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the IC3 loop and Carboxyl terminus of ETA and ETB. Additionally, we have generated a structural model of the ETA Carboxyl terminus (Residues 373-427) from NMR data both in solution and in the presence of DPC micelles. We hope that with these tools we can soon identify and develop targeted inhibitors of vasoconstriction through high throughput screening and rational design.

545-Pos Board B300

Investigating EGF Receptor Signaling Dynamics with Patterned Ligand Surfaces

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Receptor mediated signaling is often precisely regulated in both space and time, which necessitates the application of methods for simultaneously capturing both aspects with a high level of detail. The epidermal growth factor receptor (EGFR) is a prominent example as numerous, dynamic interactions exist between this receptor and its downstream effectors. To advance spatiotemporal insights on EGFR signaling, we have developed a technique by which lithographic processes and chemical modification are used to pattern micron-sized features of EGF on silicon or glass. Fluorescently labeled streptavidin, covalently attached to these surfaces, binds biotinylated EGF for the presentation of immobilized receptor ligand. NIH-3T3 cells stably over-expressing EGFR are allowed to settle on these surfaces and are subsequently fixed. Fluorescence microscopy is then used to visualize specific cellular proteins concentrating at patterned EGF features and correlation coefficient analysis measures the extent of colocalization. We have observed and quantified the recruitment of various EGFR signaling components, including Ras, MEK, and phosphorylated Erk in an F-actin and phosphoinositide synthesis-dependent manner. Further, we find that paxillin-GFP becomes visibly clustered at patterned EGF features. Pretreatment of cells with the EGFR inhibitor Iressa significantly reduces this paxillin clustering behavior and establishes the dependence of paxillin on tyrosine kinase activation. These studies reveal the formation of multi-protein EGFR signaling complexes in response to spatially defined growth factor. To measure dynamics of EGFR protein redistributions in living cells, we have recently extended this patterning technique to coverslip-thick glass substrates and two-color total internal reflection fluorescence microscopy (TIRFM). This is providing for the real time investigation of signaling proteins and their recruitment to patterned EGF. We are also applying alternative analysis methods for quantifying the redistribution of cellular components to take full advantage of this patterned ligand approach.

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Deciphering Regulatory Mechanism of the Juxtamembrane Region in Thrombopoietin Receptor Activation

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Abstract: The thrombopoietin receptor (TpoR/c-MPL) plays an important role in megakaryocyte differentiation, platelet formation and hematopoietic stem cell renewal. Its intracellular membrane-proximal region, which constitutively binds to JAK2, regulates activation of the receptor and signalling of the JAK-STAT pathway. However, the activation mechanism of TpoR at the molecular level is not understood. In our report, cell proliferation assay, fluorescence, circular dichroism and NMR spectroscopy are employed to elucidate the role of the juxtamembrane (JM) region in TpoR activation. We find that secondary structure and membrane affinity of the JM region are strongly controlled by W515 at the hinge region between the transmembrane and JM regions. Secondary structure prediction also reveals that the JM region in TpoR has a larger helical content than in other single-chained cytokine receptors. Thus, we hypothesize that TpoR, which is regulated by a more structured JM region, is strictly inactive when unliganded and can perform ligand-specific signalling.

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FGF1 and FGF2 Induced FGFR3 Dimerization in Plasma Membrane Derived Vesicles

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Fibroblast growth factor receptor 3 (FGFR3) is a receptor tyrosine kinase which resides in the plasma membrane and regulates cell survival, differentiation and angiogenesis. Fibroblast growth factors (fgfs) are a family of secreted protein ligands which bind to FGFRs to potentiate signaling. FGFR3 functions via lateral dimerization in the cellular plasma membrane. It has been shown that FGFR3 forms dimers in the absence of fgf ligands, however it is not clear how fgfs affect FGFR3 dimerization. Previously we have developed a quantitative imaging FRET (QI-FRET) technique to study membrane protein interactions in a cell-derived model system. Here we have used this method to measure thermodynamics of FGFR3 interactions in the presence of fgf1 and fgf2 in Chinese Hamster Ovary (CHO) cell derived vesicles. These measurements provide novel mechanistic insights into the role of ligand binding in receptor tyrosine kinase interactions.

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Voltage Affects the Dissociation Rate Constant of the M2 Muscarinic Receptor

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G-protein coupled receptors (GPCRs) comprise the largest protein family and mediate the vast majority of signal transduction processes in the body. Until recently GPCRs were not considered to be voltage dependent. Newly it was shown for several GPCRs that the first step in GPCR activation, the binding of agonist to the receptor, is voltage sensitive: Voltage shifts the receptor between two states that differ in their binding affinity. Here we show that this shift involves the rate constant of dissociation. We used the m2 muscarinic receptor (m2R) a prototypical GPCR and measured directly the dissociation of [3H]ACh from m2R expressed Xenopus oocytes. We show, for the first time, that the voltage dependent change in affinity is implemented by voltage shifting the receptor between two states that differ in their rate constant of dissociation. Furthermore, we provide evidence that suggest that the above shift is achieved by voltage regulating the coupling of the GPCR to its G protein.

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Cross-Signaling between the Metabotropic Glutamate 2 Receptor and the Serotonin (5-Ht) 2A Receptor in Hek-293 Cells

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Atypical antipsychotic drugs targeting the Gq-coupled 5-HT2A receptor (2AR) are widely used in the treatment of schizophrenia and psychosis. Recent studies point to a new class of potential antipsychotic drugs targeting the Gi-coupled metabotropic glutamate 2 receptor (mGluR2). We recently reported that a heteromeric complex formed between these two GPCRs integrates the actions of serotonergic and glutamatergic drugs, modulating the balance between Gi and Gq signaling and allowing us to predict the psychoactive properties of these drugs (Fribourg et al. 2011, Cell. 147(5):1011-23). Our results uncover a unifying mechanism of action of two families of antipsychotic drugs and pave the road for the development of assays for high-throughput screening of serotonergic and glutamatergic drugs. While developing such an assay, we needed to address a controversy raised by a recent study, in which co-expression of the two receptors in HEK-293 cells had no significant effect on either Gi or Gq signaling in response to several serotonergic and glutamatergic drugs (Delille et al., 2012, Neuropharmacology 62(7):2184-91). Since in Xenopus oocytes the degree of functional crosstalk between the two receptors depended on their expression levels, we generated several clones of HEK-293 cells expressing different levels of the two receptors in the background of the G-protein inwardly rectifying GIRK1/GIRK4 channel, which can serve as a reporter for both Gi and Gq signaling. Using fluorescence indicator and electrophysiological assays we were able to identify clones showing various degrees of functional crosstalk between the two receptors in response to single ligands. Our results have confirmed functional crosstalk of the two receptors. Even in cases where a single ligand failed to show crosstalk, like in the Delille et al. study, a combination of ligands targeting both receptors could elicit functional crosstalk.

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Single Proteoliposome Assay to Monitor Opsin and Cannabinoid Gpcr Homo-Oligomerization

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Oligomerization of transmembrane proteins is emerging as an important concept in cellular function(1). In the G-protein coupled receptor (GPCR) field oligomerization remains to be fully explored. The majority of assays available for measuring GPCR oligomerization are cell based and rely on ensemble readouts(2,3). As a complement to such assays we suggest to quantitatively study the oligomerization of receptors reconstituted in single synthetic liposomes (proteoliposomes). A liposome model membrane system provides a simplified