structural stability and intrinsic dynamics in POPC bilayers with a total of 15.6 μs molecular dynamics simulations. Cross-validation experiments were also carried out. These include (i) performing window-exchange umbrella sampling simulations with the inter-helical distances as the reaction coordinates to find the energetically most favorable human GHR TM dimer structures and (ii) extracting structural information from H1-N15 HSQC NMR spectra. The results of the three independent experiments well compliment each other. The active and inactive states of human GHR adopt different structural features. The difference is subtle, mostly in the interfacial residues, and is presumably caused by rotation and piston motions of the TM helices.

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Probing the Function of EGFR/EphA2 Interaction using Ligand Induced Heterodimerization

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Heterooligomerization of cell signaling molecules has been observed in many signal transduction systems and is thought to affect signaling output. For example, in the EGFR and Eph families of receptor tyrosine kinases, both homotypic and heterotypic interactions modulate receptor function. We hypothesize that these interactions alter the molecular composition of signaling clusters, which drives changes in cell behavior. Unfortunately, current experimental methods do not allow control of heterotypic interactions between receptors. We have developed a method to form heterodimers of cell signaling ligands on supported lipid bilayers and have employed this approach to study receptor cross-talk between EGFR and EphA2. These receptors are often overexpressed in metastatic breast cancer cells and have been reported to physically interact. We are currently probing the effects of EphA2 and EGFR co-clustering in MDA-MB-231 breast cancer cells using this directed heterodimerization approach. We have found that presentation of the ligands for these receptors in the form of a heterodimer modulates downstream signaling, and we will discuss these results.

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Super-Resolution Imaging of IgE Receptor Clustering Initiated by Structurally-Defined Ligands in Rbl Mast Cells

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Cross-linking of IgE bound to its receptor, FcεRI, by multivalent ligands initiates a transmembrane signaling cascade that activates allergic and inflammatory responses in mast cells. Super-resolution fluorescence localization microscopy is an effective tool for quantitative nanoscale imaging of the distribution and ligand-induced redistribution of FcεRI. We use structurally-defined trivalent ligands specific for anti-dinitrophenyl (DNP) IgE, for which the ligand size and valency is precisely controlled, to cross-link IgE-FcεRI. These ligands are based on a Y-shaped double-stranded DNA scaffold with DNP groups conjugated to each of the three 5' ends. The distance between DNP groups is fixed by the length of single-stranded oligonucleotides annealed to form the Y-shaped structure. As described previously for these Y_n-DNP_3 ligands, (Sil et al., 2007, *ACS Chem Biol*) some (but not other) cell signaling pathways depend on inter-DNP distances, ranging from 5 – 15 nm, which constrain the proximity of ligand-bound IgE-receptors. We are investigating the organization and dynamics of ligand-induced IgE-receptor clustering to gain new information about interactions of cross-linked IgE-receptors within the membrane that accompany or limit transmembrane signaling. We image fixed and live cells and compare Y_n-DNP_3 ligands with the heterogeneous ligand, DNP_{20}-BSA. IgE is labeled with a photo-switchable probe and IgE-receptor clustering is quantified in super-resolution images with spatial correlation functions. In living cells, we record single-receptor trajectories, and receptor mobility is monitored in real time through analysis of populations of tracks. Both DNP_{20}-BSA and Y_{16}-DNP_3 cause a sharp decrease in mobility upon ligand addition, but produce IgE-receptor clusters that appear distinctive in size and density. Y_{16}-DNP_3–stimulated changes occur on a shorter time scale when matched for DNP concentration with DNP_{20}-BSA. Continuing work with Y_n-DNP_3 ligands will probe clustering-dependent IgE-receptor association with membrane structures and downstream signaling partners.

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Multi-Color Single Particle Tracking of QD-IgE-FcεRI: Directly Correlating Oligomer Size with Receptor Mobility and Signaling

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IgE binds to its high affinity receptor, FcεRI, expressed on mast cells and basophils. Crosslinking of IgE-FcεRI complexes leads to intracellular signaling and eventual immune response. Although much is known about this receptor family, the precise mechanism of signal initiation remains elusive. Previous single particle tracking (SPT) experiments have revealed that small, mobile receptors are signaling competent. However, high-speed SPT had typically been limited to two-color tracking, such that aggregates larger than dimers could not be distinguished. In order to better understand the relationship between receptor mobility, aggregate size and signaling, we have developed a novel high-speed hyperspectral line-scanning microscope (HSM) to perform multi-color SPT (mcSPT) of up to 8 colors of quantum dot (QD)- labeled IgE-FcεRI simultaneously on the surface of mast cells. The HSM is unique in its capability to acquire a 4D image (x,y,λ,t) of ~30 microns $\perp$ 2 with 128 spectral channels at ~30 fps. This combined with the advantageous characteristics of QDs (high quantum yield, single source excitation, narrow emission spectra) provides an exceptional platform for mcSPT. Using the HSM, we can determine receptor aggregate size from the spectral signature, and then correlate aggregate size with mobility. Comparison of aggregate mobility in the presence of the tyrosine kinase inhibitor, PP2, reveals the influence of adaptor protein recruitment on mobility. The HSM also allows for simultaneous measurement of calcium flux (Fluo-4) while performing mcSPT, providing a direct readout of signaling in response to receptor aggregate formation and mobility. These experiments not only address important questions in IgE-FcεRI signal initiation, but also provide new biophysical insight into the long-standing debate over the relationship between receptor oligomer size and mobility in the plasma membrane.

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Single Particle Tracking using Fluorogen Activating Peptides to Investigate FcεRI Signaling Dynamics

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The high affinity IgE receptor, FcεRI, serves as the primary immunoreceptor on mast cells and basophils. Cross-linking of IgE-bound FcεRI via multivalent antigen initiates a signaling cascade that ultimately results in the release of key mediators for an allergic response. Priming with IgE has been traditionally thought of as a passive event, yet certain forms of IgE termed 'cytokinergic' IgEs (cIgE) have been shown to induce activation independent of antigen. The mechanism of action of cIgEs remains unclear. To determine whether changes in receptor mobility are associated with cIgE activation, we have developed a fluorogen activating peptide (FAP)-tagged FcεRI -subunit for single particle tracking. FAPs are genetically expressible tags that bind an exogenous fluorogen dye. The titration of the fluorogen dye allows the labeling density to be adjusted at the time of imaging without the need to photo-bleach or photo-activate a population of dye molecules. We find that even in the presence of concentrations of cIgE that induce robust signaling, FcεRI remains highly mobile.

We take advantage of the FAP system to additionally investigate the mobility of the downstream scaffolding protein Linker for Activation of T-cells (LAT). The mechanism through which FcεRI crosslinking propagates signaling to LAT remains a topic of debate as LAT has been previously shown to form clusters segregated from FcεRI after activation. However, these studies were carried out using high concentrations of multivalent allergen that resulted in large immobile FcεRI aggregates. The far-red emission of the MG2P fluorogen allows for simultaneous tracking of FAP-LAT mobility with respect to FcεRI labeled with Alexa488-IgE. Using FAP-tagged LAT, we investigate how LAT mobility and distribution change over a range of activating conditions, in which FcεRI goes from mobile to immobile crosslinked aggregates.

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Single Molecule Studies of Key Processes during the Initiation of the Adaptive Immune Response

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T cell activation is pivotal to the initiation of the adaptive immune response which confers resistance to pathogens and plays a role in numerous diseases. Over the past decades, key protein interactions contributing to T cell activation via antigen recognition have been characterized in molecular detail, in particular the interaction of T-cell receptors (TCR) with antigenic peptide bound to