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Development of a FRET-based Reconstitution Assay to Probe the Interaction Between the Rho Family GTPases and Defined Synthetic Lipid Membranes

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The Rho Family GTPases are a tightly regulated class of signaling proteins that control a number of important cellular processes. Known most prominently for their ability to remodel the actin cytoskeleton in mammalian cells, this family has been shown to play essential roles in cell migration, epithelial cell polarization, phagocytosis, secretion, and cell cycle progression. These outcomes occur in many different subcellular locations and, as such, they require the GTPase to be able to quickly access them. Rho Guanine nucleotide Dissociation Inhibitor (Rho-GDI) is a ubiquitously expressed protein that stimulates the removal of Rho family GTPases from membranes and has been shown to be important in regulating their localization. This study employs a novel FRET assay to reconstitute Rho family GTPase interactions with GDI at the membrane surface. Cdc42 was loaded with fluorescently labeled guanine nucleotides (Mant-nucleotides) and inserted into fluorescein labeled membranes that can quench Mant fluorescence by FRET. GDI's removal of Cdc42-bound Mant-nucleotide from the proximity of acceptor labeled lipids was demonstrated by the complete restoration of Mant fluorescence upon GDI addition. This assay is able to provide detailed kinetic information and shed light on the molecular basis of the Cdc42's interaction with GDI. Additionally, we are able to probe the nature of the interaction between Cdc42 and the membrane surface, using liposomes of variable lipid composition. Here, we demonstrate a direct role for PIP_2 on Cdc42's affinity for membranes and we identify the residues of Cdc42 that are receptive to this lipid, providing a more detailed understanding of Cdc42's behavior at the membrane surface in living cells.

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Preferential Binding Of cGMP Phosphodiesterase To Phospholipid Monolayers

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The light-activated rhodopsin, metarhodopsin II, forms a complex with the alpha subunit of transducin (Tα). The exchange of GDP for GTP leads to a conformational change of Tα which is released from its beta-gamma subunits and rhodopsin. Tα-GTP then activates cGMP phosphodiesterase (PDE) which hydrolyzes cGMP and leads to the closure of the cGMP-gated channels and to the hyperpolarization of the rod photoreceptors. PDE is made of two catalytic (αβ) and two inhibitory (γ) subunits (Pαβγγ). PDE is acylated with a farnesyl and a geranylgeranyl and is thus membrane bound. PDE was found to be located, at least in part, to detergent-resistant membrane microdomains. These microdomains, also called rafts, have been shown to contain a large amount of phospholipids with saturated fatty acyl chains as well as cholesterol. However, the preferential binding of PDE to saturated phospholipids has not yet been shown. The objectives of this research work were thus to characterize the membrane binding properties of PDE using Langmuir monolayers. The Pαβγγ was isolated and purified from bovine rod outer segments (ROS) of retinal photoreceptors. PDE was injected into the subphase underneath phospholipid monolayers bearing different fatty acyl chains (length and unsaturation) and polar headgroups such as those present in ROS. PDE binding was monitored by surface pressure measurements. The injection of PDE underneath phospholipid monolayers led to an increase in surface pressure which indicates its membrane binding. The surface pressure data demonstrated that the adsorption kinetics of PDE is dependent on the type of phospholipid fatty acyl chain (length and unsaturation) and headgroup. For example, on the basis of its maximum insertion pressure, PDE shows a preferential binding onto saturated phospholipid monolayers. This data is consistent with the possible localization of PDE to lipid rafts of ROS membrane.

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Lipid-protein Interactions Between α2-adrenergic Receptor Transmembrane Peptides And Model Membranes

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α2-Adrenergic receptors belong to a large family of membrane proteins, known as G-protein coupled receptors (GPCRs), involved in signalling across biological membranes. The association of GPCRs to the plasma membrane makes them susceptible to their lipid environment and in turn, these proteins are also capable of modulating the lipid structure and properties of the membranes with which they interact. To study peptide-lipid interactions, model peptides consisting of a simple repeating motif designed to form stable α-helices have been the most common approach used [1]. Our experimental design followed a novel strategy using peptides with identical sequences to the putative transmembrane segments (TM), H4, H6 and H7 helix, of the human α2-adrenergic receptor subtype C10 (P08913). P6 peptide contains the hydrophobic and the hydrophilic terminal sequence of the full TMH segment (H6), whereas P4 and P7 peptides only have the hydrophobic core of the transmembrane segments (TM) (H4 and H7). Molecular and structural parameters of peptide-DEPE membranes have been analyzed by fluorescence, DSC, X-ray diffraction and FTIR techniques. The study highlights the importance of the conceptual design of the peptide sequences using naturally derived aminoacid sequences when mimicking TM proteins as templates.

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Molecular Mechanism of an Oncogenic Mutation that Alters Membrane Targeting: Glu17Lys Modifies the PIP Lipid Specificity of AKT1 PH Domain

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The protein kinase AKT1 regulates multiple signaling pathways essential for cell function. Its N-terminal PH domain (AKT1 PH) binds the rare signaling phospholipid, phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3], resulting in plasma membrane targeting and phosphoactivation of AKT1 by a membrane-bound kinase. Recently it was discovered that the Glu17Lys mutation in the AKT1 PH domain is associated with multiple human cancers. This mutation constitutively targets AKT1 PH domain to plasma membrane by an unknown mechanism, thereby promoting PI(3,4,5)P3-independent activation of AKT1 and oncogenesis. To elucidate the molecular mechanism underlying constitutive plasma membrane targeting, the present work compares the membrane docking reactions of the isolated wild type and E17K AKT1 PH domains. In vitro studies reveal that the E17K mutation dramatically increases the affinity for the constitutive plasma membrane lipid PI(4,5)P2. The resulting PI(4,5)P2 equilibrium affinity is indistinguishable from that of the standard PI(4,5)P2 sensor, PLCδ1 PH domain. Kinetic studies indicate that the effects of E17K on PIP lipid binding arise largely from electrostatic modulation of the dissociation rate. Membrane targeting analysis in live cells confirms that the constitutive targeting of E17K AKT1 PH to plasma membrane, like PLCδ1 PH, stems from PI(4,5)P2 binding. Overall, the evidence indicates that the molecular mechanism underlying E17K oncogenesis is a broadened target lipid selectivity that allows high affinity binding to PI(4,5)P2. Moreover, the findings strongly implicate the native Glu17 side chain as a key element of PIP lipid specificity in the wild type AKT1 PH domain. Other PH domains may employ an analogous anionic residue to control PIP specificity.

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Activity And Membrane Binding Of Retinol Dehydrogenase-11 And Its Deletants

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Retinol dehydrogenases (RDHs) are enzymes that catalyze the interconversion between retinol and retinal. Not much is known concerning these RDHs. Indeed, the exact physiological role of many isoforms of RDH and their membrane binding remain unknown. In this work, we have overexpressed, purified and characterized the membrane binding of one isoform, RDH-11. The cDNA of RDH-11 and of the truncated RDH-11 (N-terminal deletion, N-del RDH-11) have been cloned in the pET28a plasmid. Those proteins have been overexpressed in *E. coli*, purified by affinity chromatography, and then concentrated by ultrafiltration. Their activity has been measured in the presence of its substrate, all-*trans* retinal, and the reaction was initiated by the addition of its cofactor, NADPH. The reaction products have been analyzed by HPLC. The data showed a very high activity of RDH-11. Indeed, 1 μg of protein was enough to convert nearly 100% of all-*trans* retinal to all-*trans* retinol. Membrane binding

measurements have been carried out by the injecting RDH-11, N-del RDH-11 and the synthetic N-terminal peptide (NTP) into the subphase of a phospholipid monolayer at the air-water interface. Their kinetics of monolayer binding, monitored by surface pressure measurements, increases as follows : NTP > RDH-11 > N-del RDH-11. Moreover, measurements by polarization-modulated infrared reflection absorption spectroscopy have allowed to confirm the alpha helical structure of the NTP and to determine its orientation as well as to compare the structure and orientation of RDH-11 and N-del RDH-11. For example, compared to the pure protein, N-del RDH-11 undergoes a conformational change upon monolayer binding.

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Calcium Independent Substrate and Product Diffusion Process of Secretary Phospholipase A2 from Taiwan Cobra

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Understanding how membrane lipids can be delivered to the active site distant from the interfacial binding surface of the enzyme and how the products got released from the active site are important to depict the interfacial enzyme reaction mechanism. Based on the crystal structure of the trimeric complex structure of the cobra phospholipase A2 (PLA2) from Naja atra with the enyzymatic substrate of diacylheptanoyl phosphatidylcholine (PC) and products of lyso PC and fatty acids (minic by SDS), we suggest that promiscuous bindings of phospholipids to the interfacial enzyme may boost the lipid desorption process via a cooperative hydrophobic interaction among the hydrocarbon chains of phospholipids and that with the interfacial surface of the enzyme. We also show that phospholipids in membranes surface with high curvature can promote the diffusion of the lipid into the substrate binding hydrophobic channel of cobra PLA2 in a calcium independent manner. The PC substrate binding site within the channel without calcium is distinct from that in the presence of calcium as one compares its binding position with that of transition binding intermediates. Interestingly, calcium appears to destabilize the binding of both substrate and product binding at the hydrophobic channel even though it is required for the enzymatic catalysis. Our results suggest that the calcium independent lipid diffusion process play an important role in the interfacial binding activation of secretary PLA2 and shed new light for the future depiction of the energy landscape.

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A New Conformation in SERCA and PMCA Ca^{2+} Pumps Revealed by a Photoactivatable Phospholipidic Probe

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The purpose of this work was to obtain structural information about conformational changes in PMCA membrane regions and their interaction with surrounding lipids. To this end, we have quantified labeling of the sarcoplasmic reticulum Ca^{2+} pump (SERCA) and the plasma membrane Ca^{2+} pump (PMCA) with the photoactivatable phosphatidylcholine analog [^{125}I]TID-PC/16, under different conditions. This probe has been used previously to analyze lipid-protein interfaces. We determined that: (1) Incorporation of the photoactivatable reagent to SERCA decreases 25% when labeling is performed in the presence of Ca^{2+} as opposed to EGTA (2) The decrease in labeling matches qualitatively with the decrease in transmembrane surface exposed to the solvent calculated by the Lee-Richards method, when comparing the known SERCA structures 2ear (E_2) (*pdb. file*) and 1su4 (E1Ca) (*pdb. file*). (3) Labeling of PMCA incubated with Ca^{2+} and calmodulin decreases by almost the same amount as compared to EGTA. However incubation with Ca^{2+} alone (no calmodulin) increases labeling by 55%. This suggests that the conformation in which the enzyme is fully active (Ca^{2+} for SERCA and Ca^{2+}-CaM for PMCA) exhibits a more compact transmembrane arrangement in both proteins. Addition of C28, a peptide containing the calmodulin binding region of PMCA, to SERCA in the presence of Ca^{2+} increases [^{125}I]TID-PC/16 incorporation, confirming the suggestion made above. The results indicate that there is an autoinhibited conformation in these P-type ATPases that affects not only the cytoplasmic regions but also the transmembrane segments.

Muscle: Fiber & Molecular Mechanics & Structure II

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***In Vitro* Study of Mechanical and Kinetic Properties of Myosin II from Frog Skeletal Muscle**

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We provide for the first time the protocol for efficient extraction and conservation of myosin II from frog skeletal muscle, a methodological achievement that makes it possible to apply single molecule techniques to the molecular motor that has been best characterized for its mechanical, structural and energetic characteristics in single muscle cells, where it works as an ensemble in each half-sarcomere. With the *in vitro* motility assay, we estimate the sliding velocity of actin on frog myosin II (V_F) and its modulation by temperature (range 4-30 °C) and substrate concentration. V_F is 8.88 ± 0.511 μm/s at 30.6 °C and decreases down to 1.6 ± 0.23 μm/s at 4.5 °C. The *in vitro* mechanical and kinetic parameters are integrated with the *in situ* mechanical and kinetic parameters of frog muscle myosin working in array in each half-sarcomere. By comparing V_F with the shortening velocity determined in intact frog muscle fibres under different loads and their dependence on temperature (Piazzesi et al., *J. Physol.*, 549:93, 2003), we find that V_F is 40-50% less than the *in situ* unloaded shortening velocity (V_0) at the same temperature and we determine the load that explains the reduced value of V_F. With the integrated approach we can define fundamental kinetic steps of the acto-myosin ATPase cycle *in situ* and their relation with mechanical steps. In particular we clarify the relation between the rate of ADP release and the rate of detachment of myosin from actin and their temperature dependence. Supported by NIH (Grant no. 5R01AR49033) and MiUR Italy.

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Construction Of Myosin Model Explaining Difference Between Experimental Results Observed With Scanning Probe And Optical Tweezers

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Single molecule measurement (SMM) techniques have been applied to myosin. Then, SMMs' results show that, during single ATP hydrolysis cycle, myosins II & V repeat several cycles of association with- and dissociation from an actin filament to generate sliding motion, suggesting that myosin can convert ATP energy by multi-step processes (MSPs). This MSPs cannot be explained by conventional ''lever-arm model'', then ''Biased Brownian motion (BBM) model'' has been proposed for a mechanism of myosin. However, the MSPs have been observed only by SMM with scanning probe (SP), and not observed with optical tweezers (OT) widely used for SMM. Because MSPs have been observed clearly with myosin II & V, it is strongly suggested that BBM is movement mechanism of myosin. Then, why have MSPs not been observed with optical tweezers? In order to answer this question, here, we construct model including characteristics of SP & OT, and simulate movement of myosin attached to measurement probes (SP or OT). Taking into account the effects of measurement probes, we construct 2-demensional potential along an actin filament, and simulate movement of myosin on the 2D potential by Monte Carlo method. For simulation, spring constants of probes parallel and perpendicular to an actin filament are set according to characteristics of each probe. As a result, sliding velocity with SP (~0.5 um/s) becomes slower than that with OT (~3 um/s), then MSPs are clearly observed with SP. This result explains well the experimental results with SP and OT.