Compartmentalized IgE Receptor–mediated Signal Transduction in Living Cells
Thomas P. Stauffer and Tobias Meyer
Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

Abstract. Several receptor-mediated signal transduction pathways, including EGF and IgE receptor pathways, have been proposed to be spatially restricted to plasma membrane microdomains. However, the experimental evidence for signaling events in these microdomains is largely based on biochemical fractionation and immunocytochemical studies and only little is known about their spatial dynamics in living cells. Here we constructed green fluorescent protein–tagged SH2 domains to investigate where and when IgE receptor (FcεRI)–mediated tyrosine phosphorylation occurs in living tumor mast cells. Strikingly, within minutes after antigen addition, tandem SH2 domains from Syk or PLC-γ1 translocated from a uniform cytosolic distribution to punctuate plasma membrane microdomains. Colocalization experiments showed that the microdomains where tyrosine phosphorylation occurred were indistinguishable from those stained by cholera toxin B, a marker for glycosphingolipids. Competitive binding studies with coelectroporated unlabeled Syk, PLC-γ1, and other SH2 domains selectively suppressed the induction of IgE receptor–mediated calcium signals as well as the binding of the fluorescent SH2 domains. This supports the hypothesis that PLC-γ1 and Syk SH2 domains selectively bind to Syk and IgE receptors, respectively. Unlike the predicted prelocalization of EGF receptors to caveolae microdomains, fluorescently labeled IgE receptors were found to be uniformly distributed in the plasma membrane of unstimulated cells and only transiently translocated to glycosphingolipid rich microdomains after antigen addition. Thus, these in vivo studies support a plasma membrane signaling mechanism by which IgE receptors transiently associate with microdomains and induce the spatially restricted activation of Syk and PLC-γ1.

The specificity and efficiency of tyrosine kinase–mediated signal transduction is thought to be controlled by differences in the structure of the catalytic domain that render a kinase selective for particular substrates and/or by the colocalization of a kinase with its substrates by direct binding interactions or by cellular compartmentalization. While enzymatic specificity and direct binding interactions can be studied by various biochemical approaches, much less is known about the dynamic colocalization of different signaling proteins that is expected to occur in living cells. We reasoned that fluorescently tagged signaling domains could potentially be used to monitor the spatiotemporal organization of signaling processes during receptor stimulation of individual cells. In the following study, we used antigen-stimulated tumor mast cells (RBL cells) as a model system to understand the local organization of the downstream tyrosine kinase–mediated signal transduction process.

Antigen-mediated crosslinking of high affinity IgE receptors (FcεRI) is a critical step for triggering the release of inflammatory agents from mast cells (12). Previous studies have shown that initial steps for mast cell activation include the Lyn-mediated tyrosine phosphorylation of IgE receptors on immunoreceptor tyrosine-based activation motifs (ITAM's; 5, 19, 25, 29, 35) and the binding of the tandem SH2 domains of tyrosine kinase Syk to these motifs (2, 13, 31). Furthermore, activation of Syk can trigger calcium signals by activation of phospholipase C (PLC)γ1 and production of inositol 1,4,5-trisphosphate (InsP3; 3). Calcium signals, together with the diacylglycerol-mediated activation of protein kinase C, have been shown to be important regulators for the secretion of histamine, the synthesis of prostaglandins, leukotrienes, and the expression of cytokines (12). Here we used green fluorescent protein (GFP)–tagged tandem SH2 domains of Syk and phospholipase C-γ1 as in vivo signaling probes to monitor FcεRI phosphorylation...
and Syk phosphorylation, respectively. We show that both tandem SH2 domains translocate from a uniform cytosolic distribution to punctuate plasma membrane microdomains after antigen stimulation. These membrane subcompartments were identified as glycosphingolipid-rich microdomains by the colocalization of GFP-tagged SH2 domains with fluorescent cholera toxin B. A surprising finding was also that fluorescently labeled IgE receptors were uniformly distributed in unstimulated cells, and only antigen stimulation induced a transient association of IgE receptor with plasma membrane microdomains. Overall, our studies show that GFP-tagged SH2 domains are powerful in vivo probes to monitor the localized phosphorylation of selective tyrosine residues in individual cells and that the transient association of receptors with microdomains is a possible mechanism by which receptors can selectively activate downstream targets.

Materials and Methods

cDNA Constructs and In Vitro RNA Synthesis

The SH2 domains of rat Syk (amino acid Met 1–Glu 265), rat PLC-γ1 (Ser 539–Gly 777), and Pleckstrin PH domain (Met 1–Gly 105) were subcloned 3′ to cycle3 GFP (4) into the eukaryotic RNA expression vector pCI 36. An additional S65T mutation was added to cycle3 GFP to increase its brightness (10). The RNA was transcribed and polyadenylated by sequential in vitro steps as described in 36. PLC-γ1 and Syk tandem SH2 domains were subcloned into the expression vector pGEX2T (Pharmacia Biotech, Piscataway, NJ) by PCR-mediated mutagenesis using primers containing additional restriction sites. Tandem SH2 domains from Syk were cloned between codons Met 1 and 256 (Glu) and tandem SH2 domains from PLC-γ1 between codons Ser 46 and 791 (Thr). The glutathione-S-transferase (GST) fusion constructs of SH2 domain from Abl and phosphorylidylinositol (PI)3-kinase were kindly provided by Dr. A. M. Pendergast (Duke University).

Expression and Purification of Recombinant SH2 Domains

The GST–SH2 domain fusion proteins were expressed in BL21 cells and purified using a glutathione Sepharose column. The GST tag was removed by thrombin cleavage, and the SH2 domains were dialyzed in a buffer containing 135 mM NaCl, 5 mM KCl, 20 mM Hepes, pH 7.4, for inhibitory experiments, or in 0.1 M NaHCO3, pH 9.0, for protein labeling.

Fluorescent Labeling of Cholera Toxin B, IgE, and SH2 Domains

The tandem SH2 domains of PLC-γ1 and Syk were labeled with Cy2 (Amersham Intl., Arlington Heights, IL). Anti-dinitrophenyl (DNP)–IgE (Sigma Chemical Co., St. Louis, MO) and cholera toxin B subunit were labeled with Cy3.5. The cholaera toxin B subunit labeled with FITC was purchased from Sigma Chemical Co. The labeling reactions were performed for 1 h at room temperature in 0.1 M NaHCO3, pH 9.0, with a twofold molar ratio of fluorescent dye to protein. The labeled proteins were separated from the unbound dye by gel filtration chromatography. The SH2 domains and the cholelra toxin B subunit were labeled in a ratio of 1:2 fluorescent labels per molecules, whereas four labels were conjugated per IgE.

Cell Preparation and Microporation of RNA and Recombinant Protein

Rat basophilic leukemia cells (2H3 type) were plated on glass coverslips at least 12 h before experiments and sensitized by incubation with 10 ng/ml DNP-specific IgE. A small volume electroporation device (a 1-μl micro- porator [33] was used for loading the proteins and mRNA (~1 μg/μl) into adherent RBL cells. RBL cells were electroporated at a field strength of 325 V/cm applied for a 40-ms period and repeated three times at 30-s intervals. For the introduction of recombinant SH2 domains, the percent uptake was determined by coelectroporation of recombinant SH2 do- mains together with fluo-3, using fluo-3 fluorescence to calibrate the approximate intracellular concentration of SH2 domains (for calibration of uptake see 23). After loading, cells were washed five times with an extra- cellular buffer containing 135 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1.5 mM MgCl2, 20 mM Hepes, pH 7.4. For experiments with recombinant SH2 do- mains, cells were left to recover for at least 5 min at 37°C. For RNA transfection, cells were returned to standard medium and placed in the incubator for ~3 h to allow for the expression of GFP-tagged domains. IgE receptors were activated by addition of variable amounts of DNP-BSA as indicated in the text.

Fluorescence Imaging and Correlation Analysis

mRNA encoding GFP-tagged SH2 domains and PH domains were elec- troporated into RBL cells. The expressed proteins were visualized before and after activation (500 ng/ml DNP-BSA) by confocal laser scanning mi- croscopy (LSM; Zeiss, Inc., Thornwood, NY). Optionally, cells were fixed after activation and costained with Cy3.5-labeled cholera toxin B subunit. For costaining experiments of IgE and cholera toxin B, RBL cells were incubated for 1 h at 4°C with 50 ng/ml Cy3.5 or fluorescein-labeled DNP-specific IgE. After a brief incubation at 37°C, the cells were activated at room temperature (~25°C) with 500 ng/ml DNP-BSA and fixed for 15 min with paraformaldehyde at different time points after activation. After wash- ing with phosphate-buffered saline, cells were incubated for 10 min with 2 ng/ml fluorescently labeled cholera toxin B and/or 1 μM FM 1-43 (Molec- ular Probes, Eugene, OR), washed with phosphate-buffered saline, and the coverslips examined by confocal laser scanning microscopy (LSM; Zeiss, Inc.).

The overlap in the fluorescence distribution of the two probes was de- termined by correlation analysis $G(\Delta x) = \sum f(i) \times g(i + \Delta x)$ with $f(i)$ as the fluorescence intensity in the green and $g(i)$ as the fluorescence intensity in the red channel. For this analysis, NIH Image 1.6 software was used to obtain the two line profiles of the plasma membrane fluorescence intensity in the red and green channels. Confocal midsections of cells were used, and the entire cell boundary around each cell was tracked in parallel for both channels.

Results

Antigen-induced Translocation of GFP-tagged SH2 Domains to Plasma Membrane Microdomains

We studied the spatiotemporal organization of antigen-mediated signal transduction in tumor mast cells (RBL cells) by using fluorescently tagged SH2 domains as in vivo signaling probes. GFP-tagged tandem SH2 domains from Syk and PLC-γ1 were expressed by microporation their in vitro transcribed and poly adenylated RNA in RBL cells, which allows for the rapid and efficient expression of fusion proteins in adherent cells (33, 36).

Addition of antigen triggered the rapid translocation of the expressed GFP-tagged Syk SH2 domain from a uniform cytosolic distribution to the plasma membrane (Fig. 1 A). A similar plasma membrane translocation was observed for the SH2 domain from PLC-γ1, while SH2 domains of sig- naling molecules not involved in this pathway (i.e., PI3 Kinase, Abl, or Grb2) failed to translocate after antigen stim- ulation, data not shown). The Syk and PLC-γ1 SH2 domains showed a marked nonuniformity in plasma membrane localization. This clustering of SH2 domains in plasma mem- brane microdomains is shown more clearly in a plasma membrane surface image in Fig. 1 B and in a line intensity profile in Fig. 1 C.

The time course of the translocation of the Syk SH2- GFP probe to the plasma membrane was monitored in individual cells by series of confocal midsections (Fig. 1 D). The arrow in Fig. 1 D points to a location where a strong
staining of the plasma membrane developed within less than 1 min after activation. The time course of the relative increase in plasma membrane translocated Syk SH2 domains is shown in Fig. 1E. Within 2 min after antigen addition, 50% of the total number of translocating SH2 domains was associated with the plasma membrane (Fig. 1E).

We tested whether the plasma membrane translocation of the GFP-tagged SH2 domains was affected by GFP by studying the translocation of fluorescently labeled recombinant Syk and PLC-γ1 tandem SH2 domains. Recombinant SH2 domains were labeled with Cy2 and loaded into RBL cells by electroporation. Fig. 2A shows that also Cy2-labeled SH2 domains translocated to the plasma membrane after antigen addition. We also determined whether the punctate distribution of translocated GFP-tagged SH2 domains is a result of membrane inhomogeneities by monitoring the distribution of plasma membrane–localized GFP-tagged pleckstrin PH domain from pleckstrin during antigen stimulation. This PH domain binds to phosphatidylinositol 4,5-bisphosphate (PIP$_2$), which is predominantly present in the plasma membrane (9). The expressed PH domains were uniformly associated along the plasma membrane and did not significantly redistribute in activated cells (Fig. 2C). A comparison of line intensity profiles of Syk SH2 and PH domains along the plasma membrane (Figs. 1C and 2C) suggests that the punctate staining observed for Syk and PLC-γ1 SH2 domains is indeed the result of a translocation to specific plasma membrane microdomains.

**Selectivity of Syk and PLC-γ SH2 Domains**

We examined whether the recruitment of Syk and PLC-γ1 SH2 domains is required for IgE receptor–mediated calcium signaling since earlier studies have suggested that
calcium signaling is mediated by Syk and PLC-γ1 activation (3). The effect of the SH2 domains on IgE receptor–mediated calcium signaling was tested by microporation of recombinant tandem SH2 domains from Syk and PLC-γ1 and single SH2 domains from Abl and PI3-kinase into RBL cells (Fig. 3A). Syk and PLC-γ1 SH2 domains, but not the SH2 domains from Abl and PI3-kinase, inhibited calcium signaling in a concentration-dependent manner (Fig. 3, A and B). Half-maximal inhibition was observed at ~200 nM of intracellular PLC-γ1 or Syk SH2 domains. This result is consistent with the hypothesis that SH2 domain interactions from Syk and PLC-γ1, but not those of Abl and PI3-kinase, are critical signaling steps for the induction of IgE receptor–mediated calcium spikes.

We then tested whether the inhibitory effect of the Syk and PLC-γ SH2 domains on calcium signaling is specific for the IgE receptor pathway by electroporating recombinant SH2 domains into RBL cells with stably transfected FMLP receptor. Calcium signaling triggered by this G protein–coupled receptor remained unaffected in the presence of the SH2 domains, while the same cells failed to respond to antigen stimulation (Fig. 3C).

To further evaluate the selectivity of SH2 domains, we competed the plasma membrane binding of fluorescently labeled Syk and PLC-γ1 SH2 domains in the presence of either unlabeled SH2 domains of Syk or PLC-γ1. While Syk SH2 domains prevented almost completely the translocation of the fluorescent Syk SH2 domain to the plasma membrane (left) upon stimulation with antigen. The translocation is suppressed in the presence of high concentrations of unlabeled Syk SH2 domains (middle) or PLC-γ1 SH2 domains (right). Bar, 10 μm.

**Figure 3.** Importance of Syk and PLC-γ1 SH2 domains for antigen-mediated calcium signaling. (A) IgE receptor–mediated calcium spiking is progressively inhibited at increasing concentrations of recombinant Syk SH2 domains. RBL cells were co-electroporated with fluo-3 and SH2 domains, and intracellular concentrations of SH2 domains were estimated by the relative fluorescence intensity of co-electroporated fluo-3 fluorescence (see reference 32 for calibration). Calcium spikes were recorded as a function of time after cross-linking of IgE receptor with DNP-BSA (50 ng/ml). The shown fluorescence intensity traces reflect calcium responses after IgE receptor activation in the presence of different concentrations of Syk SH2 domains. (B) Concentration–dependent inhibition of calcium spiking by Syk (squares) and PLC-γ1 SH2 domains (circles). SH2 domains from Abl (triangle) and PI3-kinase (cross) did not affect IgE receptor–mediated calcium signaling even at maximal concentrations. (C) The triggering of G protein–coupled calcium signals is not affected by recombinant Syk or PLC-γ1 SH2 domains. RBL cells with stably transfected FMLP receptors were activated with FMLP (10 μM) or with antigen (50 ng/ml) at different time points. Different protocols are shown with either antigen added alone, FMLP added alone, or both added sequentially (with the FMLP addition 4 min after the antigen addition).

**Figure 4.** Syk and PLC-γ1 SH2 domains bind to different binding partners. (A) The translocation of fluorescently labeled Syk SH2 domain to the plasma membrane (left) is suppressed in the presence of high concentrations of unlabeled Syk SH2 domains (middle) but not suppressed in the presence of high concentrations of unlabeled PLC-γ1 SH2 domains (right). (B) Fluorescently labeled PLC-γ1 SH2 domains translocate to the plasma membrane (left) upon stimulation with antigen. The translocation is suppressed in the presence of high concentrations of unlabeled Syk (middle) or PLC-γ1 SH2 domains (right). Bar, 10 μm.

**Syk and PLC-γ1 SH2 Domains Bind to Glycosphingolipid-rich Microdomains**

The microdomains where tyrosine phosphorylation occurred were further characterized by staining RBL cells with fluorescently labeled cholera toxin B. Cholera toxin B is a marker for glycosphingolipids with strong affinity for GM1 (20) and lower affinity for other gangliosides. Although the punctuate staining observed with cholera toxin is not identical to the microdomains targeted by the two tandem SH2 domains. Double staining experiments of GFP Syk SH2 domains (green) and Cy3.5-labeled cholera toxin B
instead of Syk SH2 domains. (E) One-dimensional plasma membrane fluorescence intensity profiles of the GFP-Syk SH2 domains and cholera toxin B-Cy3.5 of the cell shown in C. (F) Correlation analysis of cells costained with the Syk-SH2 and cholera toxin B probes 3 min after antigen activation (average of five cells). (G) Correlation analysis of cells costained with the PLC-γ1 SH2 domain and cholera toxin B probes 3 min after antigen activation (average of six cells). The relatively high correlation peak in the traces in E and F indicates a high degree of colocalization in the two images. (F and G) As a control, a comparison of the colocalization of cholera toxin B with GFP-tagged PH domains and FM 1-43 was included in F and G, respectively. Bars, 10 μm.

(red) demonstrated a near complete overlap (yellow), suggesting that GFP-tagged SH2 domains are almost exclusively localized to glycosphingolipid-rich microdomains (Fig. 5 C). A similar colocalization was observed between GFP PLC-γ1 SH2 domains and cholera toxin B (Fig. 5 D).

The colocalization was determined more quantitatively by a correlation analysis between the plasma membrane fluorescence intensity line profiles of cholera toxin B and of GFP-tagged SH2 domains (Fig. 5 E). Cross-correlation analysis is a convenient method to determine the degree of overlap between two fluororescent distributions. The higher the overlap between two distributions, the higher the cross-correlation peak amplitude (at the X-axis value of 0). The autocorrelation analysis of a profile can be used to calculate the maximal peak amplitude that would be observed if two distributions completely overlapped. The peak amplitudes were 1.26 (Syk SH2 domains; Fig. 5 F) and 1.25 (PLC-γ1 SH2 domains; Fig. 5 G) for the cross-correlation between the two profiles, compared to 1.28 for their autocorrelation. This is consistent with a near complete colocalization of cholera toxin B and GFP–SH2 domains. In contrast, the correlation between the distributions of cholera toxin B and GFP-tagged PH domains or FM 1-43 was much smaller (Fig. 5, F and G). These results strongly suggest that the recruitment of Syk and PLC-γ1 SH2 domains is confined to glycosphingolipid-rich microdomains.

Antigen-mediated Cross-linking Leads to the Clustering of the IgE Receptors

In analogy to the proposed prelocalization of growth factor receptors to caveolae (14–16, 28, 34), IgE receptors may be prelocalized to glycosphingolipid-rich regions or, alternatively, may translocate to these regions only after receptor activation (1, 6, 7, 24). We measured the plasma membrane distribution of surface IgE receptors in tumor mast cells (RBL-2H3) by monitoring fluorescently (Cy3.5) labeled surface-bound IgE in a confocal microscope. The high affinity between IgE and the IgE receptor makes fluorescently labeled IgE an ideal receptor marker. Before activation, IgE receptors were uniformly distributed (Fig. 6 A) in the plasma membrane as expected for a freely diffusing surface receptor (27). However, activation with antigen (DNP-BSA) led to the progressive accumulation of receptors in clusters over a time scale of a few min (Fig. 6 B) (see reference 30). In the bottom panels, the receptor clustering is shown in one-dimensional plasma membrane fluorescence intensity profiles. Activation leads to a marked increase in the fluorescence intensity fluctuations along the plasma membrane, indicating that the receptors become distinctly clustered.

Transient Association of Activated IgE Receptors with Glycosphingolipid-rich Microdomains

We then tested the possibility that the antigen-induced clustering of IgE receptors leads to its translocation to glycosphingolipid-rich microdomains. Fig. 7 A shows a confocal fluorescence staining of RBL cells with IgE-Cy3.5 and cholera toxin B-FITC before, as well as 3 and 10 min after crosslinking of the IgE receptor. While the colocalization between IgE (red) and cholera toxin B (green) was minimal in unstimulated cells (left), antigen addition led to a significant increase in colocalization within 3 min, as is apparent from the largely yellow membrane staining and loss of red staining in the overlay image (middle). A few minutes later, the colocalization was progressively lost, even though the IgE receptors remained clustered (right).
This transient receptor association with glycosphingolipid-rich regions was characterized more quantitatively by a correlation analysis of the plasma membrane fluorescence intensity profiles, comparing the cholera toxin B-FITC and IgE-Cy3.5 distributions. The activation-induced increase in the correlation peak amplitude between the two distributions supports the hypothesis that IgE receptors translocate to glycosphingolipid-rich microdomains (Fig. 7 B). The same analysis was then used to measure the time course of the translocation process. The IgE receptor accumulated in glycosphingolipid-rich regions for up to 4 min after activation with a $t_{1/2}$ of $<$2 min (Fig. 7 C). However, 10 min after activation, the association of IgE receptor with glycosphingolipid-rich regions was markedly decreased. This suggests that at least two translocation steps are part of the IgE receptor–mediated signal transduction cascade: a translocation of the IgE receptor from a uniform plasma membrane distribution to glycosphingolipid-rich microdomains, followed by a translocation away from these plasma membrane compartments.

**Discussion**

We investigated the spatial and temporal organization of IgE receptor–mediated signal transduction in living RBL cells by using fluorescently tagged SH2 domains of Syk and PLC-γ1. The recruitment of Syk and PLC-γ1 SH2 domains to distinct plasma membrane microdomains strongly supports the hypothesis that IgE receptor–mediated signal transduction is spatially restricted. Src family kinases such as Lyn, tyrosine kinase receptors, and other signaling proteins have been shown by biochemical studies to be localized to the detergent-insoluble plasma membrane fractions that have been termed caveolae or detergent-insoluble glycosphingolipid–enriched complexes (DIGs) (14–16, 28, 34). Could these microdomains be related to caveolae or DIGs? While the microdomains show variations in morphology and detergent solubility, they all contain a particular lipid composition (e.g., glycosphingolipids, sphingomyelins, and cholesterol [21]). Due to the lack of caveolae, and the caveolae marker caveolin in RBL cells (6, 7), we used cholera toxin subunit B to visualize the localization of potential lipid microdomains. Cholera toxin B binds to ganglioside GM1 and with lower affinity to other gangliosides. Earlier studies in RBL cells have shown a more homogenous distribution of a GD1b-specific antibody AA4 (17, 23), suggesting that cholera toxin B–labeled gangliosides and GD1b gangliosides are not equally distributed.

Our studies showed that the subcompartments stained with cholera toxin subunit B have a marked overlap with the compartments stained with the fluorescently tagged SH2 domains. This suggests that the IgE receptor as well
as Syk are specifically tyrosine phosphorylated in microdomains that contain glycosphingolipids. The existence of separate signaling compartments in the plasma membrane of RBL cells is supported by the earlier finding that the crosslinked IgE receptor associates with a detergent-insoluble cell fraction in RBL cells (7, 26). The latter study of Field et al. (7) showed that the cross-linked receptor appears in a detergent insoluble cell fraction within 2 to 3 min after antigen addition. Furthermore, this translocation to the detergent-insoluble fraction occurred in parallel with the tyrosine phosphorylation of the receptor, a process that was maximal after 3 min and subsequently decreased to <50% between 5 and 30 min. While this time course is similar to the one observed in our study, the data in our Fig. 1 D suggest a longer presence of SH2 domains at the plasma membrane. A possible explanation for the prolonged plasma membrane association of the Syk SH2 domain is a protective role of the SH2 domain in the dephosphorylation of the IgE receptor. In addition, a similar protective mechanism may also be responsible for an observed reduced receptor internalization in the presence of Syk SH2 domains within the first 15 min after antigen addition (i.e., Fig. 1 D).

While our in vivo studies with SH2 domains do not directly show a translocation of Syk and PLC-γ1 holoenzyme, our studies suggest that a tyrosine phosphorylation-mediated activation of Syk and PLC-γ1 is essential for IgE receptor–mediated calcium signaling. Nevertheless, it is important to consider possible differences in the localization between the full length protein and its SH2 domain, since full length Zap-70, a tyrosine kinase related to Syk (11), was already prelocalized to a near plasma membrane structure before activation.

Previous studies have shown that IgE receptor cross-linking leads to the Lyn-mediated phosphorylation of ITAM motifs on its β and γ subunits (5, 19, 25, 29, 35). The selective and quantitative inhibition of IgE receptor–mediated calcium signaling by Syk-SH2 domains (Fig. 3), together with the earlier finding that the SH2 domain of Syk selectively binds to ITAM motifs (2, 13, 31), supports the hypothesis that Syk has an essential function in IgE receptor–mediated calcium signaling. Similarly, the quantitative inhibition of calcium signaling by PLC-γ1 SH2 domains suggests that the Syk-mediated activation of PLC-γ1 is the main pathway in RBL cells to generate calcium signals (3). We also evaluated the specificity of SH2 domains targets by showing that unlabeled PLC-γ1 prevents the binding of fluorescent PLC-γ1 but not that of fluorescent Syk SH2 domains. In a different set of experiments, we showed that fluorescently labeled Syk SH2 domains do not bind to the tyrosine-phosphorylated PDGF or EGF receptors in NIH-3T3 cells or A431-epithelial cells, whereas activation of both receptors induced a plasma membrane translocation of fluorescently tagged PLC-γ1 SH2 domains (data not shown). Thus, our studies strongly support the hypothesis that the phosphorylation of IgE receptor and Syk are functionally important for the recruitment of Syk and PLC-γ1, respectively. Our studies also suggest that different fluorescent SH2 domains can be used as tools to selectively monitor the tyrosine phosphorylation of distinct signaling proteins in different cell types.

While the localized signaling through EGF, PDGF, and NGF receptors has been proposed to be mediated by their prelocalization to caveolae-type membrane compartments (8, 18, 22, 34), we here show that IgE receptors are uniformly localized in the plasma membrane and only transiently align with compartments stained with cholera toxin B during the activation process. This two step process for IgE receptor activation, a translocation of the IgE receptor from a uniform plasma membrane distribution to glycosphingolipid-rich microdomains, followed by a translocation away from these plasma membrane compartments a few minutes later, is likely to represent an important signaling principle by which receptors can activate signaling processes in a spatially restricted manner.

Overall, these studies show that IgE receptor–mediated signal transduction is a compartmentalized process that can be monitored in living cells. Furthermore, our studies suggest that the transient association of IgE receptors with glycosphingolipid-rich microdomains, and the sequential activation of IgE receptor, Syk, and PLC-γ1 within these microdomains, defines the specificity and efficiency of antigen-mediated mast cell activation.

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