nucleotide Exchange Factors (GEFs), including non-receptor-like GEFs, cause dynamic structural changes which facilitate substrate release. Although receptor-G Protein interactions are well known a lack of understanding of non-receptor-G protein interactions still exists, namely Gα interactions with the non-receptor GEF Ric-8A. A 10-to-100 fold decrease in nucleotide exchange rate for Ric-8A compared to receptors has been shown, which begs the question: What is the cause for the decrease between receptor and non-receptor GEF activity? To answer this, the dynamic and structural interactions of Gα-Ric-8A have been probed by fluorescence anisotropy and single-molecule Förster Resonance Energy Transfer (smFRET). Conformational changes in the ms times-scale of inter and intra-domain states and increase segmental motions of the switch (I-III) regions seem to play a role in substrate release.

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Single Molecule Imaging Reveals that Activating Kinase Domain Mutations Reduce EGFR Mobility and Enhance Dimerization

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The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases which play an important role in a number of physiological responses and have also been identified as a common signaling component in several cancer types. Physiological signaling is initiated by ligand binding which stabilizes EGFR dimers, resulting in auto-phosphorylation of the intracellular kinase domain. Mutation of EGFR is associated with tumor progression due to constitutive activity resulting from several known kinase domain variants. Our previous work has shown that ligand-induced dimerization of wild type EGFR results in decreased receptor mobility, and that this reduction in mobility is dependent on kinase activity. Therefore, we wondered whether ligand-independent activity of EGFR mutants correlates with differences in receptor mobility or dimerization kinetics.

Here, we employ multi-color, single particle tracking (SPT) of quantum dots to visualize and quantify the dynamics and interactions of individual receptors within the membrane. Using a novel, high-speed hyperspectral microscope, we are able to track up to eight spectrally distinct quantum dots for visualization and quantification of receptor interactions. From this data, we quantify receptor dimerization kinetics using a Hidden Markov Model. We find that unliganded EGFR mutants exhibit a reduced mobility and longer dimer lifetimes when compared to wild type. These results are consistent with constitutive activity of the EGFR mutants and suggest that the active kinase domain facilitates dimer formation in the absence of ligand. Moreover, we test the effects of clinical tyrosine kinase inhibitors on the single molecule behavior of EGFR mutants. We further examined the mobility and clustering of EGFR within the cellular actin cytoskeleton by hyperspectral tracking over GFP-actin, as well as two-color super-resolution microscopy.

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Total Internal Reflection Fluorescence (TIRF) Microscopy Guided Quantification of GLUT4 Translocation for the Identification of Insulin Mimetic Drugs

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BACKGROUND AND PURPOSE. Insulin stimulates the transport of glucose in target tissues by triggering the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. Resistance to insulin, the major abnormality in type 2 diabetes, results in a decreased GLUT4 translocation efficiency, leading to hyperglycemia. Thus, special attention is drawn on the search for compounds, termed insulin mimetics, which are able to enhance this translocation process in the absence of insulin.

EXPERIMENTAL APPROACH. In the present work Total Internal Reflection Fluorescence (TIRF) microcopy was applied to quantify GLUT4 translocation in highly insulin sensitive CHO-K1 cells expressing a GLUT4-myc-GFP fusion protein. Customized software was developed to further increase the throughput capability.

KEY RESULTS. Using our approach we proved the GLUT4 translocation inhibitory properties of selected substances in live and fixed cells. In addition, we validated the efficacy of known GLUT4 translocation inducers and characterized new substances, mainly phytochemicals, with potential insulin mimetic function.

CONCLUSIONS AND IMPLICATIONS. Taken together, TIRF microscopy proved to be a superior tool for the quantification of GLUT4 translocation and the search for insulin mimetic drugs.

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The Membrane Proximal Region of the Human Cannabinoid Receptor CB1 N-Terminus Allosterically Modulates Ligand Affinity

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The human cannabinoid receptor, CB1, a G protein-coupled receptor (GPCR), contains a somewhat long (~110 a.a.) amino terminus. We explored a potential role for the CB1 N-terminus in modulating ligand binding to the receptor. Although the majority of the CB1 N-terminus is not necessary for high-affinity ligand binding, previous studies have found that mutations introduced into a conserved membrane proximal region (MPR) do impair the receptors ability to bind ligand. Moreover, the highly conserved MPR (~ residues 90-110) contains two invariant cysteine residues that are conserved in all CB1 receptors. Our data suggest these two cysteines (C98 and C107) form a disulfide in human CB1, and this C98-C107 disulfide is more accessible to reducing agents than the disulfide in extracellular loop 2 (EL2). The presence of the N-terminal C98-C107 disulfide appears to modulate ligand binding to the receptor in a way that can be quantitatively analyzed by an allosteric model. The C98-C107 disulfide also alters the effects of CB1 allosteric ligands, Org 27569 and PSNCBAM-1. Our results provide new insights to how the N-terminal MPR and EL2 act together to influence the high-affinity orthosteric ligand binding site in CB1, and suggest the CB1 N-terminal MPR may be an area through which allosteric modulators can act.

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Monitoring Conformational Changes During Agonist Release from a GPCR in Real Time: Transmembrane Helix 6 Resets as All-Trans Retinal is Released from Rhodopsin

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The active metarhodopsin II (MII) conformation of rhodopsin loses activity when it releases its agonist, all-trans retinal, and decays to the inactive apoprotein, opsin. Here, we studied how the conformational change and retinal release are linked. In our experiments, we used tryptophan induced quenching of fluorescence (TrIQ) to observe movement of transmembrane helix 6 (TM6) through an attached fluorescent probe, while simultaneously measuring intrinsic tryptophan fluorescence to monitor retinal release. The data show TM6 moves from its position in the active structure back into the helical bundle in opsin (indicative of the inactive conformation) with a rate matching that of retinal exit; both processes exhibit a t1/2 of ~15 minutes at 20°C in 0.05% dodecyl maltoside at pH 6.0. We further tested the correlation of these events in two ways. First, we carried out an Arrhenius analysis and determined near identical activation energies for TM6 resetting and retinal release (~23 kcal/mol). Additionally, we tested the effect of altering the rate of retinal release on the rate of the conformational change. The release process was slowed by introducing a mutation to the Schiff base counterion, E113Q. When retinal exit was slowed, the rate of TM6 resetting decelerated to the same degree. Likewise, accelerating retinal release by using hydroxylamine to hydrolyze the Schiff base linkage caused a corresponding acceleration of the rate of TM6 movements. With these tools in hand, we have begun investigating the dynamics and energetic of other movements in the protein, with the goal of constructing a more comprehensive model for receptor deactivation following light-activation, as well as testing how mutations (such as retinitis pigmentosa), small molecules, and other proteins can effect these events.

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Engineering the Endothelin A and B Receptors using a Soluble Template for Structural Analysis and Small Molecule Screening

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With almost 1000 unique G Protein Coupled Receptors (GPCR's) mapped in the human genome, and an estimated 40% of current marketed drugs targeting these proteins, there is little question that it worthwhile to develop new methods to more efficiently screen for small molecule agonists and antagonists. One such group of GPCRs, the endothelin receptors, is home to one of the most potent vasoconstrictors, the Endothelin A receptor (ETA) which is an excellent target for selective antagonists for the treatment of pulmonary arterial hypertension. We have developed a method for screening selective antagonists which target intracellular domains of ETA and ETB using a soluble template (to mimic a region of the transmembrane domains of ETA and ETB) fused to