Spying on IgE receptor signaling: simply complex, or not?

Derek Toomre

Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520

Plasma membrane organization and the potential role, or not, of lipid raft microdomains in signal transduction is a controversial topic. Cross-correlation fluorescent correlation spectroscopy (CC-FCS) shows promise as a new approach to rapidly probe protein–protein interactions in living cells during signal transduction. CC-FCS data from studies of IgE receptor signaling challenge models of large stable lipid raft signaling domains and reveal a new complexity in the dynamic (re)organization of signaling complexes.

Signal transduction, as the name implies, involves amplification of signals and transduction of "information" into (or out of) cells. In IgE receptor signaling, antigen cross-linking of the transmembrane receptor FcεRI results in phosphorylation of its cytosolic tail by Lyn, a Src-family kinase (Holowka et al., 2005). Within seconds, a second kinase, Syk, is recruited to phosphorylated FcεRI that, in turn, activates and recruits dozens of other adapters and enzymes. Cascading signals are propagated and ultimately result in the degranulation of mast and basophil cells. Although the essential IgE receptor signaling proteins have been identified, little is known about how these molecules dynamically assemble and interact. Similar mysteries shroud most other signal transduction pathways. In an exciting new approach, Larson et al. (2005) use two-photon confocal cross-correlation fluorescent correlation spectroscopy (CC-FCS) to monitor Lyn and IgE receptor protein–protein interactions during IgE signal transduction (see p. 527 of this issue). Their observations offer surprising new twists on the mechanisms and kinetics that govern these interactions.

As allergy and asthma sufferers are acutely aware, tight regulation of mast cell signaling circuits is essential. But how are early plasma membrane signaling events organized and regulated? Are they simply governed by protein–protein interactions, or do lipid or cytoskeleton domains provide a higher-ordered spatial architecture? Clustered "lipid rafts" have been proposed to serve as signaling platforms in IgE receptor activation (Simons and Toomre, 2000; Young et al., 2003; Holowka et al., 2005). Most data supporting this model have come from studies of detergent-resistant membranes (DRMs) and biochemical manipulations of cholesterol levels. However, despite their operational utility, DRMs are nonphysiological (in that they can induce large aggregations of lipids) and not equivalent to biological rafts, which are proposed to be small (<50 nm) and mobile (Simons and Toomre, 2000). Moreover, the specificity of cholesterol manipulations in intact cells has been recently called into question (Munro, 2003). Clearly, live cell studies of native unperturbed lipid rafts are essential, but FRET, FRAP, and single molecule imaging have yielded conflicting results (Munro, 2003). Indeed, single-molecule imaging has shaken other well-known paradigms as to how the plasma membrane is organized (Singer and Nicolson, 1972).

In the classical fluid mosaic model (Singer and Nicolson, 1972), proteins freely diffuse in the plane of the membrane. However, this simple model soon encountered obstacles. FRAP studies showed that membrane proteins diffused orders of magnitude slower than predicted from Stokes-Einstein's laws of diffusion (Kusumi et al., 2005). Elegant single-molecule tracking experiments revealed that the membrane was organized by the underlying actin cytoskeleton into small ∼30–200-nm compartments (Simson et al., 1998; Tomishige et al., 1998; Kusumi et al., 2005). Long-range protein diffusion was slow and anomalous, due to transient compartmental confinement. However, when blazingly fast cameras were used (40,000 frames/s), diffusion within the corralled compartment was as fast as predicted from the free diffusion calculations (∼5–10 $\mu m^2/s$; Kusumi et al., 2005).

Another lightning-fast (sub-microsecond) approach to spy on rapid single molecule dynamics is FCS. The principle of FCS is deceptively simple (Elson, 2001). It "simply" measures the flux of a small number of fluorophores (even single molecules!) through a miniscule volume. Rapid temporal fluctuations in fluorescence are used to obtain "autocorrelation curves" from which diffusion constants can be calculated. Larson et al. (2005) used confocal FCS to investigate the mechanism of IgE receptor signaling. The ligand (IgE) and Lyn were fluorescently tagged with $Alexa^{546}$ and GFP, respectively. After signal transduction was initiated, the lateral mobilities of both the cross-linked receptor (A^{546}-IgE-FcεRI) and Lyn-GFP were decreased. However, when Lyn kinase activity was

Correspondence to derek.toomre@yale.edu

Abbreviations used in this paper: CC-FCS, cross-correlation fluorescent correlation spectroscopy; DRM, detergent-resistant membrane.

The Journal of Cell Biology, Vol. 171, No. 3, November 7, 2005 415–417
http://www.jcb.org/cgi/doi/10.1083/jcb.200510105

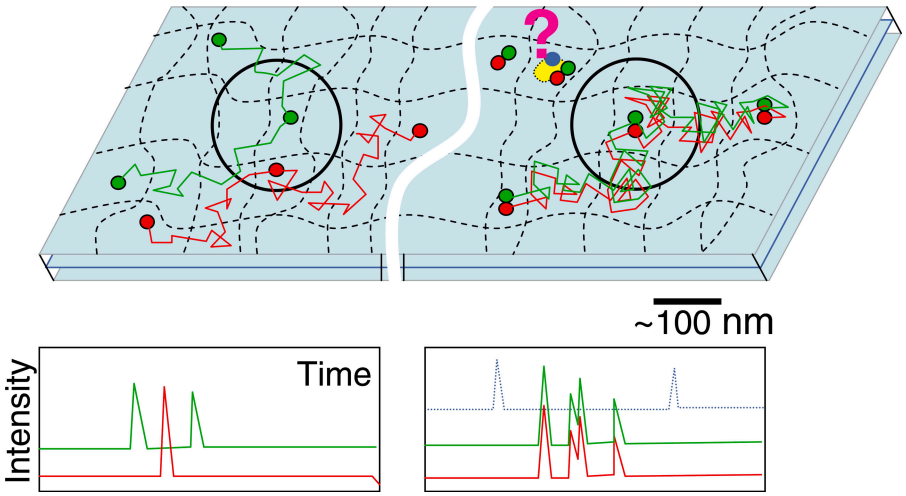

Figure 1. **Cartoon showing two-dimensional "hop diffusion" by CC-FCS.** Noninteracting (left) and interacting proteins (right) in a z100-nm compartmentalized plasma membrane (see text and Kusumi et al., 2005). A confocal CC-FCS spot detector (circle) would only show a high temporal coincidence for interacting proteins (raw data intensity trace, bottom right); this interaction need not be direct, rather simply within a complex or domain smaller than the detector volume. Protein–protein interactions and/or stable association with a lipid microdomain (yellow) could increase the residency time in the compartment and decrease the long-range anomalous diffusion. If a lipid raft reporter protein (blue) shows no correlation (dotted trace), interpretations include: partitioning to the raft was transient, lipid rafts are heterogeneous or represent a major phase, or they do not play a primary role in this interaction. Note: for demonstrative purposes this illustration is greatly simplified.

chemically inhibited, there was no decrease in Lyn-GFP mobility after activation, hinting that direct protein interactions were responsible for the slower diffusion. The mechanism was not investigated, but potentially "oligomerization-induced trapping" could decrease the inter-compartmental diffusion (Kusumi et al., 2005). Of note, Larson et al. (2005) observed 0.1–0.4 $\mu m^2/s$ FCS diffusion values, which approximates those detected by FRAP (Kenworthy et al., 2004) and indicates that the diffusion was anomalous. Some of these results and interpretations differ from earlier FCS (Pyenta et al., 2003) and confocal (Pyenta et al., 2001) studies by the authors, but may reflect methodological differences; cross-linking was previously performed for several hours at 4°C and caused large-scale "patching" of proteins.

Larson et al. (2005) then proceeded with impressive CC-FCS studies. CC-FCS is a variation of the FCS whereby two different dyes are simultaneously detected. Temporal cross-correlation of their signals can reveal whether they are part of the same complex (Fig. 1). The authors showed a high temporal cross-correlation of diffusion of cross-linked FcεRI and Lyn-GFP, substantiating that A^{546}-IgE-FcεRI and Lyn-GFP are part of the same complex. These interactions peaked several minutes after maximal phosphorylation of FcεRI by Lyn, and thus are secondary to the initial signaling (whether initial signaling occurs in lipid microdomains is still open). Surprisingly, they observed strong time-dependent variations in FcεRI and Lyn-GFP interactions over tens of minutes, suggesting multiple transient interactions. Cytochalasin D treatment perturbed these associations, suggesting that the actin cytoskeleton may play a structural or stabilizing role. Of potential relevance, earlier immuno-EM studies showed that the stimulated mast cell membrane contains multiple signaling domains of different composition in close apposition to one another with peripherally associated actin (Wilson et al., 2002).

Remarkably, Larson et al. (2005) showed that an inner leaflet raft marker that is targeted to DRMs, but lacks SH2 and SH3 domains (PM-GFP), showed no decrease in mobility or association with IgE-FcεRI after cross-linking. The simplest interpretation is that the lipid environment, as monitored by this "raft reporter," is not sufficient to drive the interactions between IgE-FcεRI and Lyn-GFP; rather, they were primarily driven by protein–protein interactions. These findings are congruent with recent FRAP (Kenworthy et al., 2004) and single-molecule (Douglass and Vale, 2005) studies. These latter authors observed ~10-fold differences in diffusion of various membrane proteins. However, the diffusion values did not correlate with the proteins' detergent insolubility, but rather correlated to the leaflet (outer, inner, or transmembrane) within which the proteins resided (Kenworthy et al., 2004). Although at odds with a model of large stable lipid rafts, these findings are compatible with models evoking no microdomains, or very small lipid rafts, or a model of dynamic partitioning, where raft proteins have short residency times (Kusumi et al., 2004; Simons and Toomre, 2000). It is also possible that early signaling interactions (within the first 3 min) or other raft markers will show different behaviors. An additional complexity is that plasma membranes are in a constant flux, and phases shown to exist in model systems may not apply to intact cells. Reduction of these complex systems to minimal interactions is a laudable aim, but as Einstein once cautioned, "everything should be as simple as possible, but not simpler."

This powerful demonstration of CC-FCS to monitor IgE receptor signaling dynamics and obtain mechanistic insight will undoubtedly inspire others to apply this method to additional signaling pathways. It may be enlightening to use CC-FCS to examine other raft proteins and lipids, including those on the outer leaflet. The IgE signaling protein LAT is another interesting candidate, as it was reported to form secondary signaling domains and cocluster with the GPI-anchored protein Thy-1 (but not GM1; Wilson et al., 2004). It will be important to determine the underlying cellular mechanism for the prolonged temporal fluctuations in FcεRI and Lyn-GFP associations. On the technical side, amplitude analysis of the raw photon count data should help reveal how many molecules are actually involved in the molecular signaling events.

Submitted: 18 October 2005
Accepted: 19 October 2005

References

Douglass, A.D., and R.D. Vale. 2005. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell.* 121:937–950.

Elson, E.L. 2001. Fluorescence correlation spectroscopy measures molecular transport in cells. *Traffic.* 2:789–796.

Holowka, D., J.A. Gosse, A.T. Hammond, X. Han, P. Sengupta, N.L. Smith, A. Wagenknecht-Wiesner, M. Wu, R.M. Young, and B. Baird. 2005. Lipid segregation and IgE receptor signaling: A decade of progress. *Biochim. Biophys. Acta.* 17:3–21.

Kenworthy, A.K., B.J. Nichols, C.L. Remmert, G.M. Hendrix, M. Kumar, J. Zimmerberg, and J. Lippincott-Schwartz. 2004. Dynamics of putative raft-associated proteins at the cell surface. *J. Cell Biol.* 165:735–746.

Kusumi, A., I. Koyama-Honda, and K. Suzuki. 2004. Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. *Traffic.* 5:213–230.

Kusumi, A., H. Ike, C. Nakada, K. Murase, and T. Fujiwara. 2005. Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules. *Semin. Immunol.* 17:3–21.

Larson, D.R, J.A. Gosse, D.A. Holowka, B.A. Baird, and W.W. Webb. 2005. Temporally resolved interactions between antigen-stimulated IgE receptors and Lyn kinase on living cells. *J. Cell Biol.* 171:527–536.

Munro, S. 2003. Lipid rafts: elusive or illusive? *Cell.* 115:377–388.

Pyenta, P.S., D. Holowka, and B. Baird. 2001. Cross-correlation analysis of inner-leaflet-anchored green fluorescent protein co-redistributed with IgE receptors and outer leaflet lipid raft components. *Biophys. J.* 80:2120–2132.

Pyenta, P.S., P. Schwille, W.W. Webb, D. Holowka, and B. Baird. 2003. Lateral diffusion of membrane lipid-anchored probes before and after aggregation of cell surface IgE-receptors. *J. Physiol. Chem. A.* 107:8310–8318.

Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1:31–39.

Simson, R., B. Yang, S.E. Moore, P. Doherty, F.S. Walsh, and K.A. Jacobson. 1998. Structural mosaicism on the submicron scale in the plasma membrane. *Biophys. J.* 74:297–308.

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science.* 175:720–731.

Tomishige, M., Y. Sako, and A. Kusumi. 1998. Regulation mechanism of the lateral diffusion of band 3 in erythrocyte membranes by the membrane skeleton. *J. Cell Biol.* 142:989–1000.

Wilson, B.S., J.R. Pfeiffer, and J.M. Oliver. 2002. FcεRI signaling observed from the inside of the mast cell membrane. *Mol. Immunol.* 38:1259–1268.

Wilson, B.S., S.L. Steinberg, K. Liederman, J.R. Pfeiffer, Z. Surviladze, J. Zhang, L.E. Samelson, L.H. Yang, P.G. Kotula, and J.M. Oliver. 2004. Markers for detergent-resistant lipid rafts occupy distinct and dynamic domains in native membranes. *Mol. Biol. Cell.* 15:2580–2592.

Young, R.M., D. Holowka, and B. Baird. 2003. A lipid raft environment enhances Lyn kinase activity by protecting the active site tyrosine from dephosphorylation. *J. Biol. Chem.* 278:20746–20752.