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Biophysical Chemistry 82 (1999) 109–119

Biophysical Chemistry

www.elsevier.nl/locate/bpc

How does the plasma membrane participate in cellular signaling by receptors for immunoglobulin E?

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Received 15 September 1999; accepted 15 September 1999

Abstract

Accumulating evidence strongly supports the view that the plasma membrane participates in transmembrane signaling by IgE-receptors (IgE–FcεRI) through the formation of lipid-based domains, also known as rafts. Ongoing biochemical and biophysical experiments investigate the composition, structure, and dynamics of the corresponding membrane components and how these are related to functional coupling between FcεRI and Lyn tyrosine kinase to initiate signaling in mast cells.

Keywords: IgE receptor; Membrane domains; Lipid rafts; Signal transduction; Lipid–protein interactions; Lipid–lipid interactions

1. Introduction

The question posed by the title of this paper reflects our view, based on evidence accumulating in a number of laboratories that plasma membrane structure plays an integral role in cellular activation processes [1]. In particular, membrane lipid composition and resulting properties affect and likely regulate the coupling of key cellular signaling proteins. We summarize below experiments leading to our current understanding of how specialized regions of the plasma membrane facilitate the initial events in transmembrane signaling by IgE–FcεRI (Fig. 1) and report on the progress of ongoing efforts to investigate the structure, dynamics, and regulation of these protein–lipid interactions.

2. Immunological operation of IgE–FcεRI

The allergic immune response centers upon immunoglobulin E (IgE) that binds tightly, via its Fc segment, to receptors (FcεRI) residing in the

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0301-4622/99/$ - see front matter
PII: S0301-4622(99)00110-6

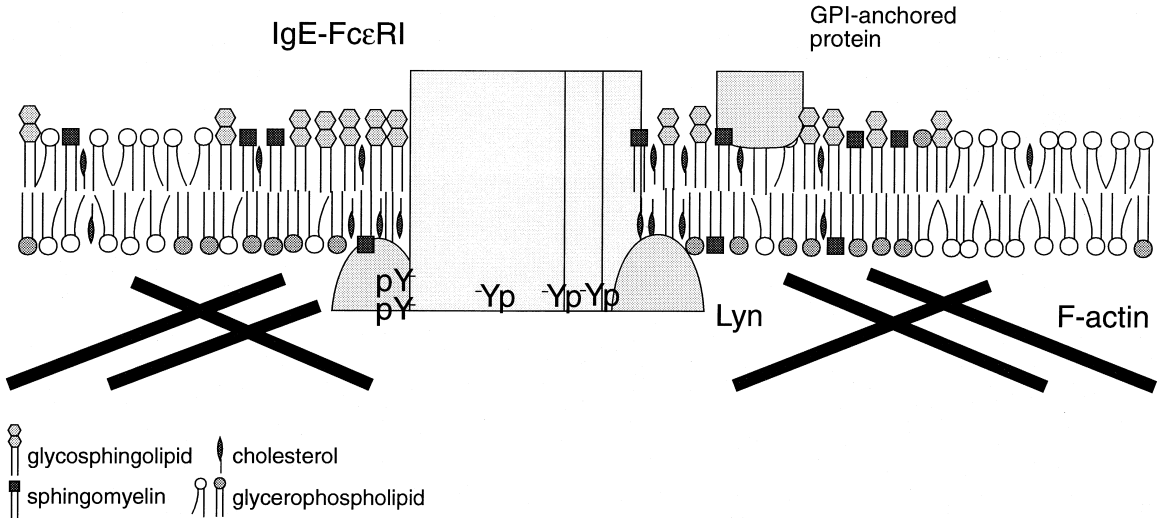


Fig. 1. Regulation of FcεRI-mediated transmembrane signaling. Plasma membrane domains of characteristic composition (also called lipid rafts) co-compartmentalize antigen-aggregated FcεRI wit Lyn resulting in phosphorylation of FcεRI and initiation of signal transduction. This lipid-based coupling is subsequently reversed by the action of the actin cytoskeleton which separates domain components from aggregated FcεRI. It is the FCεRI aggregation process that causes initial coalescence of the membrane domains, which preferentially include lipids that tend to form an L_o phase (mostly saturated sphingolipids and glycerophospholipids and cholesterol). These domains also include proteins that are anchored to the plasma membrane via saturated acyl chains including GPI-anchored proteins in the outer leaflet and dually acylated Src family tyrosine kinases such as Lyn in the inner leaflet.

plasma membrane of mast cells and basophils, thereby sensitizing the cells to the specificities of the IgE antigen binding sites located in its two Fab segments. Binding and cross-linking these IgE–FcεRI complexes by extracellular antigen triggers transmembrane signaling that ultimately leads to cellular release of chemical mediators of allergic reactions in an exocytotic process termed degranulation [2,3]. Beyond its obvious medical importance, FcεRI serves as a useful paradigm for the multi-subunit immune recognition receptor (MIRR) family, which includes other Fc receptors and antigen-specific T and B cell receptors [4,5]. Common structural features of the MIRR family include subunits with immunoglobulin domains in their extracellular antigen or Fc-binding portions and cytoplasmic segments that contain immune tyrosine-based activation motifs (ITAMs). For all these family members, phosphorylation of tandem tyrosine residues in the ITAM sequence by membrane-associated Src family tyrosine kinases is the earliest detectable signaling event that results from receptor cross-linking by antigens [6,7]. These phosphorylated ITAMs recruit the Syk/Zap70 family of tyrosine kinases to the plasma membrane by serving as binding sites for the tandem SH2 domains of the Syk/Zap70, which then become activated.

The tetrameric FcεRI binds IgE with its α subunit, and ITAM motifs are located in cytoplasmic segments of its β and two γ subunits. Cross-linking of two or more of these receptors by antigen is essential for engaging Lyn (of the Src kinase family), leading to Syk activation and the downstream signaling events that result in degranulation, prostaglandin synthesis, cytokine production, and other hallmarks of stimulated cells [8,9]. Much of the biochemical information about the operation of the IgE–FcεRI system comes from studies on a responsive mast cell line (RBL-2H3) that expresses approximately 105/cell of these high affinity receptors which can be sensitized with IgE of selected specificities.

The process by which cross-linking of IgE–FcεRI complexes by antigen leads to tyrosine phosphorylation of FcεRI ITAMs is not fully understood. Until recently, proposed mechanisms for such transmembrane signaling events cen-

tered entirely on protein–protein interactions, and this view can be supported to a large extent by molecular genetics and other experiments that examine only the protein components. Recent evidence, however, has focused upon the spatial and temporal control of targeting and regulation of FcεRI signaling by the plasma membrane and cytoskeleton. As discussed below, our results and others have pointed toward lipid–lipid and lipid–protein interactions within the context of the plasma membrane having essential roles in the initiation of FcεRI transmembrane signaling.

3. Early evidence for cross-linked IgE–FcεRI associating with larger cellular structure(s)

We began to confront this possibility with our early fluorescence microscopy observations on RBL-2H3 cells. In these experiments small aggregates of FcεRI made with covalent oligomers of IgE cluster together on the cell surface; after extended incubations at 4°C, very large patches of receptors are readily detectable by light microscopy [10]. A robust degranulation response occurs with these oligomeric IgE, with or without the cold incubation, when the temperature is raised to a permissive level, however, neither patching nor degranulation occurs when the IgE–FcεRI are not cross-linked. We also found with fluorescence photobleaching recovery (FPR) measurements of lateral diffusion that cross-linking of IgE–FcεRI with antigen at room temperature results in rapid, reversible immobilization of these complexes that correlates with the stimulation of degranulation under these conditions [11]. Phosphorescence anisotropy measurements showed that rotational motion of IgE–FcεRI is very sensitive to cross-linking by dimer-forming ligands, also indicating larger-scale interactions of IgE–FcεRI [12].

4. Formation of plasma membrane domains after cross-linking FcεRI: evidence from microscopic visualization

Lipid interactions with FcεRI on the cell surface were suggested by our unexpected observations with fluorescence microscopy that the lipid analogue 3,3-dihexadecylindocarbocyanine ($DiIC_{16}$) co-redistributes with cross-linked IgE–FcεRI on intact RBL cells after extended incubation in the cold [13]. Subsequently, we determined that several other membrane components behave similarly including: sphingolipids, such as α-galactosyl-GD_{1b} ganglioside [14]; Thy-1, a glycosylphosphatidylinositol (GPI)-linked protein anchored to the outer leaflet of the plasma membrane (D. Holowka, E.D. Sheets and B. Baird, submitted for publication); Lyn kinase anchored to the inner leaflet by means of its conjugated palmitoyl and myristoyl chains [15]. A common feature that these three endogenous membrane molecules share with $DiIC_{16}$ is that saturated or mostly saturated acyl chains facilitate anchorage to the plasma membrane. However, not all lipid-linked molecules associated with the plasma membrane co-redistribute with cross-linked IgE–FcεRI, as exemplified by Cdc42 (D. Holowka, E.D. Sheets and B. Baird, submitted for publication), a member of the low molecular weight Rho family of GTPases which anchors to the membrane via its unsaturated geranylgeranylated chain [16].

5. Involvement of plasma membrane domains in transmembrane signaling by cross-linked IgE–FcεRI: evidence from detergent-resistant membranes

The discovery by others of detergent resistant plasma membranes (DRMs) [17], and their relationship to lipid rafts [18,19] and morphologically defined caveolae [20] led us to investigate whether these putative membrane domains might be regions of localized signaling by cross-linked IgE receptors and could account for our earlier biophysical and microscopic observations. These membrane structures, which are typically isolated from Triton X-100 (TX100)-lysed cells in the form of low density vesicles after sucrose gradient ultracentrifugation, are characterized by their enrichment in lipids with high T_m, particularly sphingolipids which have mostly saturated fatty acid

chains, GPI-linked proteins, and cholesterol. Using model membranes with compositions similar to DRMs that are isolated from cells, Brown and London [21] demonstrated that the detergent insolubility correlates with liquid-ordered (L_o) phases. As characterized previously [22], an L_o phase occurs in the presence of high concentrations of cholesterol which increases both acyl chain orientational ordering as compared to the liquid-disordered (i.e. liquid crystalline) phase and also acyl chain rotational and lateral mobility as compared to the gel phase. Thus, cholesterol abolishes the gel-to-liquid-disordered phase transition of lipids, and lipid bilayers remain fluid with a high degree of orientational order over a wide-range of temperature.

A particularly interesting finding that spurred our investigation into the biological significance of DRM preparations was their enrichment in Src family protein tyrosine kinases that are dually modified with saturated fatty acids [23,24]. We found that DRMs from RBL-2H3 cells contain active Lyn kinase and that the amount of Lyn within the DRMs increases after cross-linking IgE–FcεRI [25]. Moreover, we found that a large percentage of cross-linked IgE–FcεRI associates with the DRM vesicles, whereas, uncross-linked IgE–FcεRI complexes localizes with cytoplasmic proteins and solubilized membrane proteins at higher densities in the gradient [26]. For these latter experiments the detergent concentration used for cell lysis was lowered from a typical value of 0.5 to 0.05% corresponding to the conditions observed to enhance tyrosine kinase activity associated with aggregated FcεRI in cell lysates [27]. Although cell lysates are typically maintained at 4°C for DRM preparation [17,21], we found RBL-2H3 cell lysis and sucrose gradient ultracentrifugation at 37°C yielded similar results (Fig. 2). The functional relevance of the DRM preparations was underscored by the sucrose gradient distribution of FcεRI from cells that had been activated by cross-linking receptors before lysing: virtually all of the phosphorylated β and γ subunits of FcεRI associate with the DRM vesicles; β and γ fractionating with the solubilized proteins (which also include some Lyn) are not phosphorylated. We further showed that association of cross-linked FcεRI with DRM components precedes tyrosine phosphorylation and is not dependent on it, indicating that this association is necessary for FcεRI tyrosine phosphorylation by Lyn [26].

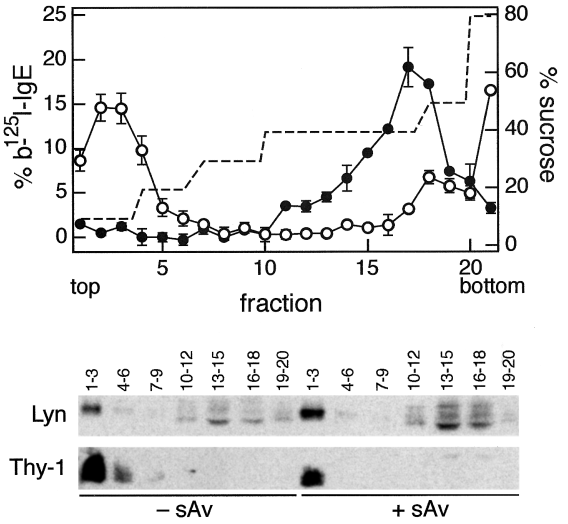


Fig. 2. Molecular distributions of IgE–FcεRI, Lyn and Thy-1 on sucrose density gradients. (a) Distribution of biotinylated-^{125}I-IgE (b-^{125}I-IgE) bound to FcεRI. RBL cells were stimulated with streptavidin (sAv) (open circles) or not (closed circles) for 5 min with 10 nM sAv at 37°C and applied to sucrose density gradients as described [15], except that stimulation, cell lysis and gradient ultracentrifugation were all carried out at 37°C. The error bars represent duplicate samples from the same experiment. These results are representative of two separate experiments. (b) Distribution of Lyn and Thy-1. Gradient fractions from the experiment shown in (a) were pooled as indicated, separated by SDS-PAGE, and immunoblotted for Lyn or Thy-1. Isolated DRM vesicles are typically found in fractions 1–9 and solubilized proteins in fractions 13–18.

The results from the DRM preparations are consistent with our fluorescence microscopy observations. They provide strong support for the hypothesis that cross-linking of IgE–FcεRI stabilizes their association with membrane domains, or rafts, on intact cells wherein coupling with active Lyn results in net receptor phosphorylation and thus initiation of transmembrane signaling. Because this view departs from the more conventionally accepted one based solely upon protein–protein interactions, a multitude of new questions are raised which demand experimental

approaches that include, and extend beyond, protein engineering. Initial information about how FcεRI, a transmembrane protein, interacts with DRMs was obtained with a variety of FcεRI mutants, chimeras and other integral membrane receptors [28]. The FcεRI β subunit and the cytoplasmic segment of the FcεRI γ subunit were found to be unnecessary for DRM association as assessed by sucrose density gradients, and the extracellular portion of the FcεRI α subunit is also insufficient for this association. Furthermore, interleukin-2 receptor α (Tac) associates with DRMs after these receptors are cross-linked by secondary antibodies, whereas an α4 integrin and the interleukin-1 Type 1 receptor do not interact with DRMs before or after cross-linking. These results indicate that association of membrane proteins with DRMs is selective for some proteins and exclude others, and that the transmembrane segments of FcεRI likely mediate its DRM interactions. A clear understanding of the structural interactions necessary for DRM association will require examining the protein for amino acid sequence-dependence and covalent and non-covalent lipid modification as well as more detailed information about the lipid and protein composition of plasma membrane domains.

6. Mass spectrometric analysis of phospholipids from RBL-2H3 cells

With the high sensitivity and resolving power of electrospray ionization Fourier transform mass spectrometry (FTMS), we compared phospholipid profiles from DRM preparations to total cell lysates and to a highly pure plasma membrane preparation [29]. Over 90 different phospholipids were spectrally resolved and unambiguously identified; mole fractions of more than two-thirds of these were determined with a precision of $\pm 0.5\%$. Compared to total lipid extracts, the isolated DRMs are substantially enriched in saturated and mono-unsaturated sphingomyelin and in glycerophospholipids which would readily form L_o phases in the presence of sufficient amounts of cholesterol. Overall, our results show that over 60% of the DRM phospholipids contain one or no double bonds, whereas, less than 30% of the phospholipids in the total lipid pool have these characteristics. Interestingly, stimulation of cells by cross-linking FcεRI prior to DRM preparation increases the amount of polyunsaturated phospholipids from 38% to $> 50\%$. This difference in the DRM preparations suggests significant alteration in the composition and/or structure of the plasma membrane after cells are stimulated.

Analysis of the plasma membrane vesicles from unstimulated cells revealed a phospholipid composition similar to the DRM preparations but with some distinct differences. In particular, the contribution of phosphatidylcholine (PC) to the plasma membranes is almost 70% greater than that for DRMs, whereas the contribution of phosphatidylinositol and phosphatidylglycerol is less than 50% and 20% of those for DRMs, respectively. These differences may suggest that there are some headgroup preferences for DRM association in addition to the anticipated preferences in acyl chain composition. Interestingly, the percentage of phospholipids in the plasma membrane that are saturated or mono-unsaturated is $\sim 50\%$, intermediate between the value for DRMs (60%) and that for total lipids ($< 30\%$), which may suggest that a substantial percentage of the plasma membrane has L_o properties.

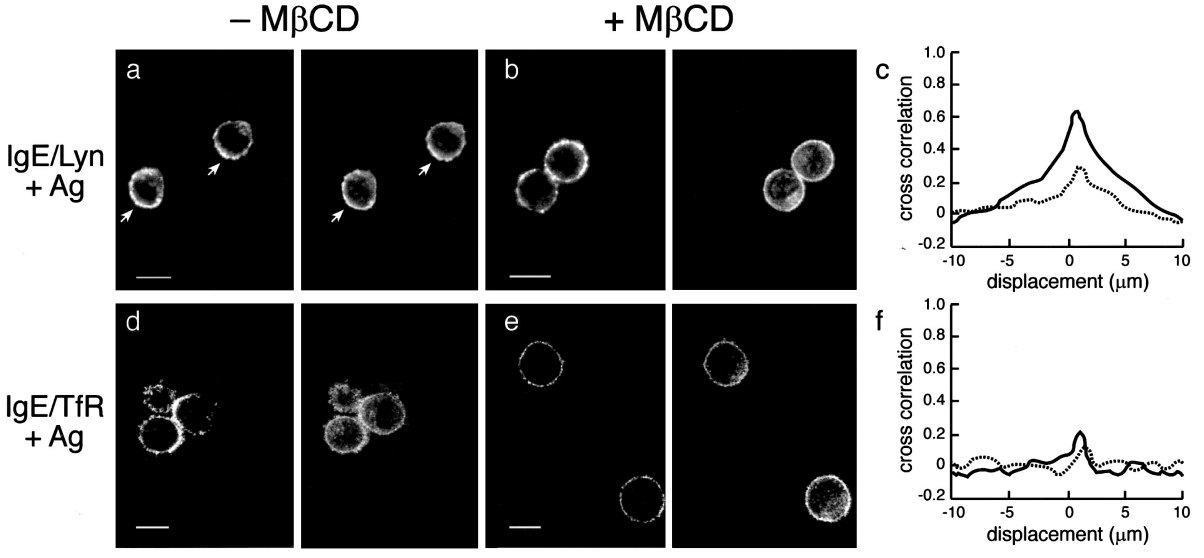
7. ESR analysis of lipid ordering and dynamics in DRMs

The L_o properties of isolated DRMs were confirmed by electron spin resonance (ESR) spectra of two PC derivatives with spin labels in their acyl chains (5-PC and 16-PC), as well as a cholestane analogue of cholesterol [30]. Order parameters (S) and perpendicular rotational diffusion rates ($R_\perp$) from DRM spectra taken at various temperatures were compared to model membranes in the gel, liquid-disordered and L_o phases. $R_\perp$ values for 16-PC in DRMs increase gradually between 15°C and 45°C and are nearly the same as those in model vesicles with a 50:50 mol% mixture of dipalmitoyl-PC (DPPC)/cholesterol that are expected to be in L_o phase for this entire temperature range; these values are sub-

stantially greater than $R_\perp$ for pure DPPC vesicles in the gel phase ($T < T_m = 41°C$). S values for 16-PC in DRMs are comparable to 50:50 mol% DPPC/cholesterol vesicles and remain roughly constant or decrease slightly between 15°C and 45°C. These are similar to S values for pure DPPC vesicles in the gel phase and greater than S values for DPPC vesicles in the liquid-disordered phase ($T > T_m = 41°C$). Preliminary ESR measurements of S and $R_\perp$ for 16-PC in plasma membranes purified from RBL-2H3 cells indicate they do not differ dramatically from the DRM. $R_\perp$ and S values for 5-PC and cholestane in DRM were compared to those values for pure SM vesicles ($T_m = 41–48°C$ for different chain lengths), and the same patterns expected for L_o were observed; that is, DRMs have higher S than the liquid-disordered phase and higher $R_\perp$ than the gel phase.

8. Importance of cholesterol in IgE–FcεRI signaling

Although cholesterol is present in the plasma membrane in the range of 30–50 mol% [31], its lateral distribution and partitioning between the inner and outer leaflets is not known. To determine the relationship between the L_o properties of DRMs conferred by cholesterol and FcεRI signaling, we used methyl-β-cyclodextrin (MβCD) which efficiently removes cholesterol from the plasma membranes of a variety of cell types [15].



	– MβCD		+ MβCD	
	ρ	n	ρ	n
IgE/Lyn	0.67 ± 0.09	12	0.29 ± 0.16	13
IgE/TfR	0.25 ± 0.16	7	0.15 ± 0.24	9

Fig. 3. Cross-correlation analysis of co-redistribution with aggregated IgE–FcεRI in immunofluorescence images. Pairs of confocal fluorescence images of dually labeled RBL cells that had been treated with MβCD (cholesterol depleted) (b, e) or not (a, d) were acquired as described elsewhere [15]. Cells are focussed at the cell equators; the left sides of panels a, b, d, and e are the distributions of IgE, and the right sides of these panels are the distributions for either Lyn (a, b) or transferrin receptor (TfR) (d, e). The cross-correlation between each pair of fluorescence intensity profiles are calculated as a function of displacement. Cross-correlations averaged over a number of dually labeled cells are plotted for IgE/Lyn (c) and IgE/TfR (f); the solid lines correspond to the untreated cells, and the dotted lines correspond to the cholesterol depleted cells. The peak of the cross-correlation plot yields the Pearson correlation coefficeint (ρ) which gives the degree of co-localization with ρ = 1 signifying perfect overlap between the two fluorescence signals. The table reports the mean ± S.D. of ρ calculated, and n is the number of cells analyzed.

We showed that depletion of 60% of the cholesterol from RBL-2H3 cells with MβCD substantially inhibits antigen-stimulated tyrosine phosphorylation of FcεRI and other proteins, whereas, more downstream activities such as calcium mobilization and stimulated exocytosis are maintained. The dramatic effect on phosphorylation correlates with the loss of cross-linked FcεRI and Lyn from isolated DRM vesicles. Furthermore, fluorescence microscopy reveals that Lyn remains associated with the plasma membrane following cholesterol depletion, but that it no longer couples with aggregated IgE–FcεRI (Fig. 3). Repletion of cholesterol with MβCD-cholesterol complexes restores tyrosine phosphorylation and DRM association of FcεRI and Lyn, demonstrating the specificity of cholesterol in this treatment. These results provide compelling evidence for the involvement of cholesterol-rich regions of the plasma membrane in both the structural and functional coupling between FcεRI and Lyn that occurs in mast cells during cell activation.

9. Quantitative microscopy of structural distributions and dynamics of plasma membrane domains on cells

The structural and functional interactions between cross-linked FcεRI and the DRM markers such as Lyn, GPI-anchored proteins and gangliosides has been further validated by fluorescence microscopy of intact cells. The structural dynamics of these interactions can now be examined in detail with a variety of quantitative fluorescence microscopy methods, including fluorescence resonance energy transfer [32,33], FPR [34], fluorescence correlation spectroscopy (FCS) [35], and image correlation spectroscopy [36].

One way of quantifying the extent of colocalization between two fluorescently labeled molecules evaluates the cross-correlation between the two fluorescence intensity traces around the equator of a cell, and many cells can be averaged to obtain a reliable overall comparison ([37]; P. Pyenta, D. Holowka and B. Baird, manuscript in preparation). As illustrated in Fig. 3, cross-correlation analysis confirms that Lyn co-redistributes with cross-linked IgE–FcεRI on control cells and that this interaction is disrupted after cholesterol depletion with MβCD. For comparison, transferrin receptors and cross-linked IgE–FcεRI show no appreciable colocalization whether or not the cells have been depleted of cholesterol.

To determine whether the co-redistribution of Lyn with cross-linked IgE–FcεRI is due to its membrane anchoring by saturated palmitoyl and myristoyl acyl chains, we constructed a green fluorescent protein (GFP) chimera containing the segment of Lyn with the membrane-anchoring acylation sites. This GFP-Lyn analogue targets efficiently to the plasma membrane when permanently expressed in RBL cells. As confirmed by cross-correlation analysis, cross-linking of IgE–FcεRI for several hours at 4°C results in co-redistribution of the GFP-Lyn analogue similar to that described above for Lyn, GD_{1b} and Thy-1, whereas, Cdc42 remains uniformly distributed under these conditions (P. Pyenta, D. Holowka and B. Baird, manuscript in preparation). We are extending these studies by examining the distribution and dynamics of membrane associated GFP constructs, in comparison to FcεRI and other membrane components, with the independent and complementary methods of FPR and FCS (P. Pyenta, P. Schwille, D. Holowka, W.W. Webb, and B. Baird, manuscript in preparation). Interpreting the data in terms of the standard free diffusion model or in terms of the anomalous subdiffusion model (which includes constrained diffusion) [34] provides valuable insight into structural changes affecting Lyn at the inner leaflet of the plasma membrane in response to IgE–FcεRI cross-linking at the cell surface.

10. Cytoskeletal regulation of IgE–FcεRI association with membrane domains

Antigen-stimulated tyrosine phosphorylation of FcεRI by Lyn in RBL-2H3 cells at 37°C typically peaks within a few minutes and then declines sharply, whereas at 4°C phosphorylation reaches a plateau that is sustained over a longer time period [26]. Interestingly, the phosphorylation of FcεRI at 37°C can be sustained, similar to that

observed at 4°C, in the presence of cytochalasin D which inhibits new polymerization of actin [38]. These findings point to functional relevance for fluorescence microscopy observations that marked co-redistribution of DRM components with cross-linked IgE–FcεRI occurs over time at 4°C but only transiently at higher temperatures [37] unless cytochalasin is present (D. Holowka, E.D. Sheets and B. Baird, submitted for publication). F-actin, visualized with rhodamine phalloidin, also redistributes with the DRM components at the lower temperature; at the higher temperature where cell activation occurs, F-actin depletion from cross-linked IgE–FcεRI patches precedes depletion of labeled gangliosides or GPI-linked proteins. Under these conditions, F-actin is seen to move to the membrane ruffles that accompany activation, whereas the cross-linked receptors do not (D. Holowka, E.D. Sheets and B. Baird, submitted for publication). These observations support the hypothesis that antigen-stimulated polymerization of F-actin at higher temperatures causes time-dependent redistribution of F-actin; DRM components, including Lyn, also redistribute towards the membrane ruffles, thereby reversing their initial association with aggregated receptors (see Fig. 1). Protein linkage between the actin cytoskeleton and membrane domains is currently an open question; possible candidates include members of the actin-binding ezrin/radixin/moesin family [39] and domain-associated CD44 [40]. Evidence that membrane lipids can affect this association comes from experiments showing that increasing sphingomyelin content sustains antigen-stimulated signaling in RBL cells and also prevents the interaction of F-actin with plasma membrane domains formed upon IgE–FcεRI cross-linking (D. Holowka, E.D. Sheets and B. Baird, submitted for publication).

11. Summary and conclusions

As the accumulated data show unequivocally, FcεRI cross-linking that stimulates transmembrane signaling changes the distribution and dynamics of membrane proteins and lipids. The detergent resistant plasma membrane domains we have characterized resemble the lipid rafts that have been described for other cells [18,19], although the definition of these is still vague. The plasma membrane changes involve non-covalent interactions among lipids and proteins such that lipids mediate protein–protein coupling. Interactions occur laterally in the outer leaflet and also transversely across the two leaflets: cross-linking of either IgE–FcεRI (transmembrane) or Thy-1 (outer leaflet) causes co-redistribution of GD_{1b} gangliosides (outer leaflet) and Lyn (inner leaflet). Thus, the coalesced membrane domains maintain a lipid composition that selectively includes, or excludes, other lipids or proteins depending on their structural properties. Although lateral interactions within bilayers have been characterized in detail with model systems and phase diagrams, the basis for the more limited inter-leaflet coupling that occurs has not yet been defined and remains an intriguing aspect of transmembrane signaling [1,21].

We have established that membrane-mediated coupling between Lyn and FcεRI accompanies and precedes the initial phosphorylation events. Compelling support for these membrane events being an integral part of the signal transduction mechanism comes from cholesterol depletion which alters the native plasma membrane structure, prevents the co-redistribution, and also severely reduces receptor phosphorylation [15]. Although not yet examined to the same extent as for IgE–FcεRI, similar membrane structures may be generally important for the function of other members of the MIRR family of immunoreceptors. For example, T cells exhibit cross-link-dependent association of T cell receptor subunits with rafts, and receptor-mediated Ca^{2+} mobilization is inhibited by cholesterol depletion [41,42]. Also, palmitoylation of the LAT protein is essential for its association with rafts and for its tyrosine phosphorylation which leads to T cell activation [43].

Membrane domains (or rafts) that coalesce with cross-linked receptors are observed on intact cells with fluorescence microscopy; these correlate with detergent-resistant membranes, which in turn have properties similar to model membranes comprising saturated lipids and cholesterol in the

L_o phase [21]. The propensity to form these domains must be present to some degree prior to receptor cross-linking. Although phase separation is useful for considering model membranes, it is not yet possible to define lipid interactions in these terms for the highly heterogeneous plasma membrane which also contain membrane-associated proteins. A more appropriate framework for thinking about biomembranes may be non-ideal mixing, i.e. FcεRI and Lyn transiently cluster with small groups of lipids of like character without the longer-range interactions inherent to a separated phase. Evidence that monomeric FcεRI associates preferentially with lipids that tend to form detergent-resistant membranes and L_o phases comes from recent microscopy observations that non-cross-linked IgE–FcεRI undergo some co-redistribution with cross-linked IgE–FcεRI after long-term incubation in the cold (unpublished results). The fact that monomeric IgE–FcεRI do not associate with isolated DRM vesicles is probably due to the greater sensitivity of these interactions to detergent-mediated disruption. Thus, selecting the amount of detergent that 'solubilizes' the membrane, yet retains critical interactions becomes problematic.

Much more information about the type and extent of lipid interactions involved is needed before the structure and dynamics required for this cooperative coupling between aggregated FcεRI and Lyn kinase can be defined. The simplest view that L_o regions of the membrane are isolated islands in a sea of disordered lipids is probably not correct. With relatively high amounts of cholesterol, the plasma membrane may be substantially liquid-ordered, a view supported by our mass spectrometry and initial ESR data. Non-ideal mixing (or phase separations) between lipids corresponding to two or more L_o compositions may be possible, although phase diagrams for even simple mixtures containing cholesterol have not yet been well characterized [44].

As pointed out by Metzger [45], a complete model for lipid-mediated coupling must ultimately be consistent with observations that stable FcεRI aggregates as small as dimers successfully engage with Lyn to activate RBL-2H3 cells and that only FcεRI within aggregates are stably phosphorylated. The first observation suggests that the size of the lipid domains associated with oligomerized receptors should be cooperatively larger than the summed size of smaller clusters of the same types of lipids that surround the monomeric receptors. Thus, there may be a threshold size for these domains composed of saturated lipids and cholesterol for sufficiently stable association of Lyn via its saturated fatty acid chains. The second observation might be explained by preferential exclusion of phosphatases from the coalesced domains as has been proposed for CD45 in T cell receptor-mediated signaling [46]. This brings up a relevant issue: interpretation of phosphorylation data is complicated by other cellular processes that proceed at physiological temperatures. In particular, results with cytochalasin suggest that the actin cytoskeleton causes the domains containing Lyn to separate from the aggregated, phosphorylated receptors, leaving them exposed to phosphatases ([47]; D. Holowka, E.D. Sheets and B. Baird, submitted for publication). Thus, it may be that cross-linking of FcεRI by covalent IgE dimers creates domains large enough to include Lyn and exclude phosphatases but not large enough to engage cytoskeletal components.

For comparison, a transphosphorylation model based upon protein–protein interactions has been proposed to account for the initial phosphorylation events [27]. In this view Lyn binds with low affinity to monomeric FcεRI at a constitutive site that does not allow receptor phosphorylation; then cross-linking of FcεRI leads to a juxtaposition of receptors which allows the transphosphorylation of the critical ITAM motifs. The simplest form of this model also raises issues that must be reconciled with the data. Although a weak binding site in FcεRI-β (but no other subunit) for Lyn has been detected [48], this association does not appear to be a requirement for receptor phosphorylation because it occurs with receptors that lack the β subunit (FcεRI-$\alpha\gamma_2$) [49]. Also, it is not yet clear why the relatively high concentration of non-bound Lyn does not phosphorylate the ITAM sequences of monomeric FcεRI (the ratio of non-bound-to-bound Lyn must be high for low affinity binding), as Lyn activity levels are only

modestly affected by receptor cross-linking. Finally, it is difficult to explain the effect of cholesterol depletion on Lyn-FcεRI coupling with this model [15]. Although transphosphorylation has been characterized for the EGF receptor and other receptors containing tyrosine kinase activity in the same polypeptide [50], this mechanism is less appealing for FcεRI which evidently associates weakly and reversibly with Lyn tyrosine kinase at its constitutive site.

The difficulty in addressing questions associated with the simplest interpretations of both types of models relates directly to the major challenge of detecting the tiny amounts of transient physical and chemical interactions that may be required to initiate the signaling cascade. Weak dynamic interactions probably orchestrate a wide array of cellular processes, thereby allowing the cell to respond rapidly and reversibly to specific ligands that bind surface receptors. The experiments presented in this review demonstrate that structural arrangements involving certain lipids in the plasma membrane can provide effective mechanisms for targeting or co-localizing the critical interactants. A variety of experimental approaches, which evaluate the structural and dynamic involvement of both proteins and lipids, must be applied to develop a realistic view of the collective events occurring during transmembrane signaling.

Acknowledgements

The work described here was supported by National Institutes of Health grants AI09838 (E.D.S.), AI22449 and AI18306.

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