aggregated by antigen at 22°C for 20 min, small patches of these are formed, and these patches often cluster together on one side of the cell. As see in Fig. 4 c, the concentration of Lyn is enhanced in these regions of patched receptors in the absence of MβCD treatment. For MβCD-treated cells, IgE–FceRI also redistributes into patches after aggregation by antigen (Fig. 4 d, left), indicating that lateral mobility is not impeded by cholesterol depletion; however, Lyn does not redistribute with IgE–FceRI under these conditions (Fig. 4 d). As indicated in the first line of Table I, these differences are statistically significant when quantified by cross correlation analysis of multiple cells. Thus, cross-link-dependent interactions between FceRI and Lyn on the cell surface are largely prevented by cholesterol depletion, consistent with the loss of interactions of these proteins with DRMs in the sucrose gradient analyses of lysed cells described above.

Fig. 4 e shows that, as previously observed (Pierini et al., 1996), cross-linking of IgE–FceRI at the cell surface results in co-redistribution of the GDb ganglioside that is labeled by Cy3-A A4 mAb. For MβCD-treated cells, we find that co-redistribution of this outer leaflet DRM marker with cross-linked IgE–FceRI is reduced compared with control cells but not completely disrupted as was the case for Lyn. Fig. 4 f shows an example of this variability, in which one cell exhibits partial co-redistribution of the labeled gangli-
oside, and the other shows a complete lack of co-redistribution with patched IgE–FcεRI. Under these conditions, ≤20% of the cells exhibited detectable co-redistribution of labeled ganglioside, whereas no detectable co-redistribution of Lyn with IgE–FcεRI patches was observed. From these results, it appears that the interaction between Lyn and FcεRI is more sensitive to cholesterol depletion than is the interaction between the ganglioside and FcεRI in the intact cells, although both are substantially prevented. The difference observed may be related to the greater sensitivity of the Lyn–DRM interactions than GD1α–DRM interactions, as shown in Fig. 3 (see Discussion).

As a further control, we compared the distribution of the transferrin receptor (CD71) to FITC-IgE–FcεRI cross-linked under the same conditions as above. Previous studies showed that this transmembrane protein does not associate with isolated DRMs (Melkonian et al., 1999), nor does it co-distribute with other DRM-associated proteins when simultaneously but separately cross-linked on BHK and Jurkat T cells (Harder et al., 1998). As seen in Fig. 4, g and h, TfRs do not co-distribute with cross-linked IgE–FcεRI, and they remain evenly distributed around the periphery of the cell, often in tiny clusters that may reflect interactions with coated pits. Quantitative analysis of the cross correlation of TfR with cross-linked IgE–FcεRI show no appreciable colocalization between these molecules whether or not the cells have been depleted of cholesterol (see Table I). These results support the significance of the co-redistribution of Lyn with cross-linked IgE–FcεRI described above, as well as its inhibition by cholesterol depletion with MβCD.

Figure 4. Immunofluorescence of the cellular distributions of FcεRI, Lyn, GD1α, and TfRs. Each panel consists of paired confocal fluorescence images of FITC-anti-DNP IgE–FcεRI on the left side and the indicated Cy3-labeled proteins on the right side. Panels a, c, e, and g show cells not pretreated with MβCD, and panels b, d, f, and h show cells pretreated with 10 mM MβCD before IgE–FcεRI cross-linking by antigen (c–h) or uncross-linked (a and b). a–d are paired images of IgE–FcεRI and Lyn, e–f are of IgE–FcεRI and GD1α, and g–h are of IgE–FcεRI and TfRs. Arrows indicate regions of co-redistribution of Lyn (c) or GD1α (e and f) with patched IgE–FcεRI. Bars, 10 μm.
Table I. Cross Correlation Analysis of the Co-redistribution of Lyn or TFR with Antigen–cross-linked FITC-IgE–FcεRI

|               | −MβCD | +MβCD |
|---------------|-------|-------|
| IgE/Lyn       | 0.67 ± 0.09 | 0.29 ± 0.16 |
| IgE/TfR       | 0.25 ± 0.16 | 0.15 ± 0.24 |

Quantitative analyses were carried out on fluorescent intensity profiles of equatorial images of individual cells as described in Materials and Methods. The peak values for each cross correlation plot (Eq. 1) were averaged to yield a correlation coefficient (ρ) for numerical comparison of the degree of co-redistribution; 1 corresponds to complete co-localization. The correlation coefficients are reported as mean ± SD, and n is the number of individual cells analyzed.

Cholesterol Repletion Restores FcεRI Tyrosine Phosphorylation Together with the Association of Cross-linked FcεRI and Lyn with DRMs

To investigate the reversibility of cholesterol effects on the functional and structural interactions of FcεRI with Lyn, we restored cholesterol levels in MβCD-treated cells by incubating them with cholesterol-MβCD complexes. The efficiency of repletion is dependent upon the incubation period of the cells with the complex, the molar ratio of cholesterol to MβCD, and the final concentration of MβCD (Christian et al., 1997; Sheets, E.D., unpublished results). To optimize repletion, we used several dilutions of cholesterol-MβCD complexes prepared as described in Materials and Methods. In our sequential depletion/repletion experiment, cells were initially left untreated (control samples) or incubated with MβCD to lower cholesterol levels as described above. During the second step, the cells were incubated for 2 h at 37°C at the indicated dilution of cholesterol/MβCD. 3 mM MβCD only, or buffer only. We found that (a) the cholesterol levels of cells depleted by exposure to MβCD in the first step did not change during the subsequent incubation in the absence of MβCD; (b) the presence of 3 mM of MβCD during the second step also did not cause additional cholesterol depletion, nor did it alter the distribution of IgE–FcεRI in the sucrose gradients; and (c) under optimal conditions of cholesterol repletion used (3-6 mM MβCD; 8:1, mol/mol MβCD/cholesterol), the cholesterol content of the repleted cells was 3.0-3.5-fold higher than that in the untreated control cells. Furthermore, TLC analyses of total lipid extracts indicated that cholesterol was the only lipid that changed detectably during the depletion/repletion treatments (data not shown).

As shown in Fig. 5, repletion of cholesterol in MβCD-treated cells results in partial restoration of antigen-stimulated tyrosine phosphorylation. In the experiment shown, maximal recovery of stimulated tyrosine phosphorylation of FcεRI β and other bands was achieved when 1:50 dilution of the preformed 8:1 MβCD/cholesterol complexes was used to give a final concentration of 6 mM MβCD during the repletion step (Fig. 5, lane 10). As seen in Fig. 5, lane 6, cells that had been treated with MβCD alone during both the depletion and repletion steps have no detectable β phosphorylation, and only a very small amount of stimulated tyrosine phosphorylation is seen in the higher molecular weight bands. Similar results to these were obtained in three separate experiments. Under the conditions of cholesterol depletion/repletion, loss of IgE–FcεRI was determined to be 77 ± 4% (n = 5), and, based upon the proportional relationship between receptor number and β phosphorylation (above), this leads us to expect that optimal restoration of FcεRI β phosphorylation should be ~23% of the stimulated control in Fig. 5, lane 2. The somewhat smaller restoration that is apparent (Fig. 5, lanes 10 and 12) suggests that other factors, such as the loss of other outer-leaflet DRM components during cholesterol depletion noted above, may reduce the maximum restoration achievable (see Discussion).

Repletion of cholesterol also results in restoration of cross-link–dependent association of IgE–FcεRI with isolated DRMs. When lysates of cholesterol-repleted cells are analyzed on sucrose gradients, cross-linked IgE–FcεRI (Fig. 3 a, △) migrates to the low density sucrose region, whereas uncross-linked IgE–FcεRI (Fig. 3 a, △) is found in the 40% sucrose region, similar to the gradient distributions from control cells (Fig. 3 a, ○ and ●). Cross-linked IgE–FcεRI from cholesterol-repleted cells migrate at slightly lower densities in the sucrose gradients than this complex in the control cells, suggesting that the average density of DRMs in repleted cells is lower than in control cells, possibly due to a decrease in the protein/lipid ratio resulting from an increased cholesterol content. Furthermore, as shown in Fig. 3 b (bottom), p56 Lyn also migrates to the low density region of the gradient (fractions 1-6) after cholesterol repletion in cells with both cross-linked and uncross-linked FcεRI. These results, in parallel with the restoration of stimulated FcεRI tyrosine phosphorylation (Fig. 5), provide strong evidence that cholesterol is important for functional coupling of FcεRI with Lyn and for their mutual association with DRMs.

Discussion

Our results demonstrate that cholesterol plays a critical role in the initial step of FcεRI signaling: antigen-stimu-
lated tyrosine phosphorylation of this receptor by the Src family tyrosine kinase Lyn. In parallel with loss of this stimulated phosphorylation (Fig. 1), reduction of cellular cholesterol by MβCD causes the loss of association of both Lyn and cross-linked FceRI with DRM s isolated after cell lysis by TX-100 (Fig. 3). Restoration of the cholesterol content of the depleted cells using preformed cholesterol–MβCD complexes restores the association of Lyn and cross-linked IgE–FceRI with DRM s (Fig. 3) and also causes partial restoration of antigen-stimulated tyrosine phosphorylation in the cells (Fig. 5). These results support the hypothesis that interactions of cross-linked IgE–FceRI with DRM s are important for the initial coupling of FceRI and Lyn that results in receptor phosphorylation. On the cell surface, the association of Lyn with aggregated IgE–FceRI is lost as the result of cholesterol depletion (Fig. 4), indicating that the interactions detected in isolated DRM s are relevant to those occurring in intact cells. Furthermore, these microscopy results argue against a direct interaction of cross-linked FceRI with Lyn as the basis for association of receptors with DRM s, and they support the view that the L₀ structure of the plasma membrane is important for FceRI–Lyn interactions.

An initially surprising finding in our studies is the apparently selective loss of FceRI and outer leaflet plasma membrane components of DRM s due to cholesterol depletion by MβCD. As indicated by Western blot analysis and fluorescence microscopy, there is a smaller loss of Lyn due to cholesterol depletion, and there is no detectable loss of other cellular proteins by silver stain analysis of whole cell lysates (data not shown). The mechanism by which cholesterol depletion causes this selective loss is not yet known, but it is interesting to speculate that vesicles containing these components may pinch off from the cells in a mechanism that depends on their local structural environment in the plasma membrane. In a recent study by Ilangumaran and Hessl (1998), a similar preferential release of DRM components by MβCD treatment was characterized in lymphocytes and endothelial cells, and evidence for release of these components in membrane vesicles was described. Our results suggest that FceRI may preferentially associate with DRM components on intact cells even in the absence of receptor cross-linking. Consistent with this, Basciano et al. showed that pre-binding of AA₄ mAb or its Fab fragment to the α-galactosyl GD₃ antigen on RBL-2H3 cells can effectively inhibit the subsequent binding of IgE to FceRI (Basciano et al., 1996). By varying the cell surface density of antigen-specific IgE in the range of 30–100%, we show that the loss of FceRI due to cholesterol depletion cannot account for the nearly complete inhibition of FceRI tyrosine phosphorylation that is observed in these cells. Furthermore, the partial restoration of antigen-stimulated tyrosine phosphorylation without an increase in FceRI expression after cholesterol repletion strengthens the evidence that cellular cholesterol critically regulates the coupling of the remaining FceRI and Lyn.

An important observation by fluorescence microscopy is that cholesterol depletion does not prevent antigen-dependent aggregation of IgE–FceRI on the cell surface, even though it prevents the co-redistribution of Lyn and inhibits stimulated tyrosine phosphorylation. We previously showed that the cholesterol-binding polyene antibiotic, filipin, prevents anti-IgE–mediated patching of IgE–FceRI (Feder et al., 1994), indicating that it may prevent aggregation of the receptor necessary to initiate signaling. Unlike MβCD, which extracts cholesterol into a water-soluble complex that can be washed away, filipin forms complexes with cholesterol in the membrane that appear to restrict lateral diffusion of at least some membrane proteins, making this reagent less useful for studying the role of cholesterol in signaling by receptors that must aggregate in response to their ligands to be effective. The rapidity with which MβCD can reduce cell cholesterol by substantial amounts without significantly compromising cell integrity, as evidenced by our degranulation results, and the capacity to restore stimulated tyrosine phosphorylation by reintroduction of cholesterol via MβCD complexes, make this an extremely valuable tool for investigating the role of cholesterol in a wide variety of receptor systems.

Recent studies used MβCD to investigate the role of cholesterol in signaling by other receptors. Pike and Miller (1998) showed that cholesterol depletion by MβCD inhibits EGF- and bradykinin-stimulated phosphatidylinositol turnover, which can be restored by cholesterol repletion with MβCD–cholesterol complexes. These receptors belong to the families of intrinsic tyrosine kinase receptors and G protein-coupled receptors, respectively, suggesting the potentially general importance of cholesterol and the L₀ structure it confers on the plasma membrane in mediating receptor signaling. Interestingly, cholesterol depletion by MβCD does not inhibit EGF-stimulated tyrosine phosphorylation of its receptor (Pike, L.J., Y. Liu, K.N. Chung, and J.A. Hessl, 1998. FASEB J. 12:1278 [abstr.]), which probably occurs via a transphosphorylation mechanism (Weiss and Schlessinger, 1998). Rather, cholesterol depletion appears to affect the compartmentalization of phosphatidylinositol 4,5-bisphosphate, the primary phospholipase C substrate in the plasma membrane, as revealed by its reduced localization with DRM s in sucrose gradients (Pike and Miller, 1998). In contrast, our results with IgE receptors indicate a role for cholesterol in the initial signaling step in which these receptors are phosphorylated by Lyn, and this finding may have general relevance for other receptors that function by interacting with Src family kinases. Indeed, Xavier et al. (1998) and Moran and Miceli (1998) showed that pretreatment of T cells with MβCD inhibits T cell receptor for antigen-mediated Ca²⁺ mobilization and tyrosine phosphorylation, respectively, providing evidence for an important role for cholesterol in the function of this related multichain immune recognition receptor family member.

Our degranulation results (Fig. 2) suggest that normal levels of cholesterol may negatively regulate downstream signaling or the exocytotic process in the RBL-2H3 cells. The enhancement of degranulation stimulated by Ca²⁺ ionophore, as well as the lack of inhibition of antigen-stimulated degranulation, despite the dramatic inhibition of tyrosine phosphorylation by cholesterol depletion, are consistent with this explanation. In addition, preliminary experiments on the effects of cholesterol depletion on Ca²⁺ mobilization by antigen indicate that this activity is inhibited less than is stimulated tyrosine phosphorylation of FceRI, consistent with differential effects of cholesterol.
depletion on different signaling steps (Holowka, D., and E.D. Sheets, unpublished results). Membrane structural changes involved in exocytosis may also be affected. As described above, the proportionality of FcεRI tyrosine phosphorylation with the number of receptors cross-linked in the range of 30–100% does not hold for more downstream signaling events, as only a small fraction of FcεRI needs to be cross-linked to achieve maximal degranulation. In future experiments, it will be interesting to explore the effects of cholesterol depletion on specific downstream signaling pathways and exocytic membrane events through bypassing receptor-mediated signaling with alternate means of activation.

The results described here are consistent with the hypothesis that cross-linked IgE–FcεRI interact with Lyn-containing DRM domains on the cell surface and that these structural interactions are integral to the initiation of signal transduction. Monomeric FcεRI probably interact dynamically with DRM components, and these transient complexes may exist in the plasma membrane of unstimulated cells as small clusters similar to those recently described for certain GPI-anchored proteins (Friedrichson and Kurzchalia, 1998; Varma and Ayyar, 1999). In this hypothesis, cross-linking of IgE–FcεRI on the cell surface causes them to cluster with DRM components, thereby creating larger L o regions containing FcεRI and Lyn that are segregated from more fluid regions of the plasma membrane. It is likely that most transmembrane proteins are more readily accommodated by phospholipids in the more fluid liquid crystalline phase, and these would then segregate from the L o regions containing FcεRI and DRM components. For example, the tyrosine phosphatase CD45 is a transmembrane protein that was shown to be largely excluded from DRM s on T cells (Rogders and Rose, 1996) and has been recently shown to negatively regulate the Src family kinase Lck (D’Oro and A shwell, 1999). Our microcopy results (Fig. 4 and Holowka, D., E.D. Sheets, and B, Baird, manuscript in preparation) indicate that segregated DRM domains occupy a large percentage of the cell surface (20–50%) as detectable within the limits of optical resolution, and earlier studies indicated that DRM phospholipids represent a similarly large percentage of plasma membrane phospholipids (Mescher and A pgar, 1985). Thus, segregation of certain proteins from others may be more important for signaling promoted by DRM interactions of cross-linked FcεRI and Lyn than an increased localized concentration of these DRM-associated proteins within domains.

In our model, cholesterol is an essential component for the L o phase, and its 60% reduction, as in the studies presented here, appears to most greatly affect the association of the transmembrane protein FcεRI and the inner leaflet component Lyn with DRM s (Figs. 3 and 4). Outer leaflet DRM components are retained in the low-density, TX-100-insoluble membrane vesicles (Fig. 3), and they still maintain a small but detectable association with cross-linked IgE–FcεRI on intact cells (Fig. 4 f and data not shown), which may reflect the continued presence of an L o environment in the outer leaflet under conditions of diminished cholesterol. It is possible that sphingomyelin and other sphingolipids enriched in the outer leaflet of the plasma membrane (Devaux, 1991) cause a preferential retention of cholesterol in this leaflet of the bilayer under conditions of limiting cholesterol, since this particular class of phospholipids may interact preferentially with cholesterol (Brown, 1998). Thus, cholesterol-dependent associations at the inner leaflet of the plasma membrane may be more sensitive to cholesterol depletion than are such interactions in the outer leaflet. A second alternative explanation for our results, it is possible that cholesterol serves as a critical boundary lipid for FcεRI that facilitates a direct interaction with Lyn. However, this explanation would not account for the association of these components with DRM s and the correlation between loss of this structural association and loss of functional coupling. The involvement of DRM s in functional coupling between FcεRI and Lyn as a means of promoting the proximity of these proteins while excluding transmembrane tyrosine phosphatases such as CD45 is an attractive hypothesis that warrants further examination.

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