Dear ESF Office Members, dear Maria

Most importantly, I would like to thank the ESF for the financial and logistic support that made the EuroMEMBRANE International Conference “Membrane Dynamics in Physiology and Disease” possible – and a great success!

More than 210 participants, who produced almost 100 posters and 43 talks, contributed to a lively and interactive meeting (for details see Program and AbstractBook files). The high ratio of contributing participants yielded a very productive atmosphere at the conference. Highlights were the lectures of the keynote speakers Pietro De Camilli and Robin Irvine. The quality of all the presentations was excellent, and especially the young researchers showed their potential, and the impressive progress in the field.

Besides the participants from the EuroMEMBRANE consortia (see participants list), the audience was very international.

The conference was held at the University of Basel, and was supported by the Basel Signaling Alliance and local private sponsors (see lipidsignaling.org; Sponsors). The lecture hall situated centrally in the town of Basel provided a comfortable venue, and ample space for poster presentations, with the possibility to merge coffee breaks and lunches with scientific discussions.

The conference covered various aspects of membrane biology grouped into the sessions “Vesicular Trafficking, Proteins & Lipids”, “Lipid Signaling in Health and Disease”, “Membrane Organization and Function”, “Lipid Dynamics, Modelling and Visualisation”, and “Measuring and Manipulating Lipids”. The presented methods and techniques were diverse, novel and original, and included genetic, biochemical, biophysical, computational, microscopy, chemical and more approaches.

The conference will have further impact, as a selected number of speakers currently prepare a mini-review series for the FEBS Journal.

Altogether, the conference was a fantastic opportunity to exchange information with leading figures in membrane biology, and has served many participants to initiate scientific collaborations beyond the ESF EuroMEMBRANE programme.

With many thanks and kind regards

Matthias Wymann

Attached: Program_EM2012.pdf; AbstractBookEM2012.pdf; ParticipantsEM2012.xls

Program

Membrane Dynamics in Physiology and Disease

Basel, June 5 - 8, 2012

Tuesday, June 5, 2012

14:00 - 16:50	Registration, Poster Set-up	
Plenary Lecture:		*Chair: Matthias Wymann*
17:00 - 18:00	Pietro De Camilli, Yale University	Membrane dynamics and phosphoinositide signaling in the endocytic pathway
18:00 -	*Poster Session, Barbeque*	

Wednesday, June 6, 2012

Versicular Trafficking, Proteins & Lipids		*Chair: Martin Spiess*
08:30 - 09:00	Scott Emr, Cornell University	Kinase Signaling Cascade Regulates Arrestin-Related Ubiquitin-Ligase Adaptor During PM Protein Turnover
09:00 - 09:30	Roger Williams, Cambridge University	Regulation of phosphoinositide 3-kinases on membranes
09:30 - 10:00	Volker Haucke, FU Berlin	Membrane scaffolds in synaptic vesicle exo-endocytosis – from molecules to systems
10:00 - 10:30	*Coffee, Postersession*	
10:30 - 11:00	Anne Spang, University of Basel	Reduction of N-glycosylation causes cell polarity-dependent loss of cell-cell contacts in the two cell-stage C. elegans embryo
Short Talks		
11:00 - 11:15	Maria Patrizia Stoppelli, Institute of Genetics and Biophysics (IGB)	Specific sphingolipids required to urokinase receptor signalling
11:15 - 11:30	Shirish Mishra, University of Fribourg	Expression of oleosin and perilipins in yeast promotes formation of lipid droplets from the endoplasmic reticulum
11:30 - 11:45	Fabien Lefebvre, Karolinska Institute	How F-BAR proteins might play a role in membrane dynamics and actin cytoskeleton remodeling coordination?
Flash Talks		
11:45 - 11:50	Thommie Karlsson, Linköping University	Localized Aquaporin 9-Mediated Water Fluxes Precede Actin Polymerization in the Formation of Filopodia
11:50 - 11:55	Aurore Levy, Institut Curie	Role of caveolin 1 trafficking in the sorting of sphingomyelin
11:55 - 12:00	Wilhelm Palm, Max Planck Institute of Molecular Cell Biology and Genetics	Effects of Lipoproteins and Dietary Lipids on Drosophila Membrane Lipidomes
12:00 - 14:00	*Lunch, Poster Session*	

Lipid Signaling in Health and Disease			*Chair: Scott Emr*
14:00 - 14:30	I	Harald Stenmark, Oslo University	PtdIns3P in membrane dynamics and tumour suppression
14:30 - 15:00	I	Matthias Wymann, University of Basel	Localized and Versatile: PI3Kγ as a Hub in Inflammation and Obesity
15:00 - 15:30	I	Mohamed Bentires-Alj, Friedrich Miescher Institute Basel	Luminal Expression of PIK3CA Mutant H1047R in the Mammary Gland Induces Heterogeneous Carcinomas
15:30 - 16:00	I	*Coffee, Poster Session*	
16:00 - 16:30	I	Takehiko Sasaki, Akita University	Identification of a novel substrate for phosphoinositide phosphatases
16:30 - 17:00	I	Walter Nickel, Heidelberg University	A Toroidal Membrane Pore Mediates Unconventional Secretion of Fibroblast Growth Factor 2
17:00 - 17:30	I	Tobias Meyer, Stanford University	Pulling and pushing: signal-induced membrane deformations during cell migration
Short Talks			
17:30 - 17:45	I	Daniel Legler, University of Konstanz	Ubiquitylation of the GPCR chemokine receptor CCR7 is key for efficient receptor recycling and cell migration
17:45 - 18:00	I	John E. Burke, Medical Research Council Cambridge	Deuterium exchange mass spectrometry used to probe membrane recruitment of the common oncogene phosphoinositide 3-kinase (p110α)
Flash Talks			
18:00 - 18:05	I	André Nadler, EMBL Heidelberg	Shining light on caged diacylglycerols reveals new insights in the mechanism of the local activation of classical PKCs
18:05 - 18:10	I	Martin Kahms, University of Münster	pH-sensitive fluorescent lipids as novel probes to visualize synaptic vesicle recycling
18:10 - 18:15	I	Marie Kolarova J Heyrovsky Institute of Physical Chemistry	A novel superresolution technique for studying biological membranes - DSOM
18:15 - 20:00	I	*Drinks, Poster Session*	

Thursday, June 7, 2012

Membrane Organization & Function			*Chair: Oleg Shupliakov*
08:30 - 09:00	I	Kai Simons, MPI Dresden	Lipids organizing cell membranes
09:00 - 09:30	I	Paavo Kinnunen, University of Helsinki	Induction of functional & pathological amyloid formation by membranes containing oxidized phospholipids
09:30 - 10:00	I	Patricia Bassereau, Institut Curie	Some Physics of Lipid Sorting
10:00 - 10:30	I	*Coffee Break, Postersession*	
10:30 - 11:00	I	Ingela Parmryd, Uppsala University	Is there any order in the plasma membrane

Short Talks			
11:00 - 11:15	I	Erdinc Sezgin, BIOTEC/TU Dresden	Differential lipid packing as the principle of functional membrane heterogeneity
11:15 - 11:30	I	Sonja Huser, University of Basel	Function of Amphiphysin in Clathrin/AP-1 coated vesicle formation
11:30 - 11:45	I	Mario Brameshuber, Vienna University of Technology	Techniques for imaging of nanoplatforms in the live cell plasma membrane
11:45 - 12:00	I	Nina Jaensch, University of Geneva	Novel endocytosis assay reveals the importance of lipid remodeling of GPI-APs for their
12:00 - 14:00	I	*Lunch, Poster Session*	
Lipid Dynamics, Modelling and Visualisation			*Chair: Patricia Bassereau*
14:00 - 14:30	I	Jürgen Klingauf, University of Münster	High resolution microscopy of synaptic vesicle recycling - A readily retrievable pool
14:30 - 15:00	I	Elina Ikonen, University of Helsinki	Control of LDL-receptor trafficking and cholesterol homeostasis by NDRG1
Short Talks			
15:00 - 15:15	I	Kevin Crosby, University of Amsterdam	Brightness analysis and super-resolution fluorescence microscopy reveals molecular-level details on the self-association and distribution of the Ca2+/phospholipid binding protein annexin A4
15:15 - 15:30	I	Xueli Guan, Swiss Tropical and Public Health Institute	Lipidomics of host-pathogen interactions: human macrophage as a cellular system to study functional implications of lipid metabolism during infection
15:30 - 16:00	I	*Coffee, Poster Session*	
16:00 - 16:30	I	Howard Riezman, University of Geneva	Sphingolipid synthesis and function
16:30 - 17:00	I	Gisou van der Goot, EPFL Lausanne	Protein palmitoylation and the consequences for ER functions
17:00 - 17:30	I	Tom McIntyre, Cleveland Clinic, Cleveland	Oxidatively Truncated Phospholipids Connect TNFα to Intrinsic Apoptosis
17:30 - 18:00	I	Round Table: How do we view membranes in the future? *Chair: Kai Simons*	
18:00 -	I	*Conference Dinner*	Departure from conference: 18:15 Departure of boat at "Schifflände": 18:35

Friday, June 8, 2012

Measuring and Manipulating Lipids			*Chair: Olivier Pertz*
08:30 - 09:00	I	Philippe Bastiaens, MPI Dortmund	The wanderings of the proto-oncogene product Ras
09:00 - 09:30	I	Carsten Schultz, EMBL Heidelberg	Tools for visualizing and manipulating cell biology

Short Talks			
09:30 - 09:45	I	Florent Beaufils, University of Basel	Chemical Development of Intracellular Protein Cross-Linkers
09:45 - 10:00	I	Rafael Fritz, University of Basel	The Slit-Robo-srGAP pathway regulates cell repulsion
10:00 - 10:30	I	*Coffee, Poster Session*	
10:30 - 11:00	I	Markus Wenk, National University of Singapore	Biochemical membrane lipidomics during Drosophila development
11:00 - 12:00	I	Plenary Lecture: Rob Irvine, Cambridge University	Metabolism and functions of PIPs: up-to-date thoughts for new and old lipids
12:00 -12:10		Conclusion of meeting	
12:15 - 13:15	I	*Lunch*	

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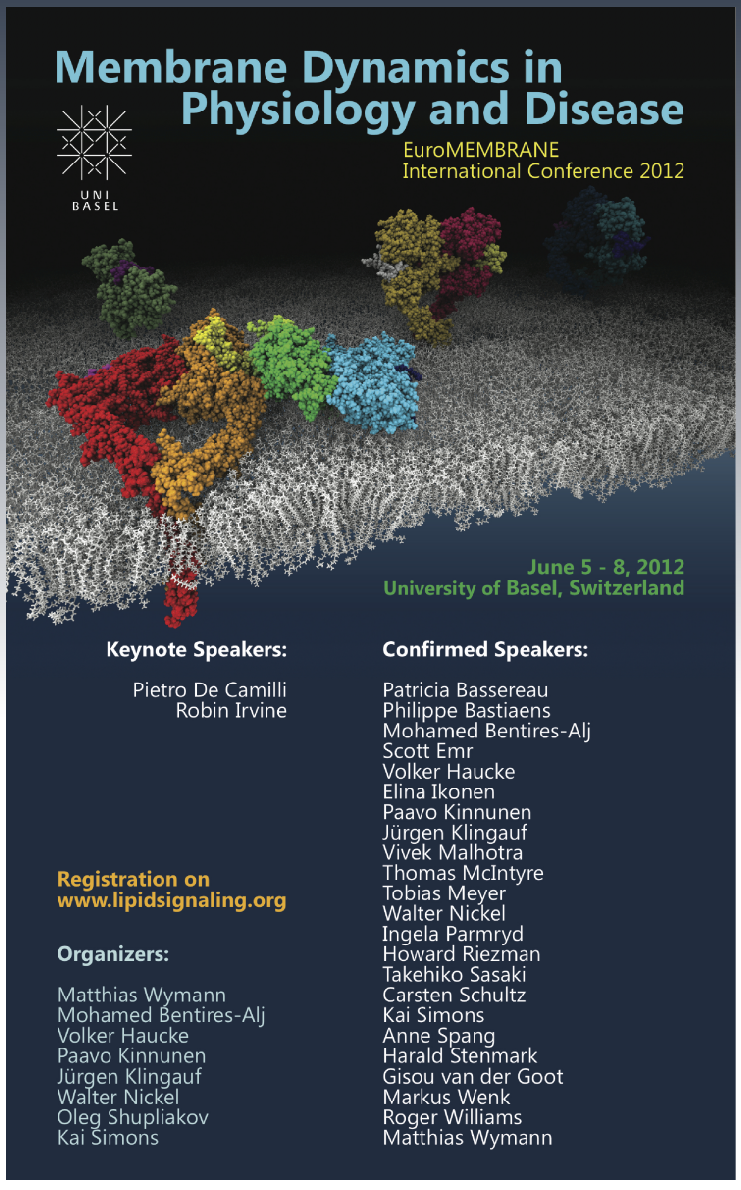
Abstract Book

Membrane Dynamics in Physiology and Disease

Basel, June 5 - 8, 2012



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Some Physics of Lipid Sorting

Patricia Bassereau

PhysicoChimie Curie- Institut Curie, Paris (France)

Membrane transport implies sequential events: membrane budding and formation of a transport intermediate (vesicle or tube), transport and eventually fusion with the acceptor membrane. During vesicle budding, sorting occurs: some lipids and proteins are selectively incorporated into these transport intermediates. It has been proposed that lipids can be dynamically sorted due to membrane bending during coat formation; the driving force for sorting in this case is the reduction in bending energy upon redistribution of the lipids between low and high curvature membranes. We have used membrane nanotubes with a controlled diameter (15-500 nm) pulled from Giant Unilamellar Vesicles to test the hypothesis. We will show that curvature-induced lipid sorting only occurs if the membrane is close to a demixing point. Lipids producing soft membranes, i.e. unsaturated phosphatidylcholine (DOPC), are enriched in membrane tubes, as compared to sphingolipids. However, using the B-subunit of Shiga toxin binding to Gb3 lipids and inducing tubular invaginations both in vivo and in vitro, we will demonstrate that the invaginations induced by STxB are enriched in sphingomyelin, and depleted from DOPC. This suggests that lipids interacting favorably with a membrane deforming protein or with its receptor can be co-sorted in curved structures, overriding the curvature-induced sorting effect.

Reference:
Callan-Jones A., Sorre B., Bassereau P. (2011). Curvature-driven lipid sorting in biomembranes. Cold Spring Harbor Perspectives in Biology 3, a004648.

The wanderings of the proto-oncogene product Ras

Philippe Bastiaens

Department of Systemic Cell Biology, Max Planck Institute for Molecular Physiology, Dortmund, Germany

Oncogenic, gain of function mutations in genes that encode signal transduction proteins, do not only change the intrinsic activity of the oncogene product but also change the “internal” state of the signal transduction network in which the oncogene product is embedded. From this point of view, oncogene products change the collective state of a multi-component network (the cytoplasmic state) such that the cells adopt a more immature/embryonic phenotype that responds less or differently to extracellular cues that maintain its original differentiated behavior. Many oncogene products occur in the early signal transduction machinery at the plasma membrane where they upset the balance of reactions such that cytoplasmic states are generated that are independent of the information contained in the extracellular milieu. Because the ability of an oncogene product to couple into a signaling network is affected by the oncogene product’s spatial distribution in the cell, and thereby determine the cytoplasmic state or activity pattern of growth factor signaling networks, it is of great value to investigate how spatially organizing reaction system affect the oncogene product’s signaling output in the cytoplasm. Based on our recent finding that the peripheral membrane proteins of the proto-oncogene Ras family are using farnesyl-binding solubilizing chaperones that help maintain their spatial organization in cells, I will discuss the underlying principles of the opposed molecular mechanisms of directional flux and diffusional randomization that pattern Ras proteins in cells and how the pharmacological modulation of this spatially organizing system can be exploited to affect the phenotype of oncogenic Ras containing cancer cells.

Luminal Expression of PIK3CA Mutant H1047R in the Mammary Gland Induces Heterogeneous Carcinomas

Mohamed Bentires-Alj

Friedrich Miescher Institute, Basel, Switzerland

The PI3K signaling cascade, a key mediator of cellular survival, growth, and metabolism is frequently altered in human cancer. Activating mutations in PIK3CA encoding the alpha catalytic subunit of PI3K occur in ~30% of breast cancers. Interestingly, more than 85% of all mutations occur in two hotspots within the gene (E542K, E545K in the helical domain, H1047R in the kinase domain). These mutations result in constitutive activity of the enzyme, transform cells in vitro and increase tumorigenicity in xenograft model but whether they are sufficient to induce mammary carcinoma in mice remains unknown. We demonstrate that expression of mutant PIK3CA H1047R in the luminal mammary epithelium evokes heterogeneous tumors that express luminal and basal markers and are positive for the estrogen receptor. Our results suggest that the PIK3CA H1047R oncogene targets a multipotent progenitor cell and show that this model recapitulates features of human breast tumors with PIK3CA H1047R.

Membrane Dynamics and Phosphoinositide Signaling in the Endocytic Pathway

Pietro De Camilli

Department of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience Neurodegeneration and Repair, Kavli Institute for Neuroscience, Yale University School of Medicine, USA

Endocytosis plays a fundamental role in all cells and a highly specialized role at neuronal synapses. In axon terminals, the efficient endocytic recycling of synaptic vesicle membranes after exocytosis makes possible the reliable function of synapses even during high frequency stimulation, in spite of their distance from the cell body, where new proteins are synthesized. Postsynaptically, endocytosis plays a key role in the regulation of the number of surface exposed neurotransmitter receptors. While much has been learned about endocytosis, our understanding of this process lags behind the field of exocytosis due in part to the multiplicity of endocytic mechanisms. We study such mechanisms using a variety of complementary approaches, which include reconstitution experiments with purified endocytic proteins and lipid membranes, broken cell preparations, intact model cells and synapses, and genetically modified mice. In my talk I will focus on studies of mechanisms underlying membrane deformation and membrane fission at early stages of the endocytic pathway, with emphasis on the role of BAR domain containing proteins, endophilin in particular. BAR domains are protein modules that bind the lipid bilayer and have curvature sensing and curvature generating properties. I will also discuss the regulatory role of phosphoinositide metabolism in the progression of membranes along early stations of the endocytic pathway.

Kinase signaling cascade regulates arrestin-related ubiquitin-ligase adaptor during PM protein turnover

Scott D. Emr, Pichiang Hsu, Yingying Zhao and Jason MacGurn

Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY

Down-regulation of cell surface receptors and transporters is mediated by a series of membrane trafficking steps including ubiquitin-mediated endocytosis, ESCRT-mediated cargo(Ub) recognition, sorting and packaging into vesicles that bud into the lumen of the endosome (MVBs), and endosome-lysosome fusion that delivers sorted receptors into the lumen of the lysosome where degradation occurs. Failure to attenuate growth-factor receptor signaling at the plasma membrane (PM) by endocytic down-regulation can lead to cancer.

In Saccharomyces cerevisiae, ubiquitination mediated by the Rsp5 Ub ligase, yeast Nedd4 homolog, is known to be required for the endocytic down-regulation of numerous PM proteins. Properly folded PM proteins, such as signaling receptors, ion channels, and nutrient transporters, exit the ER and traffic through the Golgi to the cell surface where they mediate their specific functions. Previously, we identified and characterized a family of arrestin-related trafficking adaptors, or ARTs, which recruit the Rsp5 Ub ligase to specific targets at the PM. ART proteins are key determinants of cargo selection during ubiquitin-mediated endocytosis, yet despite the emerging consensus that ARTs function as modular specificity adaptors for Rsp5, we knew very little about how the ART proteins are regulated. Here, we will present evidence that the Art1 protein undergoes a phosphoregulatory cycle. Dephosphorylation of Art1 triggers its activation and recruitment to PM cargo. Using genetics and biochemistry, we discovered that this phosphoregulatory cycle is mediated by a TORC1-Npr1 kinase signaling cascade.

Control of LDL-receptor trafficking and cholesterol homeostasis by NDRG1

Vilja Pietiäinen[1],[2]*, Boris Vassilev[1], Wei Wang[1], Nils Bäck[1], Noam Zelcer[3], Elina Ikonen[1]

[1]*Institute of Biomedicine, Anatomy, University of Helsinki, Finland*
[2]*Institute for Molecular Medicine Finland FIMM, Finland*
[3]*Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Mutations in N-myc downstream-regulated gene 1 (NDRG1) cause Charcot-Marie-Tooth Disease type 4D (CMT4D) neuropathy but the function of the NDRG1 protein is not well understood. We show that NDRG1 regulates low-density lipoprotein (LDL) trafficking via the LDL receptor (LDLR). NDRG1 silencing in A431 cells results in decreased uptake of LDL as a result of reduced LDLR plasma membrane levels. This is accompanied by LDLR accumulation in enlarged endosomes, reduced LDLR degradation and downregulation of the NDRG1 interaction partner Prenylated Rab acceptor 1 (Pra-1). Silencing of Pra-1 results in an LDLR phenotype similar to that upon NDRG1 depletion. Conversely, Pra-1 overexpression rescues the LDLR phenotype in NDRG1 silenced cells, suggesting that the diminished expression of Pra-1 may underlie the endosomal defects in NDRG1 deficient cells. Moreover, co-depletion of Idol, an E3 ubiquitin ligase that regulates LDLR ubiquitination and degradation, can rescue LDLR localization in NDRG1 silenced cells. In oligodendrocytes, a cell type relevant in the pathogenesis of CMT4D, silencing of NDRG1 results in reduced LDL uptake and downregulation of Olig2, a glial cell differentiation factor. As in A431 cells, these phenotypes are rescued by simultaneous silencing of Idol, suggesting that oligodendrocyte cholesterol balance and differentiation are controlled by ligand uptake via LDLR family members. Collectively, our findings provide evidence that NDRG1 deficiency leads to a lipid imbalance due to defective LDLR trafficking and provide insight into how the absence of functional NDRG1 may contribute to dysfunction of myelinating cells in CMT4D.

Metabolism and functions of PIPs: up-to-date thoughts for new and old lipids

Robin Irvine

Dept of Pharmacology, University of Cambridge

Phosphatidylinositol 4-phosphate (PI4P) was the first polyphosphoinositol lipid to be discovered (by Jordi Folch-Pi in 1949), and PI5P was the last (by Lucia Rameh and Lew Cantley nearly 50 years later). We have focused a significant research effort during the last few years on the PI5P 4-kinases (PI5P4Ks, also known as Type II PIP kinases), whose most likely physiological role is to regulate the levels of their substrate. We have discovered a remarkable relationship between the three mammalian isoforms, in which we believe that the highly active α isoform is targeted to different places (the nucleus and intracellular vesicles respectively) by the β and γ isoforms, which are much less active.
I will discuss our current data and thoughts on these enigmatic enzymes, and whether PI5P removal really is what they are about. These thoughts are coloured by our very recent data on a potential new function for PI4P (additional to its role as a substrate for PI4P5Ks – Type I PIP kinases) in the plasma membrane, which in turn lead to an altered perspective of the multiple functions of the common product of these PIP kinases, $PI(4,5)P_2$.

High resolution microscopy of synaptic vesicle recycling - A readily retrievable pool

Jürgen Klingauf

Dept. of Cellular Biophysics, Institute for Medical Physics and Biophysics, University of Muenster, Muenster, Germany.

Fusion of synaptic vesicles (SVs) during fast synaptic transmission is mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex assembly formed by coil-coiling of three members of the protein family: synaptobrevin 2 (syb2) and the presynaptic membrane SNAREs syntaxin-1A and SNAP-25. In order to maintain neurotransmission exocytosed SV components need to be retrieved from the surface by compensatory endocytosis. Clathrin-mediated endocytosis (CME) is thought to be the predominant mechanism of SV recycling. However, it might be too slow for fast SV recycling. Therefore, it was suggested that a pre-sorted and pre-assembled pool of SV proteins on the presynaptic membrane might support a first wave of fast CME. We monitored the temporal dynamics of such a 'readily retrievable pool' of SV proteins in hippocampal neurons using a novel probe, CypHer 5, a new cyanine dye-based pH-sensitive exogenous marker, coupled to antibodies against luminal domains of SV proteins. This way we could for the first time demonstrate the preferential recruitment of a surface pool of SV proteins upon stimulated endocytosis. Using fluorescence nanoscopy (isoSTED and FPALM) of labeled SV proteins we could resolve the spatial distribution of the surface pool at the peri-active zone. Recently, we identified dimerisation of vesicular SNARE syb2 as a first important step in self-assembly of surface nanodomains. It has been shown before that syb2 can dimerize, and a glycine residue in the transmembrane domain of syb2 was found to be important for dimerization. Fluorescence Photo-Activation Localization Microscopy (FPALM) of membranes of secretory (PC12) cells reveals re-assembly of exocytosed syb2 into nanoclusters harbouring a few ten molecules, which is abolished when the glycine residue is mutated. Likewise, in cultured hippocampal neurons, using a combination of two pH-sensitive dyes (pHluorin and cypHher) we could show that dimerization of syb2 is necessary for efficient sorting into newly endocytosing SVs.

Is there any order in the plasma membrane?

Ingela Parmryd

Department of Medical Cell Biology, Uppsala University, Sweden

The plasma membrane of eukaryotic cells contains nanodomains that are biochemically and biophysically distinct from the bulk plasma membrane. These domains are known as lipid rafts and are enriched in cholesterol, sphingolipids and phospholipids with saturated acyl chains, which make them more ordered than the surrounding membrane regions. There is a relationship between lipid rafts and actin filaments and the nature of this relationship is the main target of this talk. Using laurdan and di-4-ANEP-PDHQ, fluorophores that have emission peak shifts between ordered and disordered domains, we assessed the relative proportions of ordered and disordered plasma membrane domains in live Jurkat and primary human T cells at 37°C. The cells were subjected to treatments that affect actin dynamics, cause aggregation of membrane components or modify plasma membrane lipid content. We found a common feature in the cellular processes that increase the fraction of ordered plasma membrane domains. Using hopping probe ion conductance microscopy, we have studied the surface topography of live cells at high resolution. We found that the cell surface is neither flat nor smooth. However, topography is ignored by current models of the plasma membrane, which are based on 2D interpretations of events that occur in 3D. Simple diffusion over topographical features can then easily be mistaken for binding or transient confinement. Our results call for a conceptual change in our view of the plasma membrane, which has major implications for how we perceive cell signalling, adhesion and migration.

Identification of a novel substrate for phosphoinositide phosphatases

Takehiko Sasaki, Ken Asanuma, Midori Takasuga, Shinsuke Chida, Hiroki Nakanishi, Junko Sasaki and Shunsuke Takasuga

Department of Medical Biology, Akita University School of Medicine, Akita, Japan

Phosphoinositides are molecules that contain phosphatidylinositol, which has a glycerol backbone, two long-chain fatty acids linked to the glycerol through ester bonds, and an inositol head group that is linked to the glycerol through a phosphate group. A membrane phospholipid structurally related to phosphoinositides is phosphatidylglycerol phosphate (PGP), which is an intermediate in cardiolipin biosynthesis. PGP is dephosphorylated to yield phosphatidylglycerol, which condenses with CDP-diacylglycerol to form cardiolipin. Although hydrolytic activity toward PGP was first described in liver extracts in the early 1960's, little is known about the enzymes that catalyze this reaction. The similarity in configuration between phosphoinositides and PGP prompted us to hypothesize that there might be a bona fide PGP phosphatase among the phosphatases that have been assigned to phosphoinositide metabolism. We screened the known 29 phosphoinositide phosphatases for the activity and found 5 phosphatases that hydrolyze PGP in vitro. We will present and discuss the biochemical characteristics and in vivo roles of the putative PGP phosphatases.

Lipids organizing cell membranes

Kai Simons

Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

The lipid raft concept introduces into membrane organization the capability of dynamic subcompartmentalization based on phase separation. Rafts form dynamic platforms with a key role in regulating membrane functions. They are dynamic assemblies of sphingolipids, cholesterol and proteins that dissociate and associate. These assemblies can be induced to coalesce to form raft clusters and these are the platforms that function in membrane trafficking, cell polarization and signalling. Plasma membranes can also be brought to phase separate like model membranes but in one key property they differ. In contrast to the phase–segregating plasma membrane spheres, the transmembrane raft proteins are excluded from the Lo phase in model membranes. The selective inclusion of transmembrane proteins in the raft phase suggests that this phase possesses a quality in addition to the lipid basis for Lo-Ld phase separation seen in model membranes. Our studies suggest that transmembrane proteins become raftophilic by being lubricated by binding to raft lipids and that this capability is regulated by palmitoylation.

Reduction of N-glycosylation causes cell polarity-dependent loss of cell-cell contacts in the two cell-stage C. elegans embryo

Julia Stevens and Anne Spang

Biozentrum, Growth and Development, University of Basel, Basel, Switzerland

Cell polarity establishment is a key mechanism in the development of any multicellular organism. The one-cell stage C. elegans embryo is highly polarized, requiring sequestration of cytoplasmic polarity factors at the plasma membrane and compartmentalization of the plasma membrane itself. Compartmentalization would aid asymmetric distribution of lipids and proteins, being partially responsible for the fates of the future daughter cells. Since most plasma membrane proteins are glycosylated, we determined the effect of reduction of N-glycosylation on early development. Reduced N-glycosylation did only mildly affect polarity establishment, but cell-cell adhesion was specifically lost only at the two-cell stage. This loss-of-adhesion phenotype was rescued by interfering with either polarity establishment, indicating that polarity establishment is upstream of plasma membrane segregation. The cell adhesion proteins E-cadherin and MAGI-1 were found at contact sites between daughter cells in wild-type but not when N-glycosylation was downregulated. Importantly, they re-appeared at the cell-cell contact sites when PAR-2 function was lost.

Palmitoylation in the ER

Asvin K.K. Lakkaraju[1*], Laurence Abrami[1*], Thomas Lemmin[2], Sanja Blaskovic[1], Béatrice Kunz[1], Akio Kihara[3], Matteo Dal Peraro, F. Gisou van der Goot[1]

[1]*Global Health Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL)*
[2]*Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL)*
[3]*Faculty of Pharmaceutical Sciences, Hokkaido University, Japan*

A third of the human genome encodes N-glycosylated proteins. These are co-translationally translocated into the lumen/membrane of the endoplasmic reticulum (ER) where they fold and assemble before they are transported to their final destination. Here we show that calnexin, a major ER chaperone involved in glycoprotein folding is palmitoylated and that this modification is mediated by the ER palmitoyltransferase DHHC6. This modification leads to the preferential localization of calnexin to the perinuclear rough ER, at the expense of ER tubules. Moreover, palmitoylation mediates the association of calnexin with the ribosome-translocon complex (RTC) leading to the formation of a supercomplex that recruits the actin cytoskeleton, leading to further stabilization of the assembly. When formation of the calnexin-RTC supercomplex was affected by DHHC6 silencing, mutation of calnexin palmitoylation sites or actin depolymerization, folding of glycoproteins was impaired. Our findings thus show that calnexin is a stable component of the RTC in a manner that is exquisitely dependent on its palmitoylation status. This association is essential for the chaperone to capture its client proteins as they emerge from the translocon, acquire their N-linked glycans and initiate folding.

Biochemical membrane lipidomics during Drosophila development

Markus R Wenk

Department of Biochemistry, Yong Loo Lin School of Medicine, and Department of Biological Sciences, National University of Singapore

Once viewed simply as a reservoir for carbon storage, lipids are no longer cast as bystanders in the drama of biological systems. The emerging field of lipidomics is driven by technology, most notably mass spectrometry, but also by complementary approaches for the detection and characterization of lipids and their biosynthetic enzymes in living cells. The development of these integrated tools promises to greatly advance our understanding of the diverse biological roles of lipids (Wenk 2010 Cell 143(6):888-95).

Using liquid chromatography and mass spectrometry, we provide the most comprehensive semi-quantification of lipids during the lifecycle of Drosophila melanogaster (230 glycerophospholipids, 210 sphingolipids, 6 sterols and sterol esters, 60 glycerolipids). New biological insights are revealed through application of this new biochemical resource and will be presented.

Understanding better the fundamentals of natural variation in lipidomes as well as specific recognition of individual lipid species are main scientific aims of SLING, the Singapore Lipidomics Incubator (http://www.sling.nus.edu.sg/). Shaped by a five year competitive research program supported by the National Research Foundation and the National University of Singapore, this centre is a major global magnet for collaborating parties in lipidomics – from academia and industry – delivering new technologies and intellectual capital.

Localized and Versatile: PI3Kγ as a Hub in Inflammation and Obesity

Matthias P. Wymann

Department of Biomedicine, University of Basel

Phosphoinositide 3-kinase γ (PI3Kγ) plays a central role in inflammation [1], allergy [2], cardiovascular [3; 4], and metabolic disease [5]. Targeting of PI3Kγ attenuated rheumatoid arthritis [6], atherosclerosis [7] and allergic responses [2; 8] in mouse models. PI3Kγ complexes are composed of a p110γ catalytic subunit, and one adapter subunit of p84 or p101, which signal in a non-redundant fashion, and produce functionally distinct pools of PtdIns(3,4,5)P3 [8]. Only the p84-p110γ, but not the p101-p110γ complex, requires cooperation with activated Ras [9]. This is currently exploited to modulate cell-specific PI3Kγ activation in inflammatory, proliferative and metabolic disease. PI3Kγ in- and outputs can be controlled by upstream kinases (protein kinase A [5], and protein kinase C [Walser et al.]), and regulate PI3Kγ signaling in a cell-specific fashion. PI3Kγ plays a major role in leukocyte recruitment [10], and has thus been considered as a target to attenuate obesity-linked low-grade inflammation. Indeed, PI3Kγ null mice are protected from high fat diet-induced obesity, metabolic inflammation, fatty liver and insulin resistance. The lean phenotype of the PI3Kγ null mice has been linked to an increased thermogenesis and energy expenditure. Surprisingly, the increase in body fat was not linked to PI3Kγ in hemato-poietic cells [5]. Altogether, PI3Kγ emerges as a signaling node integrating upstream signals from G protein-coupled receptors, but can be switched to process alternative inputs. PI3Kγ signaling has now developed a signature controlling metabolic and inflammatory stress, and can provide entry points for therapeutic strategies in metabolic disease, inflammation and cardiovascular disease. For reviews see: [11-13].

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Posters and Short Talks

Small GTPases of the early secretory pathway control mitochondrial morphology and function

Karin Ackema [1], Ursula Sauder [2], Sabine Ipsen [3], Stefan Eimer [4], Jürgen Hench [3], Anne Spang [1]

[1] *Growth and Development, Biozentrum, University of Basel, Basel, Switzerland.*
[2] *Microscopy Center, Biozentrum, University of Basel, Basel, Switzerland*
[3] *Department of Neuropathology, Institute of Pathology, University Hospital Basel, Basel, Switzerland*
[4] *European Neuroscience Institute Goettingen, Germany.*

The small GTPases of the Arf family play critical roles in membrane traffic by initiating the recruitment of various coat proteins and by modulating the activity of lipid-modifying enzymes. At the Golgi, Arfs are activated by two Arf-GEF families: the Big1/2 proteins and Gbf1. In C. elegans one orthologue of each family has been identified. Similar to the yeast and mammalian counterparts, these Arf-GEFs play important roles in Golgi maintenance and secretion. Here, we report a novel function for GBF-1 in controlling mitochondrial morphology and function. Depletion of gbf-1 led to a similar phenotype as the reduction of the dynamin-related protein DRP-1, without affecting DRP-1 localization. The effect on mitochondrial morphology is evolutionary conserved and requires functional ARF-1 but not the secretory pathway in general. Interestingly, the small GTPase SAR-1, which is essential for COPII dependent trafficking at the ER, also affects mitochondrial morphology, but through a different pathway.

Role of Sec24 isoforms and GTP-hydrolysis in vesicular transport

Frank Adolf, Timo Sachsenheimer, Alexia Herrmann, Oliver Gruß, Iris Leibrecht, Gabi Weiss, Britta Brügger and Felix T. Wieland

Biochemie-Zentrum der Universität Heidelberg (BZH), Germany

COPII coated vesicles bud from the ER and mediate anterograd transport from the ER towards the Golgi. The cytosolic yeast proteins Sar1, Sec23/24 and Sec13/31 are sufficient to drive COPII vesicle formation from microsomal membranes and chemically defined liposomes. In mammals the SNARE/cargo binding subunit Sec24 is expressed in four isoforms (A-D). Recently Sec24 isoform-specific transport of distinct SNARE and cargo proteins was reported in various lines of investigation. Binding of cargo adaptors of the p24 family to the Sec23/24 complex was reported earlier.

We used a multitier-binding assay to discriminate between binding of monomeric and dimeric p24 proteins to the COPII coat. Binding of only dimeric p24 proteins, to the Sec23/24 complexes with KDs in the low affinity range of 10-20 µM.

We further investigated COPII vesicle formation utilizing mammalian semi-intact cells as donor membrane. COPII vesicles were generated in reactions with recombinant Sar1, Sec23/24 and Sec13/31 complex, without the need of other cytosolic factors as shown by western blotting and EM. In addition, COPII and COPI vesicles were efficiently generated when stable nucleotides were used to activate Sar1 and Arf1, respectively, demonstrating that GTP-hydrolysis is not required for vesicle generation in the early secretory pathway. When COPII vesicle where generated with Sec24 isoforms we observed uptake of Sec22b and p24 proteins into distinct vesicles. For a more comprehensive view we are currently investigating the content of isotopic COPII vesicles via mass spectrometry.

Integration of RNA-Seq, shotgun and top down proteomics data identify a quasi-complete expressed proteome of the human model pathogen B. henselae

U. Omasits[1], E. Qeli[1], M. Quebatte[2], B. Roschitzki[3], C. Fortes[3], S. Wu[4], L. Pasa-Tolic[4], C. Dehio[2], C.H. Ahrens

[1]*Quantitative Model Organism Proteomics, University of Zurich (UZH), Switzerland*
[2]*Biozentrum, University of Basel, Switzerland*
[3]*Functional Genomics Center Zurich, ETH and UZH, Switzerland*
[4]*Environmental Molecular Sciences Laboratory, Richland, WA, USA.*

Complete proteome discovery projects provide a wealth of unique information, including the true expression level of proteins, post-translational modifications, and ORFs missed in the genome annotation. However, in contrast to well over 3000 completed genome sequences, no complete proteome has been described so far1.

We have used Bartonella henselae, a model for bacterial-induced tumor growth and host-pathogen interaction, to explore the extent of its expressed proteome, with a particular focus on its membrane proteome. RNA-Seq data identified which genes are actively expressed by bacteria grown under two conditions that mimic the pH-dependent induction of virulence genes in the mammalian host mediated by the BatR/BatS two-component regulatory system. Cytoplasmic, total membrane, inner and outer membrane protein fractions were generated from the same samples, extensively sub-fractionated and analyzed by bottom-up and top-down proteomics on high accuracy mass spectrometers.

Using RNA-Seq data as a sensitive endpoint estimate, directed shotgun proteomics guided by our analysis-driven experimentation (ADE) feedback loop2 was applied to the sub-cellular protein fractions, targeting in particular membrane proteins, short and basic proteins. We identified roughly 85% of the proteins whose genes were expressed, including a similar coverage of membrane proteins, in particular all members of the VirB/D4 type IV secretion system. We will present insights based on strand-specific RNA-Seq, as well as results from a novel, generic proteogenomics approach that identified several previously unannotated ORFs.

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Chemical Development of Intracellular Protein Cross-Linkers

Florent Beaufils, Mirjam Zimmermann, Dominik Erhart, Ruben Cal, Olivier Jacques, Ed Constable, Matthias P. Wymann

Inst. Biochem. & Genetics, Dept. Biomedicine, University of Basel, Switzerland

Protein-protein interactions, and their localization in cellular space determine the activation status of signaling cascades in physiology and disease. A striking example for these processes is the translocation of protein and lipid kinases to the plasma membrane when cells are stimulated via growth factor-, immune- or G protein-coupled receptors. It has been established recently that cytosolic or membrane localization of signaling molecules is crucial, but in many cases a more specific integration in signalosomes and membrane micro-domains determines function. Current available molecular tools to dissect spatial signaling include a Rapamycin-inducible FKBP12/FRB dimerization system. As the FRB domain is derived from target of rapamycin (TOR), rapamycin-derivatives used here interfere with a central hub in cellular signaling, making it unsuitable to study processes involved in growth, immunity and metabolic control.

Here we present a novel protein dimerization and translocation system based on Halo-tag and SNAP-tag (HaXS). This tag combination was selected based on the high rate of reaction with their respective substrates and the absence of endogenous signaling counterparts.
The nature of the chemical spacer between the SNAP- and the Halo-tag substrate is crucial. The optimization of the chemical features of the spacer was planned by the integration of substituents modulating water solubility, cell permeability and structures isolating the two tag reactive moieties.

The resulting system integrating chemical development and matched molecular biology offers multiple opportunities to study protein-protein interactions in vitro or in a cellular environment, and should be useful for the exploration of any intra-and extracellular signaling pathways.

Tracking Brucella Infection

Houchaima Ben-Tekaya[1], Shyan Huey Low[1], Alain Casanova[1], Claudia Mistl[1], Julia Feldmann[2], Raquel Conde-Álvarez[1] and Christoph Dehio[1]

[1] *Biozentrum, University of Basel*
[2] *Swiss Tropical and Public Health Institute, University of Basel*

Brucella is a gram-negative pathogenic bacterium that causes brucellosis, a chronic infection in animals and humans. Brucella can be transmitted to humans by unpasteurized milk products or by aerosols, making brucellosis one of the major zoonosis in the world. During infection, Brucella subverts the intracellular membrane traffic routes of host cells by hijacking early endosomes, late endosomes and eventually endoplasmic reticulum exit sites to reach an ER-derived replicative niche. It was recently proposed that for egress, Brucella acquires Rab7 and autophagy initiation complexes. Despite these recent advances, our understanding of the complete life cycle of Brucella's subversion of the intracellular traffic routes remains sketchy. To tackle this issue, we are establishing multi-colour time-lapse live cell imaging approaches where we track fluorescently labelled Brucella abortus in HeLa cells that stably express markers of the membrane traffic routes. Our initial results confirmed that Brucella abortus acquired the early and late endosomal markers Rab5 and Rab7, respectively. In addition, our data indicated that Brucella initiated its replication near the Golgi apparatus, which was transiently but repeatedly reshaping around the bacteria. Intriguingly, during the replication phase, individual bacteria regained a Rab7-positive compartment; while the rest of the population replicated in a Rab7-negative compartment probably the ER-derived niche. In conclusion, the time-lapse live cell imaging approach we are establishing promises to be a powerful tool for dissecting the dynamics of Brucella trafficking, replication and egress in host cells. This will ultimately shed more light on the molecular mechanisms of Brucella's infection and spreading.

Tracking sphingosine metabolism and transport in sphingolipidoses: NPC1 deficiency as a test case

Tomas Blom[1], Zaiguo Li[2], Robert Bittman[2], Pentti Somerharju[3], and Elina Ikonen[1,4]

[1]*Institute of Biomedicine, Anatomy, University of Helsinki, Finland*
[2]*Department of Chemistry and Biochemistry, Queens College, City University of New York, Flushing, NY, USA.*
[3]*Institute of Biomedicine, Biochemistry, University of Helsinki, Finland,*
[4]*Minerva Foundation Institute for Medical Research, Helsinki, Finland*

The late endosomal/lysosomal compartment (LE/LY) plays a key role in sphingolipid breakdown, with the last degradative step catalyzed by acid ceramidase. The released sphingosine can be converted to ceramide in the ER and transported by ceramide transfer protein (CERT) to the Golgi for conversion to sphingomyelin. At the acidic pH of the lysosome the primary amino-group of sphingosine is protonated, reducing its ability to pass through the LE/LY membrane. The mechanism by which sphingosine exits LE/LY is unknown but Niemann-Pick C1 protein (NPC1) has been suggested to be involved. Here, we used sphingomyelin, ceramide and sphingosine labeled with [3H] in carbon-3 of the sphingosine backbone and targeted them to LE/LY in low-density lipoprotein (LDL) particles. These probes traced LE/LY sphingolipid degradation and recycling as suggested by 1) accumulation of [3H]-sphingomyelin-derived [3H]-ceramide and depletion of [3H]-sphingosine upon acid ceramidase depletion, and 2) accumulation of [3H]-sphingosine-derived [3H]-ceramide and attenuation of [3H]-sphingomyelin synthesis upon CERT depletion. NPC1 silencing did not result in the accumulation of [3H]-sphingosine derived from [3H]-sphingomyelin/LDL or [3H]-ceramide/LDL. Additional evidence against NPC1 playing a significant role in LE/LY sphingosine export was obtained in experiments using the [3H]-sphingolipids or a fluorescent sphingosine derivative in NPC1 knock-out cells. Instead, NPC1-deficient cells displayed an increased affinity for sphingosine independently of protein-mediated lipid transport. This likely contributes to the increased sphingosine content of NPC1 cells.

Topology of the yeast Glycerol-3-phosphate acyltransferase GAT1

A. Bochud, M. Pagac, H. Vazquez de la Mora, C. Duperrex, C. Roubaty, C. Vionnet, and A. Conzelmann

University of Fribourg, Fribourg, Switzerland

The synthesis of phosphatidic acid, the precursor of all glycerophospholipids, is assumed to occur on the cytosolic surface of the ER. In yeast, phosphatidic acid is synthesized de novo by two acyl-CoA dependent acylation reactions catalyzed by a glycerol-3-phosphate acyltransferase (Gat1p or Gat2p) and a 1-acyl-sn-glycerol-3-phosphate acyltransferase (Slc1p or Slc4p/Ale1p). The topology of microsomal Gat1p was probed by various biochemical methods, including detection of the position of dual topology reporters inserted after transmembrane helices, accessibility of native and substituted cysteines (SCAM), protease sensitivity of various inserted tags, and acyltransferase activity sensitivity to membrane impermeant reagents targeting lysine residues. By all these approaches, the conserved acyltransferase domain of Gat1p was found oriented towards the ER lumen. Thus, the first step in biosynthesis of PA in yeast appears to occur in the ER lumen, raising the question of how substrates cross the ER membrane.

Transient Targeting of PI3K Acts as Roadblock in Mast Cells' Route to Allergy

Emilie Collmann[1]*, Thomas Bohnacker[1]*, Romina Marone[1], Janet Dawson[2], Markus Rehberg[3], Rowan Stringer[4], Fritz Krombach[3], Christoph Burkhart[2], Emilio Hirsch[5], Gregory J. Hollingworth[4], Matthew Thomas[4], Matthias P. Wymann[1]

[1]*Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Switzerland.*
[2]*Novartis Institutes for BioMedical Research, Basel, Switzerland.*
[3]*Walter Brendel Centre of Experimental Medicine, Ludwig-Maximilians-Universität München, Munich, Germany.*
[4]*Novartis Institutes for BioMedical Research, Horsham, West Sussex, United Kingdom.*
[5]*Department of Genetics, Biology and Biochemistry, Molecular Biotechnology Center, University of Torino, Torino, Italy.*

The number of tissue-resident mast cells is dynamically regulated by the recruitment of mast cell (MC) progenitors from the vasculature to exacerbated tissues. Blocking MC progenitor flux provides therefore a yet unexploited strategy to alleviate inflammation and allergy. Here we demonstrate that the severity of an allergic response correlates with mast cell recruitment during IgE-mediated allergic sensitization. Gene targeted mice without functional PI3Kγ and a novel PI3Kγ-specific inhibitor (HBC520) identify PI3Kγ as a crucial element to initiate mast cell accumulation in IgE-challenged skin. PI3Kγ was essential for the release of TNF-α from IgE-decorated mast cells. Loss of PI3Kγ activity reduced the capacity of mast cells to trigger the exposure of VCAM-1 and ICAM-1 on human umbilical vein endothelial cells (HUVECs). In addition, fluorescently labeled, PI3Kγ-deficient bone marrow-derived mast cells (BMMCs) lost their capability to adhere to TNF-α treated cremaster muscle endothelia as monitored by intravital microscopy, while PI3Kδ was not required in the process. The TNF-α inhibitor etanercept (Enbrel®) blocked IgE-induced mast cell recruitment completely, linking tissue mast cell cytokine release to endothelial activation and mast cell recruitment. Although PI3Kγ is required for full-scale tissue mast cell activation in anaphylaxis, our study highlights mast cell precursor recruitment as a dominant, and facilitated target to modulate anaphylaxis, and provides a novel strategy to achieve control of allergic conditions by the elimination of a mast cell progenitor flux requiring PI3Kγ activity.

Techniques for imaging of nanoplatforms in the live cell plasma membrane

[1]M. Brameshuber , [2]J. Weghuber , [3]V. Ruprecht , [4]I. Gombos , [4]I. Horvath , [4]L. Vigh , [5]P. Eckerstorfer , [5]E. Kiss , [5]H. Stockinger , [1]G.J. Schütz

[1]*Institute of Applied Physics, Technical University of Vienna*
[2]*Upper Austria University of Applied Sciences, Wels*
[3]*Institute of Science and Technology, Klosterneuburg*
[4]*Institute of Biochemistry, Biological Research Center, Szeged, Hungary*
[5]*Institute for Hygiene and Applied Immunology of the Center for Pathophysiology, Infectiology and Immunology,*
Vienna

We recently developed a method termed TOCCSL ('Thinning out Clusters while Conserving Stoichiometry of Labeling') which allows for the first time the direct imaging of nanoscopic stable platforms with raft-like properties diffusing in the live cell plasma membrane. Our method senses these platforms by their property to assemble a characteristic set of fluorescent marker-proteins/lipids on a time-scale of seconds. A special photobleaching protocol was used to reduce the surface density of labeled mobile platforms down to the level of well-isolated diffraction-limited spots, without altering the single spot brightness. The statistical distribution of probe molecules per platform was determined by single molecule brightness analysis. For demonstration, we used the consensus raft marker glycosylphosphatidylinositol-anchored monomeric GFP and the fluorescent lipid analogue Bodipy-GM1 which preferentially partitions into liquid ordered phases. For both markers we found cholesterol-dependent homo-association in the plasma membrane of living CHO and Jurkat T-cells in the resting state, thereby demonstrating the existence of small, mobile, stable platforms containing these probes.
Since TOCCSL is suitable for characterizing the mobile fraction of observed marker-proteins/lipids, the information about slowly diffusing or immobile nanoplatforms is not addressable. To gain information on the whole population of observed molecules we will present the concept of an inverse approach ('iTOCCSL'). The fluorescent marker is substituted by a photo-activatable protein linked to the molecule of interest. By irreversibly switching a small fraction of markers from a dark into the fluorescent state single molecule brightness and diffusion analysis after activation will add to the characterization of nanodomains.

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Deuterium exchange mass spectrometry used to probe membrane recruitment of the common oncogene phosphoinositide 3-kinase (p110α)

John E. Burke, Olga Perisic, Glenn Masson, Oscar Vadas, and Roger L Williams

MRC Laboratory of Molecular Biology, Cambridge UK

Most cellular responses to extracellular stimuli have a common component of regulation arising from selective recruitment of a network of signalling complexes to membranes. However, studying these systems remains a daunting task. We have made unprecedented progress in understanding these systems by applying a synthesis of deuterium exchange mass spectrometry (DXMS), X-ray crystallography and FRET spectroscopy towards the PI3 kinase (PI3K) family of proteins. PI3Ks are lipid kinases that are involved in a variety of cellular functions, including growth, proliferation, and metabolism. The importance of regulating PI3K activity is highlighted by the fact that the PI3K p110α catalytic subunit (PIK3CA) is one of the most frequently mutated genes in cancer.

Using DXMS we have examined the activation of wild-type p110α/p85α and a spectrum of oncogenic mutants in three enzyme states: basal, RTK phosphopeptide activated, and membrane bound. Differences in amide exchange rates upon activation show that for wild-type p110α/p85α the transition from an inactive cytosolic conformation to an activated form on membranes entails four distinct conformational events. DXMS results for cancer mutants show that all upregulate the enzyme by enhancing one or more of these dynamic events. Protein-lipid FRET and lipid kinase assays showed that all mutations increased binding to membranes and basal lipid kinase activity, even mutations distant from the membrane surface. Our results elucidate a unifying mechanism in which diverse PIK3CA mutations stimulate lipid kinase activity by facilitating motions required for catalysis on membranes.

Synthesis of photocleavable chemical inducers of dimerisation

Ruben Cal, Florent Beaufils, Viktor Hoffmann, Mirjam Zimmermann, Ed Constable, Matthias P. Wymann

Inst. Biochem. & Genetics, Dept. Biomedicine, University of Basel, Switzerland
Inst. Organic Chemistry, Dept. Chemistry, University of Basel, Switzerland

Protein-protein, protein-membrane interactions and complex receptor interactions play a crucial role in most signaling cascades. Chemical inducers of dimerization (CID) have been developed to promote these interactions in a spatial and temporal way. These CIDs have two binding sites, which react with high affinity to specific protein domains. We have recently developed a new family of covalent CIDs (HaXS), which are highly reactive and specific towards Halo- and SNAP-tag, two protein tags which have no endogenous counterparts.
Here we report our synthetic efforts to produce a new library of HaXS substrates bearing a photo-cleavable linker between the two tag substrate functions. Cell permeable, photo-cleavable CID have not been reported so far. As photo-cleavable groups we chose coumarin- and ortho-nitrobenzyl derivatives due to their cleavage properties and their structural similarity to our best HaXS substrate. These photo-cleavable CIDs would allow the control of the induction of protein dimerisation (signalling pathway → on), and upon irradiation with a laser at a specific wavelength the dimerisation would be disrupted in a local and timed fashion (signalling pathway → off).
Optimization of the physico-chemical properties of the photocleavable group was performed regarding the absorbing wavelength, shifting the absorption of the molecules to lower energy (avoiding UV damages of the cell) and cleavage, achieving a cleavage of the molecules in a few minutes.
First series of photo-cleavable CIDs display excellent cell permeability and dimerization reactivity. In vitro photo-cleavage study has showed successful cleavage of over 95% of the molecule in few minutes. The biological validation of the compounds is ongoing.

Brightness analysis and super-resolution fluorescence microscopy reveals molecular-level details on the self-association and distribution of the Ca2+/phospholipid binding protein annexin A4

Kevin C. Crosby, Mark A. Hink, Theodorus W.J. Gadella

Molecular Cytology, van Leeuwenhoek Centre for Advanced Microscopy
Swammerdam Institute for Life Sciences, University of Amsterdam

The mobility, aggregation, and clustering of membrane proteins are critical elements in cell signaling. Here we present two advanced imaging techniques, brightness analysis and photoactivated localization microscopy (PALM), that allow us to study these processes in cells at the molecular level.

We utilized these methods to characterize the self-association and distribution of annexin A4 in vivo. AnxA4 belongs to a family of proteins found in most eukaryotes that have the shared trait of binding negatively-charged phospholipids in a calcium dependent manner. AnxA4 can induce changes to membrane structure and has been implicated in a range of cellular processes, including exocytosis and the negative regulation of Cl- conductance. Alterations in AnxA4 expression have been linked to certain types of cancer and seem to contribute to increased invasiveness and resistance to chemotherapy.

AnxA4 self-associates upon membrane binding, a characteristic that may be a key factor in this protein's ability to reorganize and restrict the mobility of membranes. While the self-association of AnxA4 has been tracked in living cells utilizing FRET microscopy, the molecular details of this process have thus far only been observed in vitro, using purified proteins assembled on artificial lipid layers.

Brightness analysis has allowed us to visualize the trimeric configuration of AnxA4, which we propose is the predominate mobile form of the protein at the membrane. PALM revealed a clustered distribution, suggesting another level of organization. This assembly of AnxA4 may serve to stabilize membrane domains, perhaps creating signaling platforms and/or serving as a functional scaffold for other proteins.

Molecular mechanism of metabolic branching in the synthesis of Glycosphingolipids

Giovanni D'Angelo[1,2], Michele Santoro[1], Guseppe Di Tullio[3], Elena Polishchuk[1], Antonio Varriale[2], Frances Platt[4], Peter Mattjus[5], Akihiro Harada[6], Maria Antonietta De Matteis[1].

[1]*Telethon Institute of Genetics and Medicine Via Pietro Castellino, 111 Napoli, 80131 Italy*
[2]*Institute of Protein Biochemistry National Research Council Via Pietro Castellino 111 80131 Naples, Italy*
[3]*Consorzio Mario Negri Sud, Via Nazionale 8/A, S. Maria Imbaro (Chieti), 66030, Italy*
[4]*University of Oxford, Oxford, UK*
[5]*Åbo Akademi University, Biochemistry, Department of Biosciences, Turku, Finland*
[6]*Department of Cell Biology and Anatomy, Graduate School of Medicine, Osaka University, Osaka, Japan*

Glucosylceramide (GlcCer) is the common precursor of hundreds species of Glycosphingolipids (GSLs). The synthesis of a given GSL species primarily depends on the cell-specific expression of the dedicated enzymes, however the concomitant expression of enzymes devoted to the synthesis of different species of final GSL is commonly observed in different cell types thus leaving open the question as to whether the final GSL profile is stochastically determined or whether more active mechanisms exist. We have obtained evidence indicating that the channeling of the common precursor GlcCer into the different final GSL species relays indeed on an active process that involves the mode of transport of GlcCer through the Golgi complex. In fact, from its site of synthesis at the cis-Golgi, GlcCer can be either transported via vesicular trafficking through the Golgi stack (undergoing processive glycosylation by the enzymes residing in the different Golgi cisternae) or it can be "shunted" to the trans-Golgi.network (TGN) by the glycolipid transfer protein FAPP2, which operates the non-vesicular transfer of GlcCer. We show that while ganglioside synthesis depends on vesicular trafficking of GlcCer through the Golgi stacks, globoside synthesis relies on the direct delivery of GlcCer to the TGN by FAPP2. We show that, both in diverse cell systems and in mice, the abrogation of FAPP2 function results in a specific globosides synthesis impairment with little if any effect on gangliosides production.
Our results settle a precedent for different modalities to cross the Golgi complex serving differential cargo processing by Golgi enzymes.

Spatial control of Cdc42 signaling by the Golgi matrix protein GM130

Hesso Farhan, Baschieri Francesco, Timo Trefzer

Biotechnology Institute Thurgau

The Golgi apparatus has a widely accepted role in directional cell migration. However, it remains unclear whether the Golgi plays a primary role in the establishment of directional polarity as this would necessitate local control of polarity signaling at the Golgi. In the current work we provide evidence that the Golgi for a primary role of the Golgi in cell migration.
We show that knockdown of the Golgi matrix protein GM130 reduces the activity of the small GTPase Cdc42, a master regulator of polarity. Using FRET-based reporters, we show that knockdown GM130 impairs Cdc42 activation at the Golgi, but has no appreciable effect on Cdc42 activation at the plasma membrane. Thus, GM130 controls local activity of Cdc42 at the Golgi. Several cellular effects of Cdc42 were affected. Knockdown of GM130 (i) reduced the formation of filopodia, (ii) impaired directional cell migration and (iii) affected cyst formation of Caco-2 cells in a 3D culture model. Furthermore, we identify RasGRF as a novel interaction partner for GM130 and show that these two proteins interact at the level of the intermediate compartment (ERGIC). As RasGRF is known to act as an inhibitor for Cdc42, but as an activator for Ras, we hypothesized that GM130 acts as a suppressor of RasGRF, which explains the inhibition of Cdc42 activity in GM130-depleted cells. Accordingly, knockdown of GM130 increased MAPK signaling downstream of Ras. Our findings implicate GM130 in the spatial control of Cdc42 and Ras signaling at the Golgi.

The Slit-Robo-srGAP pathway regulates cell repulsion

Rafael Fritz, Katrin Martin, Olivier Pertz

Institute of Biochemistry and Genetics, University of Basel

Cells constantly interact with their surrounding. This interaction involves an overlap between two cells. We found cell-cell contact to be a potent inducer of membrane dynamics in fibroblasts, stimulating both membrane protrusion and retraction. Within an overlap zone, the protruding part of the cell remains in contact with the matrix and is covered by the upper cell. Lamellipodia and finger-like protrusions squeeze between focal adhesions leading to their partial remodelling.
Repulsive signalling is one part of this constant interaction. We identified srGAP2 (Slit-Robo GTPase-activating protein 2) as a regulator of cell repulsion. Downregulation of srGAP2 inhibits cell repulsion and enhances both cell-cell contact duration as well as cell-cell overlap. This phenotype is driven by altered cytoskeleton dynamics with membranes being strongly protrusive and infiltrating neighbour cells more efficiently. We conducted an siRNA screen to identify further components involved in cell repulsion. Knockdown of Slit and Robo, which are involved in repulsive signalling during axon path finding, enhanced cell contact-dependent spreading and cell-cell overlap. Moreover, Slit2, Robo4 and srGAP2 are enriched in fibroblasts at protruding parts of the cell such as lamellipodia and finger-like protrusions. Thus, migrating cells may utilize a polarized cell repulsion machinery consisting of Slit, Robo and srGAP to sample their environment and to navigate within tissues. Importantly, loss of cell repulsion may be a yet unidentified mechanism behind the acquirement of invasive cell behaviour.

Morphodynamic profiling to explore spatio-temporal signaling networks regulating neurite outgrowth

Ludovico Maria Fusco[1], Kevin Smith[2], Fethallah Benmansour[2], Riwal Lefort[3], Francois Fleuret[3], Pascal Fua[2], and Olivier Pertz[1]*

[1]*Institute for Biochemistry and Genetics, Department of Biomedicine, University of Basel*
[2]*IC-CVLab, EPFL*
[3]*Idiap Research Institute*

Neurite outgrowth requires a highly sophisticated signaling program that involves co-ordinated regulation of cytoskeletal, adhesion and trafficking dynamics. Rho GTPases have the potential to co-ordinate all of these cellular events on time and length scales of tens of seconds and single microns. We have previously elucidated the neurite proteome using shotgun proteomics (1) and have used a bioinformatic approach to identify a neurite-localized 220 protein Rho GTPase interactome. The challenge is now to identify spatio-temporal signaling networks that regulate different neurite outgrowth subfunctions (neurite inititation, elongation, filopodia movement). Neurite outgrowth morphodynamics processed by timelapse imaging is highly informative to understand the spatio-temporal modularity of Rho GTPase signaling. Here we present a robust high-throughput timelapse imaging platform that allows to acquire 240 two-color timelapse movies across a 24 well plate for 24 hours with 12 minute resolution. This is currently being used for performing a siRNA screen of the Rho GTPase interactome mentioned above. In the context of a multi-disciplinary collaboration, this will be interfaced with 1. a computer vision framework that allow to automatically segment neuronal shape and extract a large variety of morphodynamic parameters, and 2. computational machine learning techniques that will allow to phenocluster the morphodynamic features associated with each molecular perturbations. Here, we present our global approach and the first phenotypes observed by targeting part of the Rho GTPase network. Identifying spatio-temporal signaling networks operating during neurite outgrowth, has the potential to greatly simplify the Rho GTPase protein interaction network and make it accessible for modeling efforts.

1. Pertz O.C., Wang Y., Yang F., Wang W., Gay L.J, Gristenko M.A., Clauss T.R., Anderson D.J., Liu T., Auberry K.J., Camp D.G., Smith R.D., and Klemke R.L. (2008) Proc Natl Acad Sci USA 105(6):1931-6

Dimeric p23 and p24 tails modulate an Arf1 activation / inactivation cycle

Iva Ganeva, Stefanie Hubich, Carolin Weimer, Frank Anderl, Inge Reckmann, Priska Eckert, Britta Brügger, Felix T. Wieland

Heidelberg University Biochemistry Center (BZH), Heidelberg, Germany

Machinery proteins required for the formation of COPI vesicles include transmembrane proteins (e.g.members of the p24 family), the small GTPase Arf1 (ADP ribosylation factor 1) and the heptameric coat complex coatomer. The antagonizing activities of ArfGEFs (Arf guanine nucleotide exchange factors) and ArfGAPs (Arf GTPase activating proteins) regulate COPI vesicle biogenesis by driving a cycle of Arf1 activation and inactivation. We have expressed and characterized GBF1, the Golgi-localized GEF for Arf1. Here we characterize initial steps of COPI vesicle biogenesis by analyzing interactions among the machinery proteins and the regulation of activities of ArfGAP1 and the ArfGEF GBF1 in vitro. We find that GBF1 forms a trimeric complex with Arf1-GDP and the dimeric cytoplasmic tails of all p24 proteins. Strikingly, only dimeric p23 and p24 modulate both ArfGAP1 and GBF1 activities in vitro.
Our results shed further light on the particular roles dimeric p23 and p24 play in COPI trafficking: they recruit Arf1-GDP to the membrane, induce coat polymerization and modulate both GBF1 and ArfGAP1 activities. These results further support a model in which the presence of these p24 proteins ensures the efficient uptake of cargo into newly formed vesicles.

mTurquoise2 is the preferred CFP variant for live cell imaging and FRET to YFP

Joachim Goedhart[1], David von Stetten[2], Laura van Weeren[1], Linda Joosen[1], Mark A. Hink[1], Antoine Royant[2], Theodorus W.J. Gadella Jr[1].

[1]*Swammerdam Institute for Life Sciences, Section of Molecular Cytology, van Leeuwenhoek Centre for Advanced Microscopy, University of Amsterdam*
[2]*Structural Biology Group, European Synchrotron Radiation Facility*

Genetically encoded probes and sensors have found a wide application for visualizing biological processes at different scales, ranging from single molecules to complete organisms. Sensors for specific small molecules (second messengers, metabolites) protein activity (kinase activity, activtion of GTPases) and protein-protein interactions are often based on Förster Resonance Energy Transfer (FRET) between spectral variants of green fluorescent protein. Cyan Fluorescent Proteins (CFPs) are widely used as donors in these FRET sensors. The prototypal CFP has long been the modestly bright ECFP. Saturation mutagenesis, guided by structural information, combined with fluorescence lifetime-based screening generated a novel variant, mTurquoise2. This is a brighter variant with faster maturation, longer mono-exponential lifetime and the highest quantum yield (0.93) ever measured for a fluorescent protein. Together, these properties make mTurquoise2 the preferable cyan variant of green fluorescent protein for long-term imaging and as donor for Förster resonance energy transfer to a yellow fluorescent protein (YFP).

Control of specific hematopoietic cell populations by targeting Ras-PI3Kγ interactions

E. Gogvadze[1], T. Bohnacker[1], F. Botindari[1], H.A. Ebhardt[2], P. Ramseier[3], M. Vogelsanger[3], J. Dawson[3], E. Collmann[1], M.P. Wymann[1]

[1]*Inst. Biochemistry and Genetics, Department of Biomedicine, University of Basel, Basel, Switzerland*
[2]*Inst. of Molecular Systems Biology, ETH Zürich, Zürich, Switzerland*
[3]*Novartis Institutes for BioMedical Research, Basel, Switzerland*

Despite the growing knowledge concerning the inherent mechanisms driving inflammatory disease, present treatments mostly alleviate symptoms or broadly suppress the immune system. They show little target cell selectivity, display adverse effects and impair host defense. In allergy, mast cell recruitment and activation initiates disease. Specific attenuation of mast cell activity without an impact on other immune cells would be of great value in allergic therapy.
PI3Kγ is a central protein in hematopoietic cell recruitment (Wymann et al. 2003, TIPS) and mast cell degranulation (Laffargue et al. 2002, Immunity). PI3Kγ is a heterodimer composed of a catalytic p110γ and either p84 or p101 adapter subunit. The adapter proteins have distinct expression pattern - p101 is highly expressed in macrophages and neutrophils, while p84 is the only adapter subunit present in mast cells. Moreover, it was recently shown that p84/p110γ complex, but not p101/p110γ, requires Ras for its activation (Kurig et al. 2009, PNAS). Therefore we hypothesized that the Ras-PI3Kγ axis could represent a promising target for specific regulation of mast cell activity. Indeed, inhibition of Ras signaling with farnesyltransferase inhibitors interfered with various PI3Kγ-dependent functions in mast cells, including activation of protein kinase B, migration, and degranulation. PI3Kγ-dependent macrophage activation and migration was however maintained under these conditions. Our results demonstrate the important role of Ras in mast cell migration and activation, and represent a proof of concept for cell specific targeting of PI3Kγ. It could open novel possibilities for treatment of allergic disease without general suppression of the immune system.

Lipidomics of host-pathogen interactions: human macrophage as a cellular system to study functional implications of lipid metabolism during infection

Xueli Guan

Swiss Tropical and Public Health Institute.

The cell biology of intracellular pathogens (viruses, bacteria, eukaryotic parasites) has provided us with molecular information of host–pathogen interactions. As a result it is becoming increasingly evident that lipids play important roles at various stages of this intricate interaction between the pathogens and their hosts. Being positioned at the cell surface, lipids contribute to the interplay between host and pathogen, acting in first line recognition and host cell signaling during pathogen docking, invasion and intracellular trafficking. Serving as a basic building block of membranes and as an energy source, lipids are critical for the growth and replication of a pathogen.

Despite the growing appreciation of the relevance of lipids in infectious diseases, many gaps remain to be filled. Combining novel lipidomics approaches with synthetic chemistry, infection biology and molecular epidemiology, we aim to link changes in macrophage lipid metabolism, membrane trafficking and immunomodulation to defined pathogens including Mycobacterium spp., and protozoa parasites as well as microbial lipids.

Caenorhabditis elegans Intestinal Structure and Function is Controlled by Hydroxylated Glucosylceramide Production

Hannich JT[1], Marza E[1], David FP[3], Entchev EV[4],Guex N[4], Nesatyy VJ[3], Cosson P[1], Kurzchalia TV[5,6], Lesa GM[2], Riezman H[1]

[1]*University of Geneva*
[2]*MRC Laboratory for Molecular Cell Biology, University College London*
[3]*Ecole Polytechnique Fédérale de Lausanne*
[4]*Vital-IT, Swiss Institute of Bioinformatics*
[5]*King's College London*
[6]*Max-Planck-Institute-CBG.*

In nematodes, as in other systems, sphingolipids have been shown to be involved in important cellular processes including stress responses such as anoxia resistance and apoptosis. Surprisingly, it was shown that complex glycosphingolipids are non-essential in Caenorhabditis elegans. In contrast, glucosylceramide production, which provides the precursors for complex glycosphingolipids, was found to play an essential role in the development of the digestive system: worms that cannot form glucosylceramide (i.e. ceramide glucosyl transferase cgt-3;cgt-1 double and cgt triple mutants) exhibit a non-functional digestive system and therefore arrest at the first larval stage and die of starvation.

To unravel the molecular mechanisms underlying the regulation of gut development, we undertook a genetic screen for synthetic lethal mutants. We have identified fatty acid α-hydroxylation as an important regulator of intestinal function. Lipidomic analysis of the fatty acid α-hydroxylation mutant (slc-1) showed a decrease of glucosylceramide levels compared to wild-type animals, indicating that SLC-1 modulates production of glucosylceramide. slc-1 mutants, like cgt single mutants, showed a mild developmental phenotype. However, slc-1;cgt-1 or slc-1;cgt-3 double mutants arrested their development due to gut defects. Interestingly, cgt-3;cgt-1 double mutants that spontaneously overcame the developmental arrest showed a significant increase in the levels of sphingomyelin, opening the possibility that overproduction of sphingomyelin could be rescuing the developmental phenotype. To test this, we fed the different double mutants with choline, a precursor of sphingomyelin. Indeed, dietary choline was sufficient to partly overcome early larval arrest of double mutants. Based on these results, we propose that the developmental arrest is caused by an accumulation of (non-hydroxylated) ceramide, which can be relieved by increased sphingomyelin production. Experiments are underway to better understand the cell-biological basis of this developmental arrest as well as the metabolic mechanisms that control sphingolipid balance during development.

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Interactions between charged gold nanoparticles $Au144(SR)_{60}$ and lipid bilayers

E. Heikkilä[1], A. Gurtovenko[2], H. Martinez-Seara[1], H. Häkkinen[3], I. Vattulainen[1,4,5], J. Akola[1,3,6]

[1]*Department of Physics, Tampere University of Technology*
[2]*Institute of Macromolecular Compounds, Russian Academy of Sciences*
[3]*Departments of Physics and Chemistry, Nanoscience Center, University of Jyväskylä*
[4]*Department of Applied Physics, Helsinki University of Technology*
[5]*Center for Biomembrane Physics (MEMPHYS), University of Southern Denmark*
[6]*Institut für Festkörperforschung, Forschungszentrum Jülich*

Gold nanoparticles are one type of nanoagents used in nanomedicine for drug delivery and bio-imaging. The interactions between nanoparticles and cell membranes are very relevant to research in the areas of nanotoxicology, biophysics and cell biology. We have performed MD simulations of systems with charged monolayer-protected gold nanoparticles with alkanethiol tail groups, Au144(SR)60, where R=C11H22, and lipid bilayers.

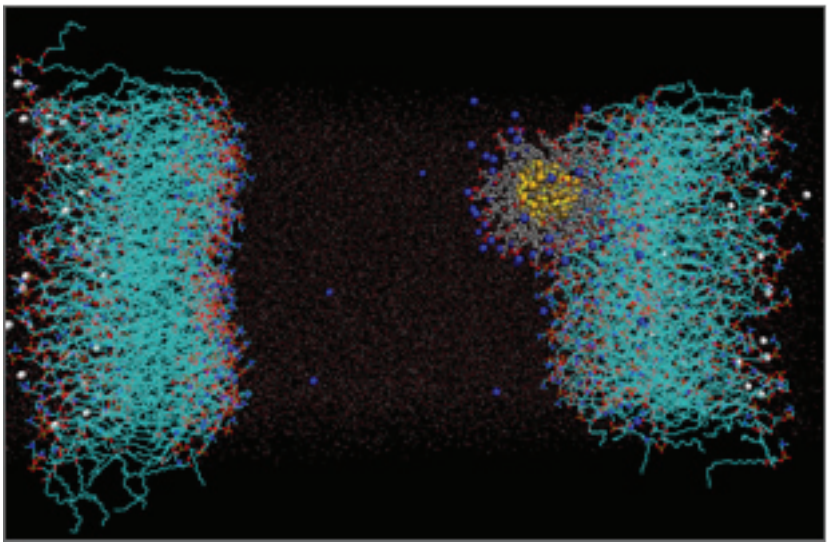


Figure 1. The anionic nanoparticle in extracellular space attaches in a negatively charged lipid bilayer at t = 38 ns.

Akt/PKB-mediated Phosphorylation of Twist1 Promotes Tumor Metastasis via Mediating Cross-talk Between PI3K/Akt and TGFβ Signaling Axes

Gongda Xue, David F. Restuccia, Qiang Lan, Debby Hynx, Stephan Dirnhofer, Daniel Hess, Curzio Rüegg and Brian A. Hemmings

Friedrich Miescher Institute for Biomedical Research

Metastatic breast tumor cells display an epithelial-mesenchymal transition (EMT) that increases cell motility, invasion and dissemination. Although the transcription factor
Twist1 has been shown to contribute to EMT and cancer metastasis, the signaling pathways regulating Twist1 activity are poorly understood. Here we show that Twist1 is phosphorylated in invasive human breast tumors. Akt-mediated Twist1 phosphorylation promotes EMT and breast cancer metastasis by modulating its transcriptional target TGFβ2, leading to enhanced TGFβ receptor signaling, that in turn maintains hyperactive PI3K/Akt signaling. Preventing phosphorylation of Twist1, as well as depletion of TGFβ2, significantly impaired the metastatic potential of cancer cells in vivo, indicating a key role of phosphorylated Twist1 in mediating cross-talk between the PI3K/Akt and TGFβ/Smad signaling axes that supports metastatic tumor development. Our results describe a novel signaling event linking PI3K/Akt hyperactivation in tumor cells to direct regulation of Twist1 activation and tumor metastasis.

Microbial agents stimulate AQP9 expression in inflammatory cells

Angelika Holm[1], Thommie Karlsson[1], Marie Hagbom[2], Vesa Loitto[1], Jonas Wetterö[3], Lennart T. Svensson[2], Karl-Eric Magnusson[1], Elena Vikström[1]

[1]*Div. of Medical Microbiology*
[2]*Div. of Molecular Virology*
[3]*Div. of Rheumatology*
Dept. of Clinical and Experimental Medicine, Linköping University

The aquaporins (AQPs) are a family of water channels involved in cell volume and shape regulation through water transport in and out of the cell, and they are thereby of importance in a number of cellular events. The aquaglyceroporin AQP9 has been suggested to play a pivotal role in inflammatory cell migration, differentiation and metabolism. Thus, we wanted to investigate the role and regulation of AQP9 in human primary immune cells in response to inflammatory stimuli, especially the effects of three distinct microbial agents, viz. Salmonella lipopolysaccharide (LPS), Rotavirus enterotoxin NSP4 and Quorum-sensing molecule C12-homoserine lactone (C12-HSL) as analyzed with molecular and imaging techniques.

qPCR analyses of human primary macrophages obtained from healthy blood donors showed a significant increase in the relative mRNA expression of AQP9 upon LPS stimulation. Analyses with Western blots further confirmed this effect at the protein level. Human primary neutrophils also responded with more AQP9 mRNA, and preliminary results from NSP4 and C12-HSL stimulations of macrophages also displayed an increase in the AQP9 mRNA expression.

These results imply participation of AQP9 in immune cell responses to diverse inflammatory stimuli. AQP9 may thus have crucial effects on cell functions, like cell volume regulation, motility and metabolism, at sites of infection and bacterial antigen deposition.

Cullin3 based ubiquitin ligases involved in endocytic trafficking

Michaela Hubner, Jatta Huotari, Ari Helenius, Matthias Peter

ETH Zürich

BTB domain containing proteins serve as substrate-specific adaptors recruiting substrates to Cullin3 (Cul3) based ubiquitin E3 ligases. Functional analysis in mammalian cells has recently revealed that Cul3 plays an important role in endocytosis, especially in endosome maturation processes.

However, the relevant BTB adaptor proteins and the substrates of Cul3 based ligases in those processes are not known so far.
Here, we show how we identified several out of the 200 known BTB adaptor proteins to possess a function in endocytosis. We applied a small- scale siRNA screening approach using image-based readouts of endocytic assays. Upon knock-down of certain BTB adaptor proteins, we see strong defects in endosome maturation and cargo trafficking at various steps of endocytosis.

These studies will help us to identify the physiological substrates of Cul3-based ubiquitin ligases in endocytosis and elucidate the molecular mechanisms Cul3 is involved.

Function of Amphiphysin in Clathrin/AP-1 coated vesicle formation

Sonja Huser, Gregor Suri, Martin Spiess*

Biozentrum, Universität Basel

Transport of cargo through the cell is generally mediated by coated vesicles. Clathrin and the adaptor complex AP-2 are the major coat components of endocytic vesicles originating from the plasma membrane. Less is known about the formation of clathrin coats at the trans-Golgi network (TGN) and endosomes, which involves the adaptor complex AP-1.

In vitro studies showed the minimal requirements for association of AP-1 to liposomal membranes to be activated Arf1, phosphoinositides, and either sorting signals or unknown cytosolic factors. We have used a liposome floatation assay to identified cytosolic proteins collaborating with AP-1 at the membrane. Separation of proteins from bovine brain cytosol with several chromatographic methods yielded an active fraction containing amphiphysin 1, amphiphysin 2, and endophilin A1. All three proteins are known to be involved in clathrin/AP-2 coat formation. They comprise a BAR domain for membrane association as well as binding sites for several endocytic proteins. The proteins were bacterially expressed, purified, and tested in the floatation assay for AP-1 membrane binding activity. Only amphiphysin 2 showed activity, both as a homodimer and as a heterodimer with amphiphysin 1. Activity depended on a motif that was shown to bind to AP-1, AP-2 and clathrin in GST pull-down experiments. In neuronal cells, amphiphysin 1 and 2 colocalized with AP-1 at the TGN. At high expression levels, amphiphysins aggregate and interfere dominantly with AP-1 localization.

Our results indicate that amphiphysin 1 and 2 function not only in endocytosis, but are also part of the machinery forming AP-1/clathrin coats at the TGN and endosomes.

Inositol analogs as probes for analyzing Phosphatidylinositol and Phosphoinositides

Shareef M. Ismail[1,3], Sandip Pasari[2], Martin J. Lear[2], Markus R. Wenk*[1,3]

[1]*NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore*
[2]*Department of Chemistry, Faculty of Science and Medicinal Chemistry Program, Life Sciences Institute, National University of Singapore*
[3]*Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore*

The molecule inositol is biosynthesized from glucose-6-phosphate or transported from culture media. Absence of inositol biosynthesis or media unavailability causes auxotrophy in eukaryotes and some prokaryotes. Inositol is conjugated with CDP-DAG for the synthesis of phosphatidylinositol (PI) lipids. Phosphorylation of PI gives rise to polyphosphoinositides which along with free inositol phosphates, participate in various signalling mechanisms.

Mass spectrometry provides a suitable technique for analyzing PI and phosphoinositides, however it is not possible to directly study their localization through microscopy. Phosphatidylinositol has never been observed in the cell and phosphoinositide microscopy has been possible only through expression of fluorescent lipid-binding protein domains (PH, FYVE, PX). This, however, raises concerns over non-lipid interactions.

We explore a method using the variations in enzyme-substrate preferences, particularly that of the primary inositol lipid synthesizing enzyme Phosphatidylinositol synthase and those involved in phosphinositide biosynthesis. Myo-inositol was modified with an azide bio-orthogonal group and supplemented into culture media. Once incorporated into inositol lipids, the azide handle can be targeted with an alkyne functionality through Azide-alkyne Huisgen cycloaddition. Saccharomyces cerevisiae, was selected as the model organism to study the effects of inositol analogues on growth, their interactions with the cellular environment and analyze their incorporation into lipids.

Azido-inositol incorporation was confirmed through mass spectrometry of lipid extracts and gave insight into the substrate requirements of enzymes involved in inositol lipid biosynthesis. Modified lipids were also fluorophore-tagged intracellularly and in lipid extracts. Azido-inositols, thus, show potential as a tool for studying the localization and trafficking of inositol lipids.

Novel endocytosis assay reveals the importance of lipid remodeling of GPI-APs for their internalization

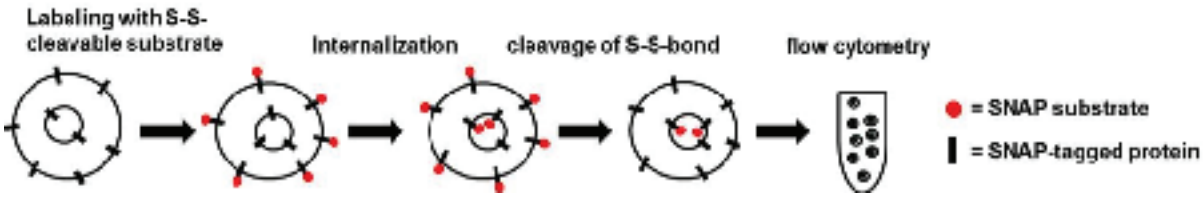
Nina Jaensch[1], Ivan R. Corrêa Jr.[2], Reika Watanabe[1]

[1]*Dept. Biochemistry Sciences II, University of Geneva*
[2]*New England Biolabs, Inc., Ipswich, MA, USA*

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are a class of lipid-anchored proteins that are expressed at the cell surface. Along the secretory pathway, GPI-APs undergo multiple structural modifications. All of them are required for proper transport and/or function of GPI-APs. Among them, lipid remodeling, which occurs in the Golgi apparatus, is a replacement of an unsaturated fatty acid with a saturated fatty acid within the lipid moiety of GPI. This reaction seems essential for GPI-APs to partition into lipid rafts, which are microdomains enriched in cholesterol and sphingolipids. GPI-APs use a unique, non-canonical endocytosis pathway, which is the GPI-enriched endocytic compartments/ clathrin-independent carriers (GEEC/CLIC) pathway.

In order to investigate the physiological role of lipid remodeling of GPI-APs in the endocytic pathway, we established a novel assay to quantitatively measure the rate of endocytosis based on flow cytometry using SNAP-tagged GPI-APs (Fig.1). We compared the rate of endocytosis of GPI-APs in wild-type Chinese Hamster Ovary (CHO) cells and a mutant CHO cell line that is defective in lipid remodeling of GPI-APs.

Interestingly, we found that unremodeled GPI-APs were endocytosed at a lower rate than properly remodeled GPI-APs. Similar to the wild-type cells, endocytosis of unremodeled GPI-APs was still dynamin-independent. Dextran and low density lipoprotein uptake did not seem to be affected in the mutant cells, suggesting the effects were specific for GPI-APs. Currently, we address whether unremodeled GPI-APs still use the GEEC/CLIC pathway. Our study will unravel how hydrophobic interactions among the membrane lipids could influence the internalization step during endocytosis.



pH-sensitive fluorescent lipids as novel probes to visualize synaptic vesicle recycling

Martin Kahms, Cora S. Thiel and Jürgen Klingauf

Department of Cellular Biophysics, Institute for Medical Physics and Biophysics, University of Muenster, Germany

In the analysis of the synaptic vesicle cycle genetically encoded pH-sensitive fluorescent proteins like pHluorin have become indispensable tools. Here, we describe the development of a new class of fluorescent lipid probes, based on pH-sensitive organic dyes coupled to phospholipids, as a promising complementary assay to genetically encoded fluorescent proteins for studying the resorting and recycling of specific lipids parallel to protein retrieval in live hippocampal synapses. These exogenous tracers allow visualizing stimulation-dependent vesicle recycling avoiding genetic perturbation. The corresponding kinetic transients, unlike in pHluorin based experiments, solely reflect membrane turnover during synaptic activity and not protein sorting. Applying different staining and stimulation paradigms, we could estimate the degree of vesicle re-use upon repeated stimulation and reveal the extent of vesicle mobilization at presynaptic terminals. Furthermore, we quantified the incorporation efficacy of different dye-labeled lipids into budding vesicles and found that putative raft-associated lipids incorporate to a lesser degree into synaptic vesicles compared to an abundant phospholipid. In conclusion, this novel class of optical tracers enabled for the first time analysis of lipid recycling in live hippocampal synapses shedding light on membrane recycling and lipid sorting during the synaptic vesicle cycle.

Localized Aquaporin 9-Mediated Water Fluxes Precede Actin Polymerization in the Formation of Filopodia

Thommie Karlsson[1], Anastasia Bolshakova[1], Marco A.O Magalhães[2], Vesa M Loitto[1], Karl-Eric Magnusson[1]

[1]*Division of Medical Microbiology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linkoping University*
[2]*Faculty of Dentistry, University of Toronto*

The interplay of a highly flexible membrane and dynamic rearrangement of the cytoskeleton enables cellular shape changes necessary to adapt to the surrounding milieu. To sense and respond to its environment the cell protrudes long finger-like projections known as filopodia. These are thought to arise from actin polymerization that pushes the membrane forward through a Brownian-ratchet mechanism. However, influx of water through membrane-anchored water channels -aquaporins (AQPs) was recently reported to be involved in this process. Here, we have investigated the role of AQP9 in the development of filopodia. We found that AQP9, when overexpressed in epithelial HEK-293 cells induces filopodia and localizes to the tip during elongation. Furthermore, when analyzing the spatial and temporal distribution of AQP9 and actin microfilaments during filopodia extension, we found that AQP9-induced elongation preceded filamentous actin polymerization. Although not necessary initially for the induction of protrusions, actin was however, essential for the stability and long-term dynamics of AQP9-induced filopodia. Based on this, we present novel evidence and a model for AQP9-induced filopodia, where water fluxes through AQP9 and actin dynamics regulate the cellular protrusive and motile machinery.

Molecular Dynamics Simulations Reveal Fundamental Role of Water As Factor Determining Affinity of Binding of β-Blocker Nebivolol to β2-Adrenergic Receptor

K. Kaszuba[1], I. Vattulainen[1,2,3], M. Karttunen[4], T. Róg[1]

[1]*Department of Physics, Tampere University of Technology, Finland*
[2]*Department of Applied Physics Aalto University School of Science and Technology*
[3]*MEMPHYS-Center for Biomembrane Physics, University of Southern Denmark, Odensee, Denmark*
[4]*Department of Applied Mathematics, The University of Western Ontario, London (ON), Canada*

The β-adrenergic antagonists (β-blockers) constitute a class of drugs that have well-established roles in treatments of various cardiovascular diseases (CVDs). Hypertension, coronary heart disease and heart failure are the most common forms of CVDs. The data collected by World Health Organization (WHO 2010) showed in 2004, 17.1 million people died from CVDs, representing 29% of all global deaths. For many years β-blockers were used for their antiischaemic, antiarryhthmic and anti-hypertensive properties. More recently, the benefit of adrenoceptor blockade was also established in patients with heart failure. Despite a 50 year history, there are two clinically important subtypes of β-adrenergic receptors (βARs) called β_1AR and β_2AR that still are promising drug targets. Our study maps the interactions between nebivolol, one of the most efficient β-blocking agents and the β_2-adrenergic receptor by simulating two optical isomers of nebivolol: ssss-nebivolol and srrr-nebivolol.The srrr-configuration binds preferentially to β_1AR and β_2AR. The ssss-form has much lower binding affinity to both of them. Our simulations suggest that experimentally observed, higher stereoselectivity of the srrr-configuration is due to interactions with water molecules, which extensively hydrate the binding site of β_2AR. By lowering the energy of binding, water enhanced the affinity of the srrr-form to β_2AR.

Oxidized Lipids Sequester Cholesterol in Membranes

Himanshu Khandelia

MEMPHYS: Center for Biomembrane Physics, University of Southern Denmark, Odense

Products of oxidation of polyunsaturated lipids (OXPLs) are implicated in various diseases. 1-palmitoyl-2-azelaoyl-ns-glycero-3-phosphocholine (PazePC) is a stable OXPL present in large amounts in diseased tissues and has also been detected in LDLs, which transport cholesterol between various tissue types.

PazePC contains a lipid acyl tail and a second shortened acyl tail with a terminal polar head (COO–). The chain reversal of the azelaoyl tail in lipid bilayers, imparts to PazePC a conical shape, making it difficult to accommodate high concentrations of PazePC in lipid bilayers. Here, the impact of the OXPL PazePC on the distribution of cholesterol in membranes and the consequent impact on properties of a model membrane have been investigated using course grained molecular dynamics (MD) simulations in ternary PLPC-cholesterol-PazePC mixtures at full hydration. Simulations on time scales of 10s of microseconds show that PazePC sequesters cholesterol into PLPC-depleted regions, and the enrichment of cholesterol in PazePC-rich regions is probably driven by the complimentary shapes of PazePC (cone) and cholesterol (inverted cone). The PLPC-depleted regions are a few nanometers in size, which may be comparable to the size of lipid domains or rafts in biological and model membranes. The cholesterol sequestering phenomena can be of significant biological impact in light of the critical role of cholesterol in modulating membrane structure, in diseases such as atherosclerosis and in driving the formation of functional lipid domains in biological membranes. Therefore, OXPLs like PazePC may exert much of their pathological impact using cholesterol as a proxy.

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A novel superresolution technique for studying biological membranes - DSOM

Marie Kolarova, Jana Humpolickova, Ales Benda, Martin Hof

J. Heyrovsky Institute of Physical Chemistry, Prague, Czech Republic

Fluorescence microscopy is an essential technique for live cell imaging. One of its drawbacks is a rather low diffraction limited spatial resolution, which is described by Abbe's diffraction law. In the last decade several methods improving spatial resolution were developed, including dynamic saturation optical microscopy (DSOM). DSOM is based on spatial monitoring of transition kinetics between bright and long-lived dark states of fluorophores. Here, the application of discontinuous irradiation causes exponential fluorescence decay which demonstrates dynamic transition from bright state to dark states of fluorophore. The higher excitation intensity is used, the faster transition to dark state occurs. By monitoring the decay rates under inhomogeneous illumination of the sample, a higher spatial resolution can be obtained. DSOM was performed on confocal microscope. Intrinsic states of molecules, such as triplet state or dim state of reversibly photoswitchable fluorescent protein Dronpa, were employed as dark states. Experiments done on supported lipid bilayer showed resolution improvement along z-axis by factor of four. Measurements on plasma membrane of living yeast provided moderate lateral resolution improvement (2x). More importantly, the out-of-focal plane fluorescence and autofluorescence were suppressed. Combination of photoswitchable proteins and mild excitation intensities makes DSOM suitable for visualisation of biological membranes.

Involvement of the novel protein Memo in the regulation of SphK1-S1P pathway

Shunya Kondo, Francisca G Maure, Nancy E Hynes

Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

Sphingosine kinases (SphKs) catalyze the phosphorylation of sphingosine to produce the bioactive phospholipid sphingosine-1-phosphate (S1P), which plays a critical role in a wide variety of processes including cellular proliferation, migration and survival. Various extracellular stimuli are known to affect SphK activity to control S1P level and its downstream processes. Here, we have uncovered a role for a novel protein, Memo (Mediator of ErbB2-driven cell MOtility), in the regulation of the SphK1-S1P pathway. Memo is a 34 kDa intracellular protein that was originally isolated as an effector of the ErbB2 receptor, based on its role in cell migration. Memo KO embryos died with hemorrhaging, which is similar to the embryos with KO of SphK1/SphK2, S1P1 (S1P receptor 1) or S1P2/S1P3. Embryos lacking Memo only in endothelial cells had similar features, indicating an essential role for Memo in these cells. In addition, a significant reduction of S1P levels was observed in Memo KO embryos. Migration of Memo KO mouse embryonic fibroblasts (MEFs) toward PDGF was less than that of WT MEFs, and was not further inhibited by blocking SphK1 or S1P1/S1P3, indicating defects in the SphK1-S1P pathway in Memo KO MEFs. Moreover, Memo KO MEFs had a defect in the regulation of SphK1 activity and the intra- and extracellular level of S1P after PDGF stimulation. These data suggest that Memo has an important role in vascular development and indicate that it has a novel molecular function in regulation of SphK1-S1P pathway.

Targeting Membrane Dynamics and Signaling: Synergistic Induction of Glioblastoma Cell Death

Serdar Korur, Brian A. Hemmings, Adrian Merlo, Matthias P. Wymann

University of Basel

Malignant Brain tumours are among the deadliest of all cancers despite intensive therapy. Current therapies are non-specific and highly cytotoxic, and rely on DNA damage causing severe adverse effects. Targeted therapies should achieve a more selective eradication of tumor cells, with less damage to normal tissue. The elucidation of molecular mechanisms underlying brain tumour progression revealed shared cellular pathways among a majority of these tumours. Targeting of such pathways at single nodes is rather inefficient, and has lead to the rapid selection of resistant clones. In order to achieve effective treatment, a simultaneous inhibition of converging signalling pathways is required. Disruptions in the normal metabolic status commonly occurs in cancer cells, which can induce abnormal expression of genes controlling cell cycle, differentiation and apoptosis, resulting in an enhanced tumorigenic potential. In this study, co-administration of compounds targeting membrane dynamics and the metabolic status of cancer cells was observed to induce highly synergistic cell death. The identification of the point of convergence of this molecular mechanism will open novel perspectives to target tumor progression.

Modeling the dynamics of the talin-integrin complex at the lipid bilayer

Sampo Kukkurainen[1], Adam Orłowski[2], Tomasz Róg[2], Ilpo Vattulainen[2], Vesa P. Hytönen[1]

[1]*Institute of Biomedical Technology & BioMediTech, Biokatu, University of Tampere, Finland*
[2]*Department of Physics, Tampere University of Technology, Tampere, Finland*

Integrin-mediated cell adhesion is a process that is critical for the functions of a healthy human body, and is involved in processes spanning from wound healing to cancer progression. Integrins are transmembrane receptors, and their cytoplasmic domains bind a variety of structural and signaling proteins. One of the key proteins binding to the cytodomain is talin that links integrin to the cytoskeleton, and acidic phospholipids are reportedly involved in its regulation.

We utilize computational tools to study the interactions of integrin and the talin head domain at the lipid bilayer. The main question in this on-going study is: How does talin interact with membrane lipids, and do these interactions drive conformational changes in talin?

To address this question, we run all-atom molecular dynamics simulations of structures of the talin head in complex with integrin transmembrane and cytoplasmic domains. The protein complex is embedded in a bilayer of dioleoyl phosphatidylcholine (DOPC) lipids or DOPC + 10% phosphatidylinositol 4,5-bisphosphate (PIP2) in water.

Our preliminary results show ionic contacts that are formed between residues of the talin head and the PIP2 lipids. We also analyze the role of PIP2 lipids in the integrin activation process.

ERM Proteins Regulate GB3 Levels and Shiga Toxin Transport

A. S. M. Kvalvaag[1, 2, 3], S. Pust[1, 3], K. Sandvig[1, 2, 3]

[1]*Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital*
[2]*Department of Molecular Biosciences, University of Oslo*
[3]*Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo*

The actin cytoskeleton is involved in cell motility, endocytosis and intracellular transport. Cytoskeletal interaction with the membrane is essential for these processes, and accessory proteins are neccesary for connecting actin filaments to different membrane components. Ezrin and moesin are members of the ERM (ezrin, radixin and moesin) protein family, known for connecting the actin cytoskeleton to membrane components such as phosphatidylinositol 4,5-bisphosphate and CD44. The ERM proteins have also been found to associate with lipid rafts and to be involved in such processes as endosomal maturation and receptor recycling. To study the role of ERM proteins in endocytosis and intracellular transport, we treated HeLa cells with ezrin and moesin specific siRNA and investigated the effect on various aspects of toxin binding and transport. Depletion of these proteins led to reduced Shiga toxin (Stx) binding to the cell surface. We also observed a decrease in the total level of the Stx receptor Gb3 and several other glycosphingolipids as well as an altered Gb3 distribution. Additionally, we found that ezrin and moesin RNAi leads to an even larger decrease in retrograde Stx transport than the decrease in binding and Gb3 level.

How F-BAR proteins might play a role in membrane dynamics and actin cytoskeleton remodeling coordination?

Fabien Lefebvre, Pontus Aspenstrom

Karolinska Institutet – Microbiology, Tumor and Cell biology department.

In all Eurokaryote organisms, coordination between membrane and cytoskeletal structure are required in order to achieve cell migration, cell division and intracellular trafficking.
Many proteins are involved in the tight regulation of membrane and cytoskeleton dynamics. The family of F-BAR proteins has been shown to have an important role in this mechanism. They are able to bind membranes and induce curvature and many of them also interact with major actors of endocytosis, actin cytoskeleton and Rho GTPases.
Mutation or defects in expression of several F-BAR proteins have been found in diseases such as cancer and mental retardation. In Drosophila, the deletion of the F-BAR protein NWK (Nervous Wreck) lead to defects in neuromuscular junction morphology and uptake of neurotransmitters.
Here, we study the function of the human F-BAR containing proteins NWK 1 and 2 . By a two hybrid screen combined with biochemical approaches, we showed that NWKs can binds to the small Rho GTPases Rac1 and RhoA. These GTPases are key regulators of actin cytoskeleton remodeling. Vav1, a regulator of Rac1 (RhoGEF) has also been found to bind to NWKs. We also indentified a major actor of endocytosis, Intersectin 1 as a binding partner of NWKs.
In addition to physical partners, we have shown that NWKs can interact with Phosphoinositides phosphate with their F-BAR domain.
Even if the function of human NWKs remains unclear, their interacting partners and their ability to bind membranes suggest a strong role in coordination between actin cytoskeleton and membrane dynamics, probably during endocytosis.

Ubiquitylation of the GPCR chemokine receptor CCR7 is key for efficient receptor recycling and cell migration

Karin Schaeuble, Mark A. Hauser, Alexandra V. Rippl and Daniel F. Legler

Biotechnology Institute Thurgau (BITg) at the University of Konstanz, Kreuzlingen, Switzerland

The chemokine receptor CCR7, a class A seven transmembrane domain G protein-coupled receptor, is essential for lymphocyte and dendritic cell homing to secondary lymphoid organs. Due to the ability to induce directional migration, CCR7 and its ligands CCL19 and CCL21 are pivotal for the regulation of the immune system. Here, we identified a novel function for receptor ubiquitylation in the regulation of the recycling process of CCR7. We discovered that CCR7 is ubiquitylated in a constitutive, ligand-independent manner and that receptor ubiquitylation regulates the basal trafficking of CCR7 in the absence of chemokine. Upon CCL19 binding, we show that internalized CCR7 recycles back to the plasma membrane via the trans-Golgi network. An ubiquitylation-deficient CCR7 mutant internalized after ligand binding but provoked a severe intracellular receptor traffic jam, manifested by CCR7 accumulation in the perinuclear compartment. Finally, we demonstrate that the lack of CCR7 ubiquitylation profoundly impaired cell migration revealing that ubiquitin-dependent recycling of CCR7 is pivotal for efficient cell migration. Our results provide clear evidence for a novel function of receptor ubiquitylation in the regulation of CCR7 recycling and migration.

Role of caveolin 1 trafficking in the sorting of sphingomyelin

A. Levy[1, 2]*, C. Lamaze[2], P. Bassereau[1]

[1]*Institut Curie, Centre de Recherche, Laboratoire Physico-chimie Curie, Paris*
[2]*Institut Curie, Centre de Recherche, Laboratoire Trafic, signalisation et ciblage intracellulaires, Paris*

Sorting of lipids and proteins is a key process allowing eukaryotic cells to execute efficient and accurate intracellular transport and to maintain membrane homeostasis. In contrast to proteins, lipid sorting is still poorly understood.

Our group had previously proposed that if a protein has a high affinity for both curved membranes (such as small vesicles or tubules) and for specific lipids, it can lead to enrichment of these lipids in transport intermediates. Since caveolin 1 (CAV1) and sphingomyelin have similar trafficking pathways between the Golgi apparatus and the plasma membrane and since CAV1 has a high affinity for sphingomyelin, we hypothesize that sphingomyelin transport can be mediated by their interaction with CAV1.

To test this hypothesis, we use two complementary approaches. First, we use a powerful in vitro system, the “giant unilamellar vesicles” (GUVs) which has been shown well suited to analyze the effect of a single protein type on the organization of lipid membranes. As a first step, we have developed an improved purification protocol of CAV1 in E Coli system. In the future, we will reconstitute CAV1 in GUVs and membrane nanotubes of controlled curvature will be pulled from them to study caveolin-mediated sorting in curved structures. Secondly, in vivo studies are performed with cells that express or not CAV1. We analyze the distribution of BODIPY-ceramide in order to study the intracellular trafficking of sphingomyelin as a function of the expression of CAV1.

A molecular insight into the impact of oxidized phospholipids on membranes, mimicking oxidative stress conditions in mitochondria-mediated cell death

Martin Lidman, Marcus Wallgren, Quoc Dat Pham, Gerhard Gröbner

Department of Chemistry, Umeå University, Umeå, Sweden

The intrinsic apoptotic pathway is mediated by the mitochondrion, which is the organelle where the Bcl-2 protein family members interact and determine the fate of the cell. A trigger for apoptotic cell death is oxidative stress, under which biological membranes undergo severe changes due to the formation of oxidized phospholipid (OxPl) species, possessing molecular properties differing from their parental lipids. The molecular mechanisms by which OxPls exert their apoptotic action in the mitochondria (e.g. altering membrane protein function) are however poorly understood.

To gain insight into the impact of OxPls on membrane systems we used differential scanning calorimetry (DSC) and solid state nuclear magnetic resonance (ssNMR) spectroscopy to study the effects of OxPls on the organization and biophysical properties of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) membrane systems. Incorporation of OxPls with either an aldehyde (PoxnoPC) or a carboxyl group (PazePC) at their truncated sn-2-chain ends enabled us to reveal OxPl dependent differences.

In the temperature region between 293 K (onset of L-phase) and 298 K two overlapping 31P ssNMR spectra reflect the co-existence of two liquid-crystalline lamellar phases (presumably one reflecting OxPl-poor domains and the other OxPl-rich domains). Deconvolution of DSC profiles also revealed these two partially overlapping thermal events.

Next, ex vivo experiments will be performed on isolated mitochondria derived from healthy, apoptotic and cancer tissue, to which Bcl-2 family proteins will be titrated. In addition, reconstitution of these proteins into mitochondria-mimicking liposomes will provide further mechanistic information about the membrane-mediated apoptotic cell death.

Electrostatic and active process in biomembranes.Effects on the mechanical and dynamical properties of Biomembranes

B. Loubet, H. Khandelia, M. A. Lomholt

MEMPHYS, center for biomembrane physics

In a typical biomembrane system the lipids, the water, the proteins and the ions all exhibit to a certain degree a no zero charge distribution. The lipid head group can be charged and shows a non zero electrical dipole, the water is also polarizable, and the proteins themselves can be charged or exhibiting some electrical dipole. All these charges will interact with each others contributing to the mechanical properties of the membrane, such as the energy it costs to bend or stretch the membrane. We investigate the modification of the membrane mechanical parameters by these electrostatic effects. To this aim we develop a Debye Hückel theory of the membrane including a charge distribution inside the membrane. We then calculate the renormalisation of the tension, bending rigidity and spontaneous curvature in this description of the membrane. We also investigate the effect of a single charge, somehow in the process of being dragged trough the membrane, on the dynamics and fluctuations of the membrane. Such a charge can be thought of to be an ion pumped by an ion pump or a charge permeating trough the membrane. Finally we explore the effect of active processes on the membrane shape fluctuations. We consider different possible origins of the activity such as the cytoskeleton pushing on the membrane or integrated proteins pushing on the lipids and the surrounding bulk fluids. We extend other works on this subject by explicitly taking into account the constrain of the membrane area while the membrane is active. In particular we give the renormalisation of the tension of the membrane due to different active processes under a constraint on the total area. We then compare our theory to experiments showing that in some case the excess area might not be conserved.

Lipid requirements for adenovirus infection

Stefania Luisoni, Maarit Suomalainen, Lukas Tanner, Markus Wenk, Nina Wolfrum, Nathalie Steinhoff, Bettina Cardel, Urs F. Greber

University of Zürich

Viruses carry genetic information between cells and individuals. For infection they overcome cellular membranes and deliver their DNA or RNA genome to the cytosol. Non-enveloped viruses, such as Adenoviruses have evolved distinct mechanisms to breach membranes, including pore formation, or local or global membrane disruption. The viral factors involved in membrane penetration are usually laid-open from the virus by specific cellular cues, such as receptor binding or particular ionic conditions in endosomes. Human adenoviruses (HAdV) are internalized by clathrin-mediated endocytosis and directed to early endosomes where the membrane-lytic virus protein VI is exposed. The binding of the virus to the receptor CAR (coxsackievirus adenovirus receptor) and the movement of the receptor-virus complex together with virus binding to immobile integrin co-receptors support the exposure of protein VI on the viruses during entry. From a druggable genome-wide RNAi screen we identified acid sphingomyelinase (ASM) to be important for HAdV-C2/5 infection. During early stages of entry, HAdV-C2 shows a different ceramide profile compared to the penetration-defective mutant HAdV-C2_TS1. Chemical inhibition of ASM inhibited infection but not virus binding or internalization. These results raise the possibility that ceramides are involved in virus penetration into the cell.

Two-Photon Time-Lapse Microscopy of BODIPY-Cholesterol Reveals Anomalous Sterol Diffusion in Chinese Hamster Ovary Cells

Frederik W. Lund[1], Michael A. Lomholt[2], Lukasz M. Solanko[1], Robert Bittman[3], Daniel Wüstner[1]

[1]*Department of Biochemistry and Molecular Biology*
[2]*MEMPHYS Center for Biomembrane Physics, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark*
[3]*Department of Chemistry and Biochemistry, Queens College, The City University of New York*

Cholesterol is an important membrane component, but our knowledge about its transport in cells is sparse. Previous imaging studies have established that vesicular and non-vesicular transport modes both contribute to sterol trafficking from the plasma membrane to intracellular organelles. However, so far in depth analysis of sterol transport was limited by significant photobleaching. Here we apply a combination of two-photon microscopy and a newly introduced fluorescent cholesterol analog, BODIPY-cholesterol, for prolonged live-cell imaging of sterol transport. Using advanced methods for image analysis we show that non-vesicular BODIPY-cholesterol transport is governed by transient binding to intracellular organelles leading to spatially heterogeneous concentrations and diffusion constants. Tracking of BODIPY-cholesterol vesicles revealed a transition from confined diffusion on time scales < ~5 sec with a transition to superdiffusion on longer time scales. We present a mathematical model which capture both diffusional modes. The mobility of sterol-containing vesicles on the short time scale could reflect dynamic rearrangements of the cytoskeleton, while directed transport of sterol vesicles occurs along cytoskeletal filaments. Interestingly, disruption of microtubules or actin filaments both resulted in a decreased anomalous diffusion constant and velocity. Thus, indicating that both types of filaments are important for the overall dynamics of sterol containing vesicles.

Molecular modeling of the PEGylated bilayer as a model for the PEGylated liposome surface in the bloodstream

Aniket Magarkar[1], Michal Stepniewski[1], Esra Karakas[1], Tomasz Rog[2], Marjo Yliperttula[1], Arto Urtti[1], Alex Bunker[1,3]

[1]*Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland*
[2]*Department of Physics, Tampere University of Technology, Tampere, Finland,*
[3]*Department of Chemistry, Aalto University, Espoo, Finland*

The criteria for effectiveness of drug delivery liposomes (DDLs) are structural stability, site specific targeting, and lifetime in the bloodstream. Often, to increase the bloodstream lifetime, the DDL is coated with poly(ethylene glycol) (PEG). Although this helps to improve the lifetime, there exists plenty of room for improvement in bloodstream lifetime efficacy. The search for an alternative to PEG is a very active field of research, but to apply rational design to this, a knowledge of the mechanism through which PEG functions in a superior fashion to other superficially similar polymers must be determined, and currently our understanding of this is incomplete. We have used molecular dynamics simulation of a set of PEGylated membranes in varying conditions to gain insight into this. We have also performed MD simulation with the Cholesterol as a formulation component of DDL at its effect on stability of the PEGylated DDL. Lastly we looked at the factors in the targeted delivery of the novel targeting moiety identified with phage display experiments. The moiety couldn't increase the targeting efficacy of the DDL when tested invitro and invivo. By MD we could identify the factors responsible for this by investigating the surface structure of the DDL. We show that, addition of salt slightly expands the PEG layer and expands the region of the PEG layer where the Na+ ions are located with various binding affinities to decreasing binding affinity to Na+ K+ and least to Ca_{2+}.

Protein-Phospholipid Interactions: From Biophysics to Therapeutics

Ajay K Mahalka, Paavo KJ Kinnunen

Helsinki Biophysics and Biomembrane Group, Department of Biomedical Engineering and Computational Science, Aalto University, Espoo, Finland

Recently, several lines of evidence have merged to suggest that membranes containing negatively charged and/or oxidized phospholipid (oxPL) cause the accumulation of the cytotoxic peptides onto the membrane surface and induce their aggregation and subsequent conversion to amyloid, with intermediate cytotoxic oligomers being responsible for killing the cells and causing loss of tissue function1,2. Interestingly, the same underlying mechanism involving lipids has been recently concluded to be responsible also for the targeting of host defence proteins3.We also demonstrated that 1-palmitoyl-2-(9′-oxononanoyl)-sn-glycero-3-phosphocholine (PoxnoPC), an aldehyde-derivatized oxPL, accelerates Finnish type familial gelsolin amyloidosis in vitro4.

In addition to counteracting protein aggregation, Hsp70 promotes cell survival by inhibiting the permeabilization of lysosomal membranes5. Hsp70 binds to bis-monoacylglycero phosphate (BMP) that activates the lysosomal acid sphingomyelinase (aSMase), whose activity is essential for the downstream cytoprotective effect, which can be blocked by an antibody against BMP 5. Along these lines we also showed Hsp70 to activate PLA2 in vitro and suggested this activation to result from Hsp70 prolonging the lifetime of the high activity oligomers, counteracting its inhibition by conversion to amyloid6.

Our recent data also suggests that Hsp70 has specifically associates with BMP, cardiolipin, and phosphatidylserine, respectively, in lysosomes, mitochondria, and the outer plasma membrane surface of cancer cells involving pathological conditions and these interactions controlling the orientation and conformation of Hsp70 in membrane7. In conclusion protein-phospholipid interactions open novel venues for the development of therapeutics for amyloid, lysosomal storage disorders (Niemann-pick disease), and cancer.

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Characterization of growth-factor induced rapid fibroblast cell migration

Katrin Martin[1], Noo Li Jeon[2], Olivier Pertz[1]

[1]*Institute of Biochemistry and Genetics, Dept. of Biomedicine, University of Basel*
[2]*School of Mechanical and Aerospace Engineering, Seoul National University*

We have previously shown that different Rho GTPase signaling programs occur at the leading edge of fibroblasts that extend pseudopods in response to an extracellular matrix (ECM) substrate versus PDGF growth factor stimulation(1). This suggests that different actin and adhesion dynamics occur in these two cell migration modes (hapto- versus chemokinesis). In order to study the dynamics of these different cell migration events, we engineered assays that standardize cell shape and morphodynamics and allow robust induction of cell motility. For that purpose, we used microfabrication techniques to pattern 1D fibronectin lines that mimick the anisotropy inherent to fibrillar structures present in the 3D ECM.

Total internal reflection live cell imaging with the Lifeact-GFP probe revealed that this was the result of a drastic remodeling of the actin cytoskeleton in which there was: i) a dramatic loss of stress fibers, ii) formation of a discrete zone at the leading edge with highly dynamic, short lived podosomes and loss of actin retrograde flow observed in the chemokinetic mode, and iii) relocalization of the actin retrograde flow to the lateral sides of the cell behind the podosome zone moving inwards. This cell migration mode is characterized by exquisite coordination between the front and the back of the cell. We are now characterizing these two cell migration modes by visualizing the dynamics of a variety of cytoskeletal and adhesion components. Because the cell morphodynamics on the 1D substrates are exquisitely stereotypic, this will allow to build integrated models of both cell migration modes.

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Regulation of Ergosterol Biosynthesis during the Adaptation to Osmotic Stress in the Yeast Saccharomyces cerevisiae

Fernando Martínez Montañés[1], Amparo Pascual-Ahuir[2], Markus Proft[2], Roger Schneiter[1].

[1]*Department of Biology, Biochemistry Div., University of Fribourg, 1700 Fribourg, Switzerland.*
[2]*Instituto de Biología Molecular y Celular de Plantas UPV-CSIC, Universidad Politécnica de Valencia, 46022 Valencia, Spain*

Hyperosmotic stress triggers a complex adaptive response which is dominantly regulated by the Hog1 MAP kinase in yeast. Here we characterize a novel physiological determinant of osmostress tolerance which involves the Hog1 dependent transcriptional down regulation of ergosterol biosynthesis genes (ERG). Yeast cells considerably lower their sterol content in response to high osmolarity. The transcriptional repressors Mot3 and Rox1 are essential for this response. Both factors together with Hog1 are required to rapidly and transiently shut down transcription of ERG2 and ERG11 upon osmoshock. Mot3 abundance and its binding to the ERG2 promoter is stimulated by osmostress in a Hog1 dependent manner. As an additional layer of control, the expression of the main transcriptional activator of ERG gene expression, Ecm22, is negatively regulated by Hog1 and Mot3/Rox1 upon salt shock. Oxidative stress also triggers repression of ERG2 and ERG11 transcription and a profound decrease in total sterol levels. However, this response was only partially dependent on Mot3/Rox1 and Hog1. Finally we show that the upc2-1 mutation confers stress insensitive hyperaccumulation of ergosterol, overexpression of ERG2 and ERG11 and severe sensitivity to salt and oxidative stress. Our results indicate that transcriptional control of ergosterol biosynthesis is an important physiological target of stress signaling.

Glycocalyx

Hector Martinez-Seara[1], Reinis Danne[1], Tomasz Róg[1], Andrey Gurtovenko[2], Ilpo Vattulainen[1]

[1]*Tampere Univeristy of Tecnology*
[2]*Institute of Macromolecular Compounds, Russian Academy of Sciences*

Glycocalyx is a highly charged layer of membrane-bound biological macromolecules attached to a cell membrane. This layer functions as a barrier between a cell and its surrounding. Glycocalyx also serves as a mediator for cell-cell interactions and protects a cell membrane from the direct action of physical forces and stresses allowing the membrane to maintain its integrity. Glycocalyx is also involved in development and progression of many diseases.

Glycocalyx is composed of glycosaminoglycans, proteoglycans and other glycoproteins bearing acidic oligosaccharides and terminal sialic acids. Most glycocalyx associated proteins are transmembrane that can be linked to the cytoskeleton. This linkage not only restricts their position and constitutes the foundation of the glycocalyx structure, but it also allows signal transduction from the external to the internal parts of a cell. Here we focus on the endothelial cells' glycocalyx.

The aim of this project is to employ Molecular Dynamics simulations to gain insight into the molecular structure and functions of the glycocalyx. This choice is justified by the fact that experimental studies of the glycocalyx are to a large extent restricted by its dynamic and soft nature driven by thermal fluctuations, implying that the glycocalyx has no unique state or structure but it instead is expected to fluctuate significantly in time. Only recent progress in the development of molecular simulation techniques and the availability of larger computer resources have fostered studies of complex biological systems such the glycolalyx itself.

Expression of oleosin and perilipins in yeast promotes formation of lipid droplets from the endoplasmic reticulum

Nicolas Jacquier, Shirish Mishra and Roger Schneiter*

Department of Biology, Division of Biochemistry, University of Fribourg, Switzerland

Most cells store neutral lipids in a dedicated compartment, the lipid droplet (LD). These LDs are structurally and functionally conserved across species. In higher eukaryotes, LDs are covered by abundant scaffolding proteins, such as the oleosins in plants and perilipins (PLINs) in animal cells. To analyze a possible function of these proteins in the biogenesis of LDs, oleosin and perilipin family members (PLIN1, ADRP/PLIN2, and TIP47/PLIN3) were expressed in yeast cells and their targeting to LDs, membrane association, and function in neutral lipid homeostasis and LD biogenesis was analyzed. When expressed in wild-type cells, these proteins were correctly targeted to LDs. However, when expressed in yeast mutant cells that lack LDs, oleosin was localized to the ER where it was rapidly degraded. Perilipin family members, on the other hand, did not localize to the ER membrane in the absence of LDs, but they concentrated in punctuate possibly cytosolic structures and lost their membrane association. Photobleaching experiments revealed that PLIN2 and PLIN3 rapidly exchanged their LD association but PLINs did not move over the LD surface as quickly as integral membrane proteins, such as oleosin. Interestingly, expression of these foreign LD proteins in cells containing elevated levels of neutral lipids/TAG in the ER membrane resulted in the induction of LD formation. These results suggest that these LD scaffolding proteins promote the sequestration of neutral lipids within the ER membrane and thereby induce the formation of LDs from the endoplasmic reticulum.

SBP1 gene is a suppressor of ret1-1 mutation in a gene encoding α-subunit of COPI coat complex

Mrozowska Paulina[1,2], Kamińska Joanna[2]

[1]*Warsaw University of Technology, Faculty of Chemistry, Warsaw, Poland*
[2]*Institute of Biochemistry and Biophysics PAS, Warsaw, Poland*

Sbp1 and Scd6 are characterized as proteins that inhibit translation through binding of their RGG domains to the translation initiation factor eIF4G (Rajyaguru et al., 2012).
We show that overexpression of SBP1 gene restored growth at elevated temperature of the ret1-1 thermosensitive mutant with defect in α-COPI subunit and defective retrograde transport from the Golgi apparatus to the endoplasmic reticulum. In contrast, overexpression of SCD6 did not influence the growth of this mutant. Analysis of polysome profiles performed on ret1-1 mutant overproducing Spb1 did not show significant inhibition of general translation initiation. This suggests that the mechanism of suppression is not related to reduced translation. Instead, Western blot analysis showed that overproduction of Sbp1 prevents the decrease of α-COPI level in ret1-1 mutant after the shift to non-permissive temperature. Further analysis revealed that this effect is not connected with elevated stability of the α-COPI protein. Additional experiments are carried out to determine the mechanism involved in Sbp1 effects on the vesicular transport.

Flotillins as ErbB2 stabilizing proteins in breast cancer

Sascha Pust[1,2], Tove Irene Klokk[1,2], Nagham Musa[1,2], Monica Jenstad[2,3], Bjørn Risberg[2,3,4], Bjørn Erikstein[5], Lionel Tcatchoff[1,2], Knut Liestøl[2], Håvard Danielsen[2,3], Bo van Deurs[6], Kirsten Sandvig[1,2,7].

[1]*Department of Biochemistry, Institute for Cancer Research, Oslo University Hospital, 0379 Oslo, Norway.*
[2]*Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, 0379 Oslo, Norway.*
[3]*Institute for Medical Informatics, Oslo University Hospital, 0379 Oslo, Norway.*
[4]*Department of Pathology, Oslo University Hospital, Norway.*
[5]*Oslo University Hospital, 0450 Oslo, Norway*
[6]*Department of Cellular and Molecular Medicine, The Panum Building, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark.*
[7]*Department of Molecular Biosciences, Faculty of Mathematics and Natural Sciences, University of Oslo, Oslo, Norway.*

Dysregulation and overexpression of the receptor tyrosine kinase ErbB2 is highly implicated in cancer. In breast cancer, high ErbB2 levels are associated with poor patient prognosis. ErbB2 is the preferred dimerization partner for the other ErbB family members and is highly expressed at the plasma membrane; causing constitutive signaling that promotes cell growth and anti-apoptotic effects. In contrast to the other ErbB receptors, no natural ligand has so far been identified for ErbB2. Here, we describe a novel mechanism for interfering with ErbB2 signaling in breast cancer. We can demonstrate a positive correlation between the expression levels of ErbB2 and the flotillin scaffolding proteins in both SKBR3 breast cancer cells and in clinical breast samples. Flotillins are involved in endocytic mechanisms and cellular trafficking, and are mainly localized at the membrane and endosomal/lysosomal compartments. Interestingly, we show that flotillins are involved in stabilization of ErbB2 at the plasma membrane. Depletion of flotillin-1 and flotillin-2 leads to destabilization and internalization of ErbB2, followed by downregulation and reduced activation of the receptor. We have evidence that flotillins are in a complex with ErbB2 and Hsp90. Depletion of one of these proteins results in disruption of the complex and destabilization of ErbB2 at the membrane, resulting in internalization and degradation of the receptor. In summary, our data reveal a new mechanistic connection of flotillin proteins and ErbB2, which is important for the understanding of receptor regulation but also for the development of new clinical drugs.

Shining light on caged diacylglycerols reveals new insights in the mechanism of the local activation of classical PKCs

André Nadler, Gregor Reither, Suihan Feng, Frank Stein & Carsten Schultz*

EMBL Heidelberg

Diaclyglycerols (DAGs) act as second messengers in a number of important cellular processes including PKC activation and growth factor signaling.1 They are part of the calcium-based signaling network and some species serve as precursors for the biosynthesis of endocannabinoids.2,3 So far, DAG induced signaling has largely been mimicked by the application of nonphysiological analogs such as phorbol esters or 1,2-dioctanoylglycerol. However, this approach does not account for the diversity of DAG fatty acid compositions nor do these compounds allow for studying the effect of locally elevated DAG levels. To address these drawbacks, we synthesized a set of photoactivatable (caged) DAGs and investigated their properties in single cell uncaging experiments. We found that a single species, 1,2-steaoryl-arachidonylglycerol (SAG), was significantly more potent in triggering Trp-channel mediated calcium influx in comparison with various other DAG species. Local photoactivation of SAG induced a stable DAG gradient, which was accompanied by locally elevated calcium levels and is most likely maintained by a feed-forward loop that depends on the local accumulation of PKCα. We propose that the physiological equivalent of this stimulus is triggered by the local hydrolysis of phosphoinositides, which finally accounts for elevated SAG levels, whereas DAGs derived from other pathways will be less effective in triggering similar events.

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Chemistry in Caenorhabditis elegans

Ulrike Pässler[1], Ratni Saini[1], Sider Penkov[2], Sebastian Boland[2], Teymuras V. Kurzchalia[2], Hans-Joachim Knölker[1]

[1]*Department Chemie, Technische Universität Dresden, Dresden, Germany*
[2]*Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany*

The life cycle of C. elegans from embryo to adult via different larval stages has been extensively studied. Of special interest is the dauer stage in which larvae endure times of harsh environmental conditions, such as overcrowding or starvation.[1] In this stage the reproductive live cycle is arrested and dauer larvae with special morphology are formed.

PART A – Synthesis of Dafachronic acids

The chemistry which is responsible for entering the dauer stage is still not fully understood. Dafachronic acids, steroidal ligands for the hormonal DAF-12 receptor, play a crucial role in this process. The synthesis of three new dafachronic acids is discussed (Figure 1).[2]

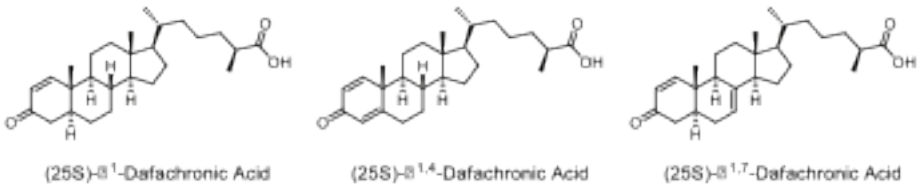

Figure 1. Three new dafachronic acids, synthesized from 3β-hydroxy-Δ5-cholenic acid.

PART B – Synthesis of Maradolipids

The transition from normal larva stage to dauer stage is associated with global changes in the lipid composition.3 A lipid mixture from temperature-sensitive mutant dauer larvae was analyzed by thin layer chromatography and a novel lipid class, now called maradolipids, has been discovered. By degradation experiments (saponification, oxidation, etc.), HPLC TOF/MS, and extensive NMR experiments (1H, 13C with DEPT, COSY, HSQC, HMBC, NOESY, ROESY) the structural assignment led to 6,6'-di-O-acyltrehaloses. Comparison of the spectral data of synthetical diacyltrehaloses (Figure 2) with those of the natural mixture confirmed the assigned structure.4 The synthesis of maradolipids and some analytical results are discussed.

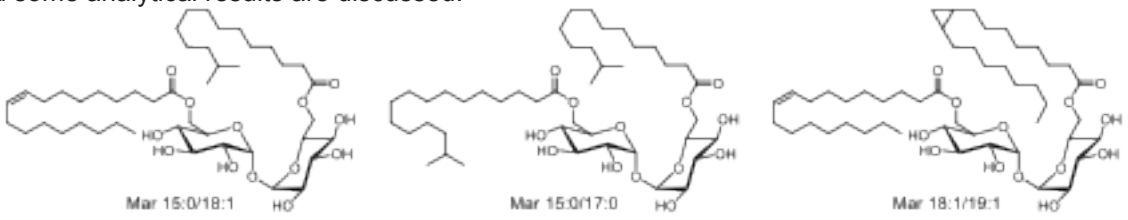

Figure 2. Three examples of synthesized unsymmetrical 6,6'-di-O-acyltrehaloses.

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Effects of Lipoproteins and Dietary Lipids on Drosophila Membrane Lipidomes

Wilhelm Palm, Julio L. Sampaio, Maria Carvalho, Andrej Shevchenko, Suzanne Eaton

Max Planck Institute of Molecular Cell Biology and Genetics

Lipids form the bilayer of cellular membranes, and their composition affects membrane biophysical properties as well as membrane protein function. In complex animals, the precise lipid mixture of cell membranes varies between different tissues and cell types. Tissues can derive lipids from cell-autonomous synthesis, but also acquire them from other organs or dietary sources through circulating lipoproteins. However, the influence of inter-organ lipid transport on polar tissue lipids is not well characterized. To which extent do tissues autonomously maintain their polar lipid composition when challenged with alterations in external lipid supply? Here, we use shotgun mass spectrometry to quantitatively investigate the influence of inter-organ lipid transport and dietary lipids on the membrane lipidomes of individual tissues in Drosophila. We show that Drosophila differentially regulates intestinal uptake, lipoprotein-mediated transport and tissue accumulation of specific sterols. In addition to sterols, lipoproteins provide a significant fraction of specific phospholipid species to cellular membranes. Fatty acids derived from the diet have a direct effect on phospholipid fatty acid residues in tissues throughout the organism. Moreover, the diet indirectly affects the proportions of different phospholipid classes. These studies reveal an unexpected plasticity of membrane lipid composition, which changes both in response to perturbed lipoprotein transport and dietary conditions. They further suggest that *Drosophila* tissues regulate bulk membrane properties rather than the abundance of individual lipid classes.

Morphological and biochemical characterization of the membranous hepatitis C virus replication compartment

David Paul[1], Simone Hoppe[1,2], Inés Romero-Brey[1], Jacomine Krijnse-Locker[1,2], Ralf Bartenschlager[1]

[1]*Department of Infectious Diseases, Molecular Virology, Heidelberg University*
[2]*Electron Microscopy Core Facility, Heidelberg University*

Hepatitis C virus (HCV) is a major human pathogen, persistently infecting more than 170 million individuals worldwide. Like all other plus-strand RNA viruses, HCV also induces rearrangements of cellular membranes, most prominently double-membrane vesicles (DMVs), in order to facilitate its replication. However, the molecular mechanisms of membrane alterations, as well as the composition of DMVs and their exact role in HCV replication, remain poorly understood. In order to gain further insights into DMV constitution we inserted an HA affinity tag into non-structural protein 4B (NS4B), the supposed scaffold protein of the HCV replicase complex. This insertion only slightly delayed RNA replication but otherwise did not affect the viral replication cycle. By using the HA-affinity tag we isolated membranes from cells stably replicating subgenomic HCV RNA, employing a combination of sucrose gradient centrifugation, and subsequent affinity purification. Biochemical analysis of purified membranes revealed co-purification of viral RNA and viral NS3 and NS5A. By using electron microscopy analyses we detected predominantly DMVs, which contained NS3, NS5A and viral RNA. These results underline the central role of DMVs in the HCV replication cycle and suggest that DMVs are the most likely site of viral RNA replication.

Employing lipid droplet heterogeneity to identify novel regulators of cholesterol storage

Simon Pfisterer, Kristiina Kanerva and Elina Ikonen

University of Helsinki

Excessive accumulation of cholesterol esters (CE) in macrophages is one of the hallmarks of atherosclerosis. Also certain carcinomas display marked increases in cellular CE storage. Therefore, investigation of the mechanisms of cholesterol ester storage in mammalian cells promises novel insights into the pathogenesis of prevailing human diseases. Cytoplasmic lipid droplets (LDs) represent the major site of CE and triacylglycerol (TAGs) storage in mammalian cells. Lipid deposition in LDs and their mobilization are regulated by LD-associated proteins. However, the molecular details of how these proteins affect CE storage are not well understood. In S. ceresisiae, CE-enriched and TAG-enriched LDs display distinct profiles of LD-associated proteins (Czabany, T. et al., JBC, 2008), highlighting the heterogeneity of CE- and TAG-enriched LDs. This raises the possibility that LD subtypes are coated by unique sets of regulatory proteins. We have successfully generated either CE- and TAG-enriched LDs in RAW 264.7 macrophages. These CE- and TAG-enriched LDs will enable us to investigate the molecular function of LD-associated proteins in CE storage and mobilization. We will study the association of cholesteryl esterifying and ester hydrolyzing enzymes with LDs and their regulation. Further, we will employ CE- and TAG-enriched LDs in a proteomic approach for the identification of novel LD-associated proteins regulating cholesterol ester storage.

A 3´-Phosphoinositide switch governs the kinetics of Clathrin-mediated Endocytosis

York Posor[1], Marielle Eichhorn-Gruenig[1], Dmytro Puchkov[1], Carsten Schulz[2], Volker Haucke[1]

[1]*Institute of Chemistry und Biochemistry, Freie Universität Berlin*
[2]*Cell Biology and Cell Biophysics Unit, EMBL Heidelberg*

Clathrin-mediated endocytosis (CME) is a key pathway for regulating the surface levels of plasma membrane proteins, including signalling receptors, adhesion molecules, ion channels and nutrient receptors. The view on the role of lipids in this process has been dominated by the importance of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) in the nucleation and assembly of clathrin-coated pits at the plasma membrane. Here, we show that CME requires the activity of the class II PI3K α. Cells depleted of PI3K C2α display reduced internalization of transferrin and severely increased lifetimes of clathrin-coated pits. In reconstitution experiments we show that PI3K C2α requires both its kinase activity and the ability to accept PI(4)P as a substrate in order to sustain CME. Using different tools to manipulate phosphoinositide levels in cells we provide further evidence for a previously undescribed requirement of PI(3,4)P2 in late stages of clathrin-coated pit formation.
Taken together, this new role for PI(3,4)P2 represents the identification of a novel lipid requirement in CME and illustrates how membrane identity of endocytic vesicles is converted towards the PI(3)P positive endosomal compartment already during carrier formation.

Monoubiquitination of Syntaxin 3 regulates its basolateral endocytosis and sorting to intraluminal vesicles in MDCK cells

Elena Reales, Bhattaram Pallavi, Thomas Weimbs

Department of Molecular, Cellular, and Developmental Biology and Neuroscience Research Institute, University of California

Epithelial cells maintain a polarized membrane organization that is essential for the development of multicellular organisms. SNARE protein syntaxin 3 localizes exclusively to the apical plasma membrane and plays an essential role in apical trafficking pathways. Syntaxin 3 misorting has been described to be essential for maintaining the cell polarity. Although many intracellular processes have been associated to be dependent on unique SNARE proteins, how these proteins are sorted and maintain into the correct vesicle or organelles is largely unknown. Here we show that a fraction of Syntaxin 3 is monoubiquitinated in Madin-Darby canine kidney (MDCK) cells. By mutagenesis we identify that ubiquitination happens in a conserved polybasic region of syntaxin 3, closed to the transmembrane domain. We show that ubiquitination-deficient mutant (syntaxin 3-5R) is partially retained to the basolateral membrane and its basolateral endocytosis is diminished. Importantly, using a Rab5Q79L mutant that leads to the formation of giant endosomal structures, we can detect syntaxin 3 wild type in intraluminal vesicles, however, the ubiquitination-deficient mutant cannot be incorporated into these endosomes. Furthermore, we identify by western-blot that syntaxin 3 is present in kidney epithelial and human urine exosomes, and ubiquitination on syntaxin 3 is essential for its efficient secretion via exosomes. Altogether, our data show that monoubiquitination on syntaxin 3 is a signal for endocytosis and sorting to intraluminal vesicles and suggests that ubiquitination on some SNARE proteins may be a general pathway for accurate distribution of these proteins to the specific surfaces, being crucial for maintaining the cell polarity.

The functional role of dynamin targeting to sites of endocytosis in synapses

Kathryn A Rees, Åsa M E Winther, Olga Vorontsova, Elena Sopova, Oleg Shupliakov

Kathryn A Rees, Åsa M E Winther, Olga Vorontsova, Elena Sopova, Oleg Shupliakov
Karolinska Institutet, Stockholm, Sweden

The fission GTP-ase dynamin is localized to the synaptic vesicle pool at rest and is accumulated at the site of endocytosis, the periactive zone (PZ), during synaptic activity. The molecular mechanism controlling its delivery and its role in synaptic function remains illusive. Here we demonstrate that in Drosophila neuromuscular junctions (NMJs) this function is to a large extent controlled by the scaffolding protein, Dap160. In dap160 mutants expressing dap160 lacking dynamin-interacting SH3 domains, dynamin is no longer accumulated in the PZ and is freely dispersed in the nerve terminal. Dap160 and Eps15 are however still targeted to the PZ as in wild type NMJs. Inability to concentrate dynamin at the PZ is accompanied by a reduction in evoked EJP´s amplitude following prolonged high frequency (10Hz) stimulation in 3rd instar larvae, while the amplitude of mEJPs and EJPs induced by low frequency (0.3Hz) stimulation were found to be the same. In addition, a decrease in FM1-43 dye uptake following stimulation was observed, thus indicating that the synaptic vesicle membrane recycling was impaired. High-resolution microscopic analysis of stimulated NMJs in dap160 mutants lacking dynamin-interacting SH3 domains, revealed an accumulation of large endosome-like structures and a decrease in the number of SVs in nerve terminals. Taken together our data indicate that proper targeting of dynamin at the PZ is essential for bulk endocytosis during prolonged high-rate synaptic activity.

Generation and characterization of Rac1 and Cdc42 sensors

Andreas Reimann, Anna Soriguera, Olivier Pertz

Department for Biomedicine, University of Basel

FRET (fluorescence resonance energy transfer) based biosensors allow for studying cell signaling in high spatial and temporal resolution. Rho GTPases have been shown to regulate many cellular mechanisms such as gene expression, proliferation, apoptosis and in particular actin dynamics. Three classes of GTPase regulators are known: inhibitory GDIs (guanine nucleotide dissociation inhibitors) and GAPs (GTPase activating proteins) and activating GEFs (guanine nucleotide exchange factors).

For both Rac1 and Cdc42, two Rho GTPase members, 25 FRET probes were generated by a standardized cloning strategy. To screen for the best sensor, the constructs were co-expressed with Rho GDI or p50Rho GAP and the dynamic range of the probe was obtained by fluorometry. The results show that Rho GDI and p50Rho GAP inhibit both biosensors. To speed up the screening and characterization process, automated ratiometric imaging was performed. The two best Rac1 and Cdc42 probes were co-transfected with different GAPs and GEFs in a 96 well plate and show a decrease (GAPs) or increase (GEFs) in FRET. Importantly, the results obtained by microscopy were consistent with those from fluorometry. Furthermore, the results were validated on a single-cell basis by using stable Rac1 and Cdc42 cell lines.

The simple to use sensor design strategy combined with the automated high-throughput microscopy provide a valid and efficient approach to clone and characterize FRET-based sensors.

Tracking and manipulation of phosphoinositide pools during cell division and autophagy

Kay Oliver Schink[1,2], Axel Norgren[1,2], Camilla Raiborg[1,2], Hilde Abrahamsen[1,2] and Harald Stenmark[1,2]

[1]*Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo*
[2]*Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo University Hospital*

Phosphoinositides, phosphorylated derivatives of phosphatidylinositol, play a critical role in the regulation of membrane trafficking and cytoskeletal organization. The phosphoinositide PtdIns(3)P has been shown to be a key regulator of endosomal trafficking, membrane sorting and autophagy.
Recently, a novel role for PtdIns(3)P during cell division has emerged. Several PtdIns(3)P-binding proteins have been identified that play a role during cytokinesis, the final step of cell division that leads to the physical separation of the dividing cells.
We are interested in the dynamic localization of PtdIns(3)P during cell division and autophagy. In order to elucidate the role of this lipid in these biological processes, we used PtdIns(3)P-binding domains as probes for this lipid in living cells. Time lapse imaging revealed that lapse imaging revealed that PtdIns(3)P-positive structures undergo rapid reorganization during cell division. During cytokinesis, PtdIns(3)P-positive structures accumulate at the cleavage furrow and adjacent to the intercellular bridge between the two dividing cells, possibly providing membrane material necessary for the physical separation of the two cells. Furthermore, PtdIns(3)P accumulates next to the midbody of dividing cells directly prior to abscission, suggesting that PtdIns(3)P-binding proteins are recruited here to ensure correct cell division. We found that this accumulation coincides with the localization of Rab family GTPases, which might locally regulate the synthesis of PtdIns(3)P.
Another important process regulated by PtdIns(3)P is the regulation of autophagy. We found a distinct PtdIns(3)P pool during autophagy that is not recognized by the probes that allow tracking of endosomal PtdIns(3)P.
Currently, we are trying to elucidate the role of PtdIns(3)P during these processes by acute perturbation of this lipid. By site-specific depletion of PtdIns(3)P using a phosphatase system anchored to different organelles, we are trying to analyze the contribution of the distinct PtdIns(3)P pools for different cellular processes.

Critical role of Phosphoinositide 3-kinase γ (PI3Kγ) in microglial phagocytosis

Caroline Schmidt, Nadine Schneble, Matthias P. Wymann, Jörg P. Müller, Reinhard Wetzker

University Hospital of Jena

Phosphoinositide 3-kinases (PI3K, lipid kinases) are central players in intracellular second messenger signalling and control diverse cellular processes including migration, proliferation and differentiation. Here we attempt to investigate the regulatory functions of PI3Kγ in microglial cells, the resident brain macrophages implicated in inflammatory processes of the central nervous system.
These processes are triggered by PAMPs (pathogen associated molecular pattern molecules) or DAMPs (damage associated molecular pattern molecules) leading to the activation of microglia cells. As a consequence microglia change their morphology, produce cytokines, migrate into inflammatory lesions and express their phagocytic function. Following these data we performed a comparative study of the phagocytotic activity of wild type microglia cells isolated from mouse brain, of PI3Kγ deficient microglia (PI3Kγ k.o.) and cells, expressing a lipid kinase deficient mutant of PI3Kγ (PI3Kγ KD).
We could show that PI3Kγ k.o. microglia display a strongly reduced phagocytosis in comparison to wild type and PI3Kγ KD cells. Different pharmacological treatment confirmed an essential role of PI3Kγ in phagocytic processes of microglia cells. The stimulation of microglia with LPS (PAMP prototype) and UDP (DAMP) induces increased phagocytic activity in wild type and PI3Kγ KD cells.
Based on the results that PI3Kγ KD microglia showed the same phenotype as wild type cells we suggest a regulatory function of PI3Kγ independent on its lipid kinase activity. In addition to the lipid kinase activity the scaffold function of PI3Kγ might play an important role in the control of microglia phagocytosis. In cardiomyocytes PI3Kγ has been shown to suppress cAMP level via direct interaction with and activation of PDE3B. Our data indicate similar regulatory function of PI3Kγ in microglia.
Together these data unveil PI3Kγ as a key mediator of the phagocytosis function of microglia.

Using protein micropatterning to probe lipid-mediated protein interactions in the plasma membrane of live cells

Eva Sevcsik, Stefan Sunzenauer and Gerhard Schütz

Institute of Applied Physics, Vienna University of Technology, Vienna, Austria

Micropatterning is a powerful tool to study protein-protein interactions on the live cell plasma membrane. Cells are plated on microstructured surfaces partly covered with ligands (antibodies) targeted against membrane proteins (bait), and the co-localization with a fluorescently labeled protein of interest (prey) is monitored. This assay has been successfully employed to characterize the interaction between CD4, a major co-receptor in T cell signalling, and Lck, a protein tyrosine kinase essential for early T cell signaling (Schwarzenbacher, et al., Nat Methods, 2008).

Here, we employ this technique to probe indirect protein-protein interactions, namely of the lipid-raft associated GPI-anchored protein CD59 and a glycosylphosphatidylinositol-anchored GFP. Interestingly, antibody-mediated micropatterning of CD59 leads to a copatterning of GPI-GFP (and vice versa). The mechanism of this can be envisioned as follows: By patterning of one GPI-anchored protein, a certain membrane microenvironment is created and the other GPI-anchored protein preferentially localizes into this environment. We employ different compounds that have been reported to act as ‘raft’ or “non-raft’ markers to further characterize the nature of these interactions.

Differential lipid packing as the principle of functional membrane heterogeneity

Erdinc Sezgin, Ilya Levental, Ronald Dirkx, Alf Honigmann, Christian Eggeling, Unal Coskun, Michele Solimena, Kai Simons, Petra Schwille

BIOTEC/TU Dresden

Heterogeneity in the cell membrane plays an important role in cellular signaling. However, the physicochemical nature of this heterogeneity is still controversial. Although nanodomains called rafts which are enriched with saturated lipids and sterols are believed to exist in the live cell membrane and form patches upon cellular triggers, the complete picture of the cell plasma membrane remains to be solved. Here, we propose a new concept for the cellular membrane heterogeneity, differential lipid packing. We show that the isolated cellular plasma membrane has the ability to form domains with different lipid packing which modulates the partitioning of the molecules in the coexisting phases. Moreover, it regulates the geometrical structure of the molecules in the membrane. These two features influenced by the lipid packing have been found to be functionally important in receptor-ligand binding. We have shown that Choleratoxin binding to its lipid receptor GM1 is highly regulated by the lipid packing of the domains. Furthermore, lipid order has an important role in Interleukin-4 ligand binding to its receptor. Finally, we have shown that cells can regulate the plasma membrane lipid packing by different mechanisms.

TORC1 Regulates de novo Synthesis of Complex Sphingolipids via Npr1-mediated Phosphorylation of Orm Family Proteins

Mitsugu Shimobayashi[1,2], Wolfgang Oppliger[1], Suzette Moese[1], Yasunori Kozutsumi[2], Paul Jenö[1], Michael N. Hall[1]*

[1]*Biozentrum, University of Basel*
[2]*Graduate School of Biostudies, Kyoto University*

The evolutionarily conserved ORM genes encode transmembrane proteins of the endoplasmic reticulum. In humans, ORMDL3 is a risk factor gene for childhood asthma. Orm proteins regulate sphingolipid homeostasis by inhibiting the activity of serine-palmitoyltransferase (SPT), the first and rate-limiting enzyme in sphingolipid biosynthesis. The homologous yeast proteins Orm1 and Orm2 are phosphorylated in response to a decrease in intracellular sphingolipids, resulting in Orm inhibition and SPT activation. However, the function of the Orm proteins and the signaling pathways modulating Orm phosphorylation are incompletely characterized. We demonstrate that the Orm proteins in Saccharomyces cerevisiae are phosphorylated on both rapamycin-responsive and -unresponsive sites, as revealed by mass spectrometry and subsequent mutagenesis analysis. Target of rapamycin complex 1 (TORC1) negatively regulates phosphorylation of the Orm proteins in a nutrient-dependent and rapamycin-sensitive manner. TORC1 inhibits the kinase Npr1 which directly phosphorylates the Orm proteins. Npr1-dependent Orm phosphorylation triggered by rapamycin, an allosteric inhibitor of TORC1, does not affect SPT activity. Rather, TORC1-inhibited phosphorylation of the Orm proteins is required for de novo synthesis of complex sphingolipids downstream of SPT. Rapamycin-unresponsive Orm phosphorylation, which is mediated by nutrient-independent and rapamycin-insensitive TOR complex 2, activates SPT. Thus, our findings reveal a signaling pathway controlling the Orm proteins in response to nutrients, and indicate that the two TOR signaling branches have partly antagonistic roles in controlling sphingolipid synthesis.

CD1d favors MHC neighborhood and relishes cholesterol domains in the membrane of a B lymphocyte

Dilip Shrestha, György Vereb, Attila Jenei, János Szöllősi

Department of Biophysics and Cell Biology, Medical and Health Science Center, University of Debrecen, Hungary

Cluster of Differentiation 1 (CD1) represents a family of proteins which is recognized for its important roles in innate and adaptive immunity. These proteins expressed on antigen presenting cells (APC), like B-cells, stimulate T cells through lipid antigens. Among the categories of CD1 receptors, CD1d is able to activate a subset of T cells termed as invariant Natural Killer T cells (iNKT). Until now, no such reports on the topological distribution of these receptors in the membrane of a B lymphocyte exist. Therefore, we used fluorescence based microscopic and flow cytometric methods, like Fluorescence Resonance Energy Transfer (FRET) to determine the neighborhoods of CD1d. We observed significant FRET between CD1d and MHC I heavy chain, β2-microglobulin and MHC II molecules suggesting their co-existence in the membrane domain of a lymphocyte. In addition, usage of statin, a cholesterol biosynthesis inhibitor, that depletes intracellular cholesterol or methyl-β-cyclodextrin, that extracts cholesterol from the membrane of a cell, implied that CD1d profoundly existed in cholesterol rich regions. Reduction in FRET between CD1d and GM1 ganglioside, a marker for lipid rafts, was observed on application of both the compounds. Since, MHC proteins were also enriched in GM1 regions, we presume that these proteins can form intricate protein-protein complex. Indeed, our triple co-localization experiment between CD1d and MHC receptors strongly supported this possibility. In the future, we plan to perform a co-culture assay and relate the effect of lipid raft disruption in APCs to changes in cytokine secretion function of iNKT cells.

Distinct roles of p101 and p87 subunits in regulating phosphoinositide 3-kinase γ by Gβγ and H-Ras

A. Shymanets[1], M. R. Ahmadian[2], E. Krause[3], R. Wetzker[4], C. Harteneck[1], B. Nürnberg[1]

[1]*Department of Pharmacology and Experimental Therapy, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Tübingen*
[2]*Institute of Biochemistry and Molecular Biology II, Medical Faculty, Heinrich Heine University*
[3]*Leibniz-Institute for Molecular Pharmacology Berlin*
[4]*Department of Molecular Cell Biology, Center for Molecular Biomedicine, Jena University Hospital*

Class I_B phosphoinositide 3-kinase γ (PI3Kγ) controls a plethora of cellular responses. PI3Kγ, a heterodimer formed by non-catalytic p101 or p87 and catalytic p110γ subunits, is regulated by Gβγ and Ras. Earlier we postulated that p101 binds to receptor-released Gβγ to translocate cytosolic PI3Kγ to the plasma membrane, enabling direct activation of p110γ by Gβγ (Brock et al., J. Cell Biol. 2003). However, the p87 subunit does not function as Gβγ adapter (Kurig et al., PNAS, 2009). Since the impact of each non-catalytic subunit in regulating p110γ by Gβγ and Ras still remains elusive, we studied their role in detail. Our findings revealed that the non-catalytic p87 and p101 subunits implement discrete functions in respect to (i) membrane recruitment of PI3Kγ and (ii) regulation of PI3Kγ enzymatic activity by Gβγ and H-Ras.

Orientation of membrane-embedded BODIPY-cholesterol determined by two-photon polarization microscopy and Fourier-space image analysis

Lukasz Michael Solanko[1], Frederik W. Lund [1], Henrik Skov Midtiby*, Jonathan R. Brewer[1], Zaiguo Li[3], Robert Bittman[3] and Daniel Wüstner*[1]

[1]*Department of Biochemistry and Molecular Biology*
[2]*MEMPHYS Center for Biomembrane Physics, Department of Chemistry and Physics, University of Southern Denmark*
[3]*Department of Chemistry and Biochemistry, Queens College, The City University of New York*

Fluorescent BODIPY-tagged cholesterol with the fluorophore at carbon-24 in the side chain (commercial name, TopFluor-cholesterol) is a promising probe for studying cholesterol transport in living cells. We present a new integrated approach to determine the molecular orientation of TopFluor-cholesterol compared to a new BODIPY-cholesterol with differently linked BODIPY-group in model and cellular membranes. The orientation of the transition dipole of the fluorescent BODIPY-cholesterol analogs relative to a rotating excitation field vector is monitored by 2-photon excitation microscopy and analyzed using a new image analysis strategy. We show that the strength of the orientation of both BODIPY-cholesterol analogs in model membranes is dramatically increased in the presence of cholesterol. Neither TopFluor-cholesterol nor new synthesized Bodipy-cholesterol formed an excimer with red-shifted emission upon concentration in membranes. We demonstrate that our analysis is capable of detecting probe orientation in the plasma membrane in regions of varying surface curvature.

PI3Kγ within a Non-Hematopoietic Cell Type Negatively Regulates Diet-Induced Thermogenesis and Promotes Obesity and Insulin Resistance

Barbara Becattini[1], Romina Marone[2], Fabio Zani[1], Denis Arsenijevic[3], Josiane Seydoux[4], Jean-Pierre Montani[3], Abdul G. Dulloo[3], Bernard Thorens[5], Frederic Preitner[5], Matthias P. Wymann[2], Giovanni Solinas[1]

[1]*Laboratory of Metabolic Stress Biology, Division of Physiology, Dept. of Medicine, University of Fribourg*
[2]*Institute for Biochemistry and Genetics, Department of Biomedicine, University of Basel,*
[3]*Division of Physiology, Dept. of Medicine, University of Fribourg*
[4]*Department of Basic Neurosciences, Faculty of Medicine, University of Geneva*
[5]*Mouse Metabolic Facility (MEF), Center for Integrative Genomics, University of Lausanne*

Obesity is associated with a chronic low-grade inflammation, and specific antiinflammatory interventions may be beneficial for the treatment of type 2 diabetes and other obesity-related diseases. The lipid kinase PI3Kγ is a central proinflammatory signal transducer that plays a major role in leukocyte chemotaxis, mast cell degranulation, and endothelial cell activation. It was also reported that PI3Kγ activity within hematopoietic cells plays an important role in obesity-induced inflammation and insulin resistance. Here, we show that protection from insulin resistance, metabolic inflammation, and fatty liver in mice lacking functional PI3Kγ is largely consequent to their leaner phenotype. We also show that this phenotype is largely based on decreased fat gain, despite normal caloric intake, consequent to increased energy expenditure. Furthermore, our data show that PI3Kγ action on diet-induced obesity depends on PI3Kγ activity within a nonhematopoietic compartment, where it promotes energetic efficiency for fat mass gain. We also show that metabolic modulation by PI3Kγ depends on its lipid kinase activity and might involve kinase-independent signaling. Thus, PI3Kγ is an unexpected but promising drug target for the treatment of obesity and its complications.

CORVET and HOPS complexes in C. elegans have distinct functions in endosome maturation

Jachen A Solinger, Anne Spang

Biozentrum, University of Basel

The small GTPases RAB-5 and RAB-7 coordinate traffic from the plasma membrane through early and late endosomes to lysosomes. Early to late endosome transport requires a maturation event, which involves the exchange from RAB-5 to RAB-7 on the endosome surface. The evolutionarily conserved SAND-1/Mon1 is a critical switch in Rab conversion. SAND-1/Mon1-dependent displacement of the RAB-5 GEF, RABX-5 from early endosomes causes the interruption of RAB-5 activation, while SAND-1/Mon1 interaction with the homotypic fusion and protein sorting (HOPS) complex helps recruiting RAB-7. Although the role of HOPS complex as a tethering factor during fusion of RAB-7 positive late endosomes to lysosomes has been well characterized, its function in conjunction with SAND-1/Mon1 on endosomes is not well understood. In yeast, the Class C core vacuole/endosome tethering (CORVET) complex and HOPS complex share core class C VPS (vacuolar protein sorting) proteins, but seem to be able to exchange the accessory units that interact with either RAB-5 (CORVET) or RAB-7 (HOPS). To define the function of C. elegans CORVET and HOPS complexes during endosome maturation in conjunction with SAND-1, we analyzed genetic interactions of all subunits with a sand-1 mutant. We found that the CORVET complex exists also in C. elegans and is involved in controlling fusion at the early endosome. It acts therefore upstream of SAND-1 and Rab conversion.

Crosslinking-Induced Nanodomains in Model Membranes Have 8nm in Radius

Štefl, M .[1] , Šachl, R.[1,2], Humpoličkova, J.[1], Cebecauer, M.[1], Machaň, R.[1], Kolařova, M.[1], Johansson, L.B.-A.[2], Hof, M.[1]

[1]*Department of Biophysical Chemistry, J. Heyrovsky Institute of Physical Chemistry of the Academy of Sciences of the Czech Republic, Prague*
[2]*Department of Chemistry: Biophysical Chemistry, Umea University Sweden*

Cell membrane organization has been subject of intensive research for last few decades. It was even more actual when revealed membrane rafts and their contribution to cell functioning. Crosslinking of raft components using various proteins resulting in microscopic phase separation in model membranes has been already confirmed. Crosslinking of raft gangliosides GM1 with cholera toxin (CTxB) forming micrometer-sized structures is example of such induced phase separation, however, the CTxB-GM1 complexes forming a minimal lipid units are still subject of ongoing cell membrane research and characterization of such subdiffraction sized structures in terms of dynamics and size has never been successfully realized.
Using two-color z-scan fluorescence correlation spectroscopy we show strong evidence of existence of nano-sized domains in model membranes containing lower amount of sphingomyelin (Sph) than needed for micro-sized domain formation. We also show two types of Sph dependent membrane nanostructures which we characterized by means of fluorescence resonance energy transfer in combination with Monte Carlo simulations. Modeling of donor decay we calculated the domain radius of approximately 8 nm, which increases with higher Sph content. Observed two types of differently behaving domains suggest a dual role of the crosslinker: first, local transient condensation of the GM1 molecules compensating lack of sphingomyelin and second, coalescence of existing nanodomains ending in large scale phase separation.

Synthetic dimeric lipids as tools to investigate cooperativity in protein-lipid binding

Frank Stein, André Nadler, Carsten Schultz

Cell Biology and Biophysics Unit, EMBL, Heidelberg, Germany

Lipids exist in great diversity and occur in a non-random distribution within membranes throughout the cell while exhibiting manifold functions[1]. They do not only form the backbone of cellular membranes and separate cell compartments, but also play a crucial role in cell signaling by interacting with proteins[2]. These interactions are mostly mediated by lipid binding domains[3]. Recently, it was shown, that proteins do not only interact with a single lipid at a time but bind several lipids in a cooperative way to increase the binding affinity to either a single lipid species or to a specific cell compartment with a certain lipid composition[4][5]. In order to study protein-lipid binding, we propose covalently linked lipids as a tool to investigate these cooperative binding events. Using synthetic organic chemistry, we extended the glycerolipid backbone with PEG linker in different length to enable lipid dimerization by means of click chemistry. We chose the signaling lipid diacylglycerol (DAG) as the first target to examine it's interaction to the C1 domain of protein kinase C (PKC). Furthermore, a photolabile protection group (cage) was attached to the DAG headgroup to allow uncaging and thereby liberating the signaling lipid in a defined time and space. Here we report the synthesis of a covalently linked caged lipid dimer based on distearyl glycerol derivatives. Moreover, we evaluated binding of the caged monomers and the dimer to the C1a domain of PKC in a cell based assay using a genetically encoded FRET sensor for DAG[6]. We found, that our synthetic DAG derivatives were still accepted as a binding partner for the C1 domain and therefore be well suited to study cooperativity in future experiments.

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Local Nanomechanical Chracterization of Solid Supported Lipid Bilayers by Atomic Force Microscopy

Frank Stetter

TU München

Widely used model system for the lipid part of biomembranes are solid supported lipid bilayers since they are easily accessible with surface sensitive techniques like Surface Plasmon Resonance, Total Internal Reflection Fluorescence and Atomic Force Microscopy.
We characterized the biomechanical responses of solid supported lipid bilayers. To this end we employed force spectroscopy and AFM imaging to test the effect of certain additives on the stability of the bilayer. The force necessary to puncture the lipid bilayer changed noticeably when additives where present in the buffer or in the DOPC bilayer: Short chain Alkanols decrease bilayer stability while bola lipids and the Parkinson Disease-related protein Alpha Synuclein point towards a stabilization of model membranes.
The technique used in these experiments extends our understanding of the nanomechanical (as opposed to micromechanical) responses of solid supported biomembranes and therefore contributes to characterization of these important model systems.

Specific sphingolipids required to urokinase receptor signalling

P. Franco[1], G. D'Angelo[2], M.A. Nappo[1], A. De Vincenzo[1], I. Iaccarino[1], D.Corda[2] and M. P. Stoppelli[1]

[1]*Institute of Genetics and Biophysics*
[2]*Institute of Protein Biochemistry, National Research Council, Naples, Italy.*

The GPI-anchored urokinase receptor (uPAR) signals upon binding to the serine protease urokinase (uPA), thus modulating cell migration, adhesion and survival. The uPA/uPAR system is active in physiological and pathological conditions, with a relevant role in cancer invasion and metastasis. To investigate whether GM3 and Gb3 are involved in uPAR signaling, membrane sphingolipid content was manipulated through biosynthetic enzyme depletion /overexpression. In HeLa cells, siRNA silencing of SIAT9 (syalyltransferase, GM3 synthase) or A4GALT (galactosyltransferase, Gb3 synthase) transiently reduced the corresponding ganglioside and globoside, respectively, as confirmed by lipid chromatography of isolated membranes. In GM3- and Gb3-depleted cells, uPAR total expression levels were unchanged and the extent of surface uPAR did not decrease, as shown by FACS analysis. However, radioreceptor competition assays showed that specific binding of 125I-ATF (residues 1-135 of human uPA), as well as the affinity of ligand-receptor interaction were markedly reduced. In agreement with this finding, all transfectants exhibited a strongly reduced ability to migrate toward uPA and a delayed uPA-dependent short-term cell adhesion. Chemotactic response to uPA could be recovered in a time- and concentration-dependent manner, if cells silenced for GM3 synthase were supplied with exogenous GM3. In conclusion, in the absence of GM3 or Gb3 sphingolipids, the uPAR is still localised on cell surface, but exhibits a reduced availability to ligand binding and uPA-dependent cell responses are reversibly impaired. These data indicate that optimal levels of specific sphingolipids are required for the high affinity uPA/uPAR interaction as well as for uPAR signaling.

Caspase-1-dependent stress-induced unconventional protein secretion

Gerhard E. Strittmatter, Pascal Schlage, Ulrich auf dem Keller, Lars E. French, Hans-Dietmar Beer

University Hospital Zürich

Most eukaryotic proteins destined for secretion leave the cell via the classical secretory route through the endoplasmic reticulum (ER) and the Golgi apparatus. These proteins usually contain an N-terminal or internal signal sequence. However, there are nuclear and cytosolic proteins lacking a signal sequence, which are secreted in an unconventional manner bypassing the ER-Golgi pathway. The pro-inflammatory cytokine IL-1β belongs to this group. It is produced as inactive precursor, which has to be proteolytically activated by the cysteine protease caspase-1. Not only processing but also secretion of IL-1β and several other unconventionally secreted proteins are dependent on caspase-1 activity. The activation of caspase-1 itself occurs in multiprotein complexes termed inflammasomes, which assemble in response to a wide spectrum of danger and stress signals. For unconventional protein secretion (UPS) several models have been proposed. However, about the molecular mechanism of the caspase-1-dependent stress-induced UPS not much is known. Caspase-1 and other components of the inflammasome are secreted alongside IL-1β, suggesting that critical players in the caspase-1-dependent secretory pathway are to be found among secreted substrates of caspase-1. We performed a screen for secreted caspase-1 substrates using a proteomics approach and could identify 114 proteins which are secreted in a caspase-1-dependent manner and proteolytically modified. Using RNAi for 41 of the identified proteins, we found 11, whose reduced expression led to reduced secretion of mature IL-1β, both upon UVB irradiation and poly(dA:dT) transfection, two known activators of different inflammasome complexes. These proteins might be implicated in the molecular mechanism of caspase-1-dependent UPS.

A Novel Tool to Study Mycobacteria Dormancy

Madhu Sudhan R.[1], Yang Pengyu[1], Srinivasa Rao[2], Yok Hian Chionh[1], Andrej Trauner[3], Yao S. Qin[1], Markus R. Wenk[1]

[1]*National University of Singapore*
[2]*Novartis Institute for Tropical diseases, Singapore*
[3]*Harvard School of Public Health, Boston*

Mycobacteria, like some other prokaryotic species, are able to accumulate large amounts of neutral lipid (triglycerol, TG) forming structures called lipid droplets (LD). These LDs are probably involved in the bacterial life cycle, as recent reports have shown that tubercular bacilli in lung granulomas are enriched in LD. These TG deposits are consumed when hypoxic dormant bacilli are reactivated, suggesting that lipid storage may contribute to mycobacterial survival during the latent and/or reactivation phase. In fact, the TG degradation process catalyzed by lipolytic enzymes may release free fatty acids, which can be utilized as a carbon source during growth and infection processes. Several studies have explored the possibility of inhibitors targeting these lipid-catabolizing enzymes as a target in latent tuberculosis. Our group has previously shown that tetrahydrolipstatin (THL), an irreversible inhibitor of serine esterases, attenuates regrowth of dormant mycobacteria by preventing TG breakdown. To better understand THL activity and its biological role, we used a novel chemical proteomic strategy to identify specific mycobacterial targets and to validate some of their biological activities. The protein bound THL-analogues are then visualized/or enriched by ‘click-reaction’ (Huisgen cycloaddition reaction) with fluorescent dye or biotin tag. We report that THL targets α, β-hydrolase family proteins, including ‘lip gene family’ enzymes. Furthermore, we show that these genes play a crucial role during mycobacterial resuscitation through their action on storage and membrane lipids and serve to fulfill the critical energy requirements of mycobacteria.

Drifting motions of the adenovirus receptor CAR and immobile integrins initiate virus uncoating

Christoph J. Burckhardt, Maarit Suomalainen, Philipp Schoenenberger, Karin Boucke, Silvio Hemmi and Urs F. Greber

Institute of Molecular Life Science, University of Zurich, Switzerland

Pathogens use cell surface molecules for attachment and uptake into a host cell. In this study, we demonstrate that the cell surface receptors for human adenoviruses Ad2 and Ad5 do not merely provide attachment sites for the virus or mediate virus uptake, but virus binding to the receptors elicits virus motions that are crucial for initiating virus uncoating. Ad2 and Ad5 enter host cells by using two receptors, the primary receptor CAR (coxsackievirus adenovirus receptor) and the secondary receptor αv-β3/5 integrins. The integrin interaction is followed by clathrin-mediated uptake of the virus and penetration of the incoming virus from endosomes to the cytoplasm. This endosome escape step requires exposure of the internal capsid protein VI, which is a membrane-lytic factor and mediates disruption of the endosome membrane. We used fluorescently-labeled viruses and live cell imaging to follow interaction of Ad2/5 with its receptors at the plasma membrane. Our results indicate that virus binding to CAR gives rise to actomyosin-2-dependent drifting motions, whereas integrin-targeted viruses are spatially more confined. The opposing CAR-mediated drifts and integrin-mediated confinements exert a mechanical strain on the virus particle which leads to structural changes on the virus capsid, and these structural changes in turn enable exposure of the membrane-active protein VI. Thus the receptor-induced virus motions at the plasma membrane initiate virus uncoating and also prime the incoming viral particle for escape from endosomes.

Influenza virus infection impacts host cell choline lipid metabolism

Lukas B Tanner[1,2], Charmaine Xuemei Chng[2], Markus R Wenk[1,2,3]

[1]*NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore*
[2]*Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore*
[3]*Department of Biological Sciences, National University of Singapore*

In this study, we harnessed a comprehensive lipidomics approach using mass spectrometry to elucidate the role of lipids during influenza virus replication. The levels of several host sphingo- and glycerophospholipids, especially choline containing species, were altered in human lung epithelial cells during the course of an infection. Specifically, saturated sphingomyelin and ether linked phosphatidylcholine species (ePC) were elevated with a concomitant decrease in ester linked phosphatidylcholine (aPC) species in infected cells. The same trend was observed in purified influenza virus particles suggestive of a functional link between choline lipid metabolism of the host cell and viral replication. The specific remodeling of PC species was unique to influenza virus as opposed to other enveloped viruses such as human immunodeficiency virus (HIV), Semliki forest virus (SFV) and vesicular stomatitis virus (VSV). Influenza virus production was impaired in cells either treated with a sphingomyelin synthase inhibitor and in ether lipid deficient cells. Consistent with a role of peroxisomes (the site of ether lipid biosynthesis) we also found decreased catalase activity in infected cells. We further show that the non-structural protein (NS1) of influenza virus harbors a highly conserved putative peroxisome targeting sequence 2 (PTS2). Strikingly, we identified NS1 as a major determinant for host cell lipid metabolism underlined by distinct sphingolipid and phosphatidylcholine profiles of two closely related influenza virus strains differing only in a non-conservative point mutation (D135G) in the effector domain of NS1. Based on these findings and published data, we propose a model whereby influenza virus redirects glycolytic flux into the biosynthesis of ether linked- and sphingolipids to facilitate virion replication and morphogenesis in the exocytotic pathway which correlates with virus pathogenicity.

Molecular simulations of a fluorescent probe in a lipid bilayer

Stepan Timr, Lukasz Cwiklik, Mario Vazdar and Pavel Jungwirth

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Academy of Sciences of the Czech Republic

We investigate the properties of a 3-hydroxyflavone based dye F2N12S sensitive to changes in cell membrane surface charge during cell apoptosis. Classical molecular dynamics of the probe in a POPC bilayer is performed with the goal to determine the location and, most importantly, the orientation of the fluorophore relative to the surface of the bilayer. The impact of cholesterol addition on the location and orientation of the fluorophore is examined. Moreover, one-photon transition dipole moment of the first excited state of the dye in its environment is calculated. The results obtained from a combination of classical molecular dynamics and quantum calculations are compared to experimental data on absorption anisotropy acquired from polarization microscopy of giant unilamellar vesicles.

The Role of Plasma Membrane Phosphatidylinositol 4,5-bisphosphate in G Protein-coupled Receptor Endocytosis

József T. Tóth[1], Dániel J. Tóth[1], László Hunyady[1], Tamas Balla[2], Péter Várnai[1]

[1]*Department of Physiology, Semmelweis University, Budapest, Hungary*
[2]*NICHD, National Institutes of Health, Bethesda, U.S.A*

Receptor endocytosis plays an important role in regulating the responsiveness of cells to specific ligands. Phosphatidylinositol 4,5-bisphosphate (PtdInsP2) was shown to be critical for many endocytic processes, but its role in G protein-coupled receptor internalization has not yet been investigated.
We used luciferase-labeled type 1 angiotensin (AT1), type 2C serotonin (5HT2C) or β2 adrenergic (β2A) receptors and fluorescently tagged proteins (β-arrestin 2, plasma membrane-targeted Venus, Rab5) to follow the endocytic route of the receptors in HEK293 cells using bioluminescence resonance energy transfer (BRET) and confocal microscopy. To reduce plasma membrane PtdInsP2-levels, we applied our previously developed rapamycin-inducible heterodimerization system, in which a 5-phosphatase domain is recruited to the plasma membrane and acutely degrades PtdInsP2. By using various target sequences we tried to perform microdomain-specific PtdInsP2 depletion.
We found that ligand-induced interaction of AT1, 5HT2C and β2A receptors with β-arrestin 2 was unaffected by PtdInsP2 depletion. However, arrival of receptors to Rab5-positive early endosomes was completely abolished, whereas removal of receptors from the plasma membrane was reduced but not eliminated after PtdInsP2 depletion. Compared to the “raft” microdomain (L10), recruitment of the 5-phosphatase to the “non-raft” microdomain (S15) led to reduced PtdInsP2 depletion (measured by PLCδ1PH). However, inhibition of AT1R endocytosis was similar in both cases.
Our data suggest that the internalization of AT1, 5HT2C and β2A receptors is dependent on plasma membrane PtdInsP2. In the absence of PtdInsP2, their endocytosis is initiated but cannot be terminated. Our present microdomain-specific 5-phosphatase targeting approach was found to be insufficient to detect any pool-specific effects of PtdInsP2 on the endocytosis of AT1R.

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Coordination of intra-endosomal dynamics by Rab7

Katarina Trajkovic, Jean Gruenberg

Department of Biochemistry, University of Geneva, Switzerland

Transport of endocytosed material from early to late endosomes is mediated by multivesicular bodies and is regulated by Rab GTPases. These include in particular Rab5 and Rab7 on early and late endosomes, respectively. Intralumenal vesicles (ILV) are formed at all stages of the pathway by inward budding of the endosomal limiting membrane. Late endosomes are highly motile compartments organized into vesicular and dynamic tubular domains. In this compartment, the opposite processes of ILV formation and back-fusion with the limiting membrane take place.

The goal of our study is to elucidate the mechanisms that coordinate traffic along endocytic pathway with intra-endosomal dynamics. Rab7 together with Mon1 which is responsible for its recruitment is a likely candidate for this coordination. Our objective is to analyze the role of these proteins by using in vivo and in vitro approaches.

We found that Rab7 knock-down attenuates transport of solutes and EGFR along the endocytic pathway. Yet, in vitro invagination assay (1) showed that ILV formation was not affected after Rab7KD. Moreover, we made use of our observations that, after VSV endocytosis, viral RNA release into the cytoplasm requires ILV back-fusion (2), and found that Rab7KD also doesn't affect this process. Conversely, we found that the dynamic properties of Snx16-containing tubular regions of late endosomes were lost and tubule biogenesis inhibited. We conclude that Rab7 is required for endocytic membrane transport and late-endosomal membrane dynamics, but not ILV formation and back-fusion. Furthermore, early-to-late endosome traffic is functionally coupled, by Rab7, with the dynamic properties of late-endosomal membranes.

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Visualization of the membrane environment surrounding the insulin receptor

Miwa Takahashi-Umebayashi[1], Luc Reymond[2], Kai Johnsson[2], Howard Riezman[1]

[1]*University of Geneva*
[2]*EPFL*

Cellular membranes are composed of numerous different lipids and proteins and are not always homogeneous. Certain sets of lipids and proteins can be preferentially associated with one another to form distinct membrane domains. For example, sterols, sphingolipids and specific proteins are thought to be assembled into microdomains, called lipid rafts. It has been considered that lipid rafts act as platforms for many biological events such as signal transduction, membrane traffic and protein sorting. Due to the mode of association between sterols and sphingolipids, lipid rafts can be highly ordered and segregated from liquid-disordered domains, at least in vitro.

To investigate protein-raft interactions, a biochemical approach based on detergent insolubility has been used extensively in the past, but seems to be unreliable. Attempts have also been made to visualize lipids and proteins in microdomains in vivo by sophisticated microscopic techniques, but to date the lipid environment and the protein have not been visualized together. To address this challenging problem, we have developed a method to observe the local membrane environment surrounding a membrane protein of interest.

In principle our technique covalently links a membrane environment-sensitive fluorescent dye via a linker of variable length to a membrane protein. We found that the SNAP-tag is useful for the crosslinking to the dye. As a test system, the insulin receptor was tagged with SNAP and then labeled with an environment sensitive dye. We have succeeded in monitoring the membrane environment surrounding the insulin receptor.

A 14-3-3γ dimer-based scaffold bridges CtBP1-S/BARS to PI4KIIIβ to regulate post-Golgi carrier formation

C. Valente, G. Turacchio, A. Luini, D. Corda

Institute of Protein Biochemistry, National Research Council, Naples, Italy

Membrane fission is required during formation of intracellular transport carriers, a central process in membrane trafficking. CtBP1-S/BARS (BARS) has a fundamental role at the Golgi complex, in macropinocytosis and in fluid-phase endocytosis. Our search for BARS-interacting proteins identified 14-3-3γ, which interacts with phosphatidylinositol 4-kinase (PI4K)IIIβ in both mammals and yeast. PI4KIIIβ and phosphatidylinositol 4-phosphate are fundamental to the organisation of the Golgi complex and post-Golgi transport in mammalian cells. In pull-down assays, 14-3-3γ bound BARS and PI4KIIIβ, although BARS did not bind PI4KIIIβ directly. In COS7 cells expressing a GFP-tagged temperature-sensitive mutant of the G protein of vesicular stomatitis virus (VSVG) as a cargo reporter, RNA interference against 14-3-3γ inhibited trans-Golgi network (TGN)-to-plasma membrane transport of VSVG. Here, a decrease in post-Golgi transport carriers was accompanied by formation of long VSVG-containing tubules (5-30 μm) from the TGN, which elongated (and retracted) at 0.5 μm/s, consistent with microtubule-based motility. Thus, 14-3-3γ is involved in BARS mediation of TGN-to-plasma membrane transport through control of the formation/ fission of constitutive post-Golgi carriers. Förster resonance energy transfer analysis shows that at the Golgi complex, 14-3-3γ dimers bridge the fission-inducing protein BARS with PI4KIIIβ. This complex is stabilised by phosphorylation of Ser294 in PI4KIIIβ and Ser147 in BARS, by the kinases PKD and PAK, respectively. Disruption of this complex inhibits fission of elongating post-Golgi carrier precursors, thus providing a functional link from PI4KIIIβ-dependent tubulation/ post-Golgi carrier formation to BARS-induced fission. Regulated assembly of this complex provides new insights into the molecular organisation of post-Golgi carriers.

Dynamics of GPCR activation: measuring Gaq-mediatedsignaling in living cells

J. van Unen, N.Reinhard, T.W.J. Gadella Jr & J. Goedhart

Swammerdam Institute for Life Sciences, Section of Molecular Cytology, van Leeuwenhoek Centre for Advanced Microscopy, University of Amsterdam

The Gaq signaling pathway has been implicated in many important cellular processes, including proliferation, cell migration and cytoskeletal organization. The classical downstream pathway through PLCβ and calcium mobilization has been extensively studied to date. More recently, another effector of Gaq is identified, p63RhoGEF, which has been proposed to activate the RhoA signaling cascade. Activation of this novel, competing pathway leads to actin polymerization and translocation of the transcription factor MKL2 to the nucleus. Using advanced microscopy techniques like Förster resonance energy transfer (FRET), we measured different downstream effectors with different temporal activation kinetics. Starting at the plasma membrane on the millisecond time-scale with receptor G-protein interaction and G-protein activation, followed by seconds time-scale dynamics of calcium fluxes and finally nuclear translocation of MKL2 on the minute time-scale. Our preliminary results hint on the importance of the different temporal dynamics in this important signaling pathway.

Behavior of 4-hydroxynonenal in Phospholipid Membranes

Mario Vazdar[1], Piotr Jurkiewicz[2], Martin Hof[2], Pavel Jungwirth[1], and Lukasz Cwiklik[1,2]

[1]*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, and Center for Biomolecules and Complex Molecular Systems*
[2]*J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic*

Under conditions of oxidative stress, 4-hydroxy-2-nonenal (4-HNE) is commonly present in living systems. This cytotoxic and highly reactive compound is generated by peroxidation of lipids in membranes. Moreover, it can be easily transferred from a membrane to both cytosol and the extracellular space. By using time-dependent fluorescence shift (TDFS) method and molecular dynamics simulations, we found that 4-HNE is stabilized in the carbonyl region of a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer. Therefore, 4-HNE is able to react with cell membrane proteins and lipids. Stabilization in the membrane is moderate and a transfer of 4-HNE to either extra- or intracellular space occurs on a microsecond timescale. These molecular-level details of 4-HNE behavior in the lipid membrane rationalize the experimentally observed reactivity of 4-HNE with proteins inside and outside the cell.

Membrane interactions of oxidized phospholipids in cultured macrophages

U. Stemmer, F. Vogl, D. Koller, L. Marlingapla Halasiddappa, E. Zenzmaier, A. Hermetter

Institute of Biochemistry, Graz University of Technology, Graz Austria

The oxidized phospholipids 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl -sn-glycero-3-phosphocholine (PGPC) are biologically active components of oxidized low density lipoprotein and as such contribute to the cytotoxicity of this particle in vascular cells. To understand the molecular basis of lipid-induced cell death we studied the membrane interactions of the pure compounds with cultured macrophages. POVPC and PGPC are toxic to these cells, but the mechanisms of their toxicities are entirely different due to the small structural differences in their fragmented sn-2 acyl chains. POVPC containing an ω-aldehyde function in this position can chemically bind to its target molecules via Schiff base formation, whereas PGPC carrying a carboxy function can only undergo physical interactions. The resulting lipid and protein targets represent the initial subcellular platforms for intracellular signaling by POVPC and PGPC. The primary protein targets of a fluorescent POVPC analogue were identified by MS/MS analysis. The identified polypeptides are associated with lipid signaling and metabolism, stress response, apoptosis, and membrane transport. POVPC toxicity is mainly due to these direct interactions on the lipid and protein level. From microarray experiments, it can be inferred that this phospholipid shows only a minor effect on the transcriptional level compared with PGPC which induces up- and down regulation of hundreds of genes. A key feature of POVPC and PGPC toxicity is the formation of ceramide, which is catalyzed by acid sphingomyelinase and specific ceramide synthase isoforms in a time-dependent manner. The causal role of ceramide as a mediator of lipid-induced cell death is currently subject to investigation.

The oxidized phospholipid PazePC modulates interactions between Bax and mitochondrial membranes

Marcus Wallgren[1], Martin Lidman[1], Quoc Dat Pham[1], Konrad Cyprych[2], Gerhard Gröbner[1]

[1]*Department of Chemistry, Umeå University, Umeå, Sweden*
[2]*Institute of Biomedical Engineering and Instrumentation, Wroclaw University of Technology, Wrocław, Poland*

For a long time lipids have been seen as merely structural membrane units with proteins doing the actual work. This view has changed in recent years where it has been shown that lipids are also directly involved in numerous physiological processes. However, how membranes change under intracellular oxidative conditions, which e.g. trigger mitochondria-mediated apoptotic cell death, is still a mystery. Oxidative stress leads to the formation of oxidized phospholipids (OxPls), which have a great impact on mitochondrial membrane integrity. To obtain insight into the role of oxidized OxPls on the regulative interplay of the pro-apoptotic Bcl-2 family protein Bax with membranes, mitochondria-mimicking liposomes with and without the OxPl Palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazePC) were studied in the presence of the protein. Differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy were used to characterize these protein-membrane interactions. DSC experiments showed that the presence of PazePC improved the association and incorporation of Bax as well as increased the thermostability of the protein. CD measurements further supported the greatly enhanced interplay of Bax with PazePC-containing membranes, and also revealed a possible conformational change of the protein at physiological temperature (37 ºC).
In summary, the presence of PazePC seems to alter the mitochondrial membrane organization, which facilitates Bax recruitment to the mitochondria and presumably its insertion into the membranes, prerequisites for the onset of mitochondrial apoptosis.

Non-raft associated, lyso Glycosylphosphatidylinositol-anchored proteins are targeted apically in polarized epithelial cells

Guillaume Alain Castillon, Laetitia Michon, Reika Watanabe

Department of Biochemistry, University of Geneva

In the polarized epithelial cells, Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are mainly localized at the apical surface. This polarized localization has been proposed to be driven by GPI-AP selective partitioning into specific membrane domains enriched in sphingolipids and cholesterol, named lipid rafts. In the Golgi apparatus GPI-APs undergo fatty acid remodeling that enables their association with lipid rafts. We established a mutant Madin-Darby canine kidney cell line where all GPI-APs are in lyso form due to the defect of GPI lipid remodeling. Lyso GPI-AP expressed in this mutant cells are not incorporated into lipid rafts but are still targeted apically, where they are released into the culture medium. The results suggest an essential role of lipid remodeling for the stable localization at the plasma membrane more than a directional targeting along the secretory pathway. Based on previous and current studies, alternative models for apical targeting of GPI-APs are discussed.

Release of ATP feedback triggers local diacylglycerol spiking in insulin-secreting β-cells

Anne Wuttke, Anders Tengholm

Uppsala University, Sweden

Diacylglycerol (DAG) controls numerous cell functions by membrane recruitment and activation of C1-domain-containing proteins, including protein kinase C (PKC). In insulin-secreting β-cells the lipid is generated in the plasma membrane in response to receptor and nutrient stimuli, but little is known about its spatio-temporal dynamics. Here, we used total internal reflection microscopy and fluorescent translocation biosensors and demonstrate that glucose and depolarizing stimuli evoke pronounced DAG spiking in the plasma membrane of β-cells. The spikes lasted ~7 s and occurred in spatially confined membrane regions. DAG spiking was suppressed after inhibition of exocytosis, but was nevertheless unrelated to insulin. ATP is co-secreted with insulin and the nucleotide was found to trigger dose-dependent increases of plasma membrane DAG-levels. Consistent with involvement of ATP, the glucose- and depolarization-induced DAG spiking was abolished by P2Y1-receptor antagonism. Each DAG spike caused local PKC activation with resulting dissociation of the protein “Myristoylated alanine-rich C-kinase substrate” from the plasma membrane. DAG-spiking was important for the magnitude of insulin release, as inhibition of DAG-spiking by P2Y1-receptor antagonism suppressed the β-cell secretory response to glucose. We conclude that transient DAG signaling microdomains occur in the plasma membrane of β-cells and that they result from release of ATP and autocrine purinoceptor activation. They translate into local and transient activation of PKC and the underlying feedback activation of purinergic receptors is important for appropriate control of insulin secretion.

Design principles of thermosensitive liposomes for local delivery of doxorubicin

Vladimir Zamotin, Roberto Tejera-Garcia, and Paavo K. J. Kinnunen

Helsinki Biophysics and Biomembrane Group, BECS Department, School of Science, Aalto University, Espoo, Finland

The efficient use of thermosensitive liposomes as carriers for the targeted drug delivery into localized tumor areas requires the optimization of their production process. Liposomes encapsulating reasonable amounts of this drug should be able to release it rapidly in the specific region at temperatures low enough to avoid the necrosis of the surrounding cells (< 44°C) and high enough to guaranty the absence of drug leaking in other body parts (> 39°C). We have analyzed the possible issues appearing in the engineering of this new type of carriers.

Despite low drug leakage and quick drug release belong to the class of mutually exclusive issues we demonstrated that incorporation of lipid with intrinsic negative curvature, DPPE, and/or well-known modulator of elasticity, cholesterol, can be served as fine tuning tools to solve this problem. Employing differential scanning calorimetry, dynamic light scattering and diverse fluorescence techniques we investigated effect of lipid variation in liposomal content on the required parameters described above.

We have tested series of low thermosensitive formulations modified by DPPE and cholesterol in different ratio and our results have showed almost instant drug release at the temperature corresponding the phase transition of certain formulation and reasonable stability of liposomes at the conditions close to physiological ones.

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Covalent tag-chemistry targeting receptor dynamics and signaling

Florent Beaufils, Mirjam Zimmermann, Dominik Erhart, Ruben Cal, Olivier Jacques, Ed Constable, Matthias P. Wymann

Inst. Biochem. & Genetics, Dept. Biomedicine, University of Basel, Switzerland

Complex receptor interactions, protein-protein and protein-membrane interactions are crucial in cellular signaling. The use of chemical inducers of dimerization (CID) to dynamically force receptor or signaling molecule proximity is an exciting approach to study signaling events in a spatial and temporal way. CID are small organic molecules that bind to specific tag protein domains, and thus force two proteins of interest (POI) into proximity. The most common dimerizer approach is based on rapamycin, which naturally functions as to dimerize FKBP12 and the FRB domain of mTOR. Since rapamycin has an inhibitory effect on endogenous mTOR, this dimerisation system can obscure output parameters in studies of cell growth and metabolism.

Here we show the development a new class of CID, crosslinking two protein tags devoid of endogenous signaling counterparts. These highly selective CID (dubbed (HaXS) react with Halo- and SNAP-tags. An extensive pharmacochemical development of these dimerizers finally yielded highly cell permeable molecules that dimerize tagged proteins intra-cellularly. Using HaXS dimerizers it is possible to selectively activate signaling pathways: as a demonstration we show that the translocation of a tagged iSH2 domain derived from the p85 regulatory subunit of PI3K to a tagged membrane anchor at the plasma membrane activates the PI3K/PKB/mTOR pathway. Similarly, targeting of tagged proteins to distinct cellular structures can be achieved, which allows investigation of organelle-specific events.

The novel dimerizer system operates to form covalent complexes, and allows therefore a direct monitoring and quantification of dimerization efficiency. The rate of dimerization can be controlled in a HaXS dose-dependent fashion. A modular approach to HaXS chemical synthesis opens the way to further develop the dimerizer molecules, change chemical properties and add novel tracing functionalities.

Regulation of mTORC1 by PtdIns(3)P and PtdIns(3,5)P2

Anna Melone1, Romina Marone1, Devaraj Subramanian2, Carsten Schultz2, Matthias P. Wymann1

1DBM, University of Basel, Basel, Switzerland; 2EMBL, Heidelberg, Germany

The mammalian target of rapamycin (mTOR) serine/threonine kinase is the catalytic component of two functional distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 integrates signals emerging from growth factors and nutrients such as amino acids to regulate cell proliferation and cell growth. Here we analyzed two different phosphoinositides, PtdIns(3)P and PtdIns(3,5)P2, in the amino acid-dependent regulation of mTORC1 signaling. The role of PtdIns(3)P was analyzed by targeting PtdIns(3)P-generating enzymes, human vacuolar protein sorting 34 (hVps34) and class II phosphatidylinositol 3-kinase (PI3K). In contrary to previous reports, we found that downregulation of hVps34 does not completely blunt mTORC1 signaling. Unexpectedly, silencing of class II PI3Ks results in an isoform-specific activation of mTORC1 where PI3K-C2 □ knockdown increases, while PI3K-C2β knockdown modestly decreases mTORC1 activity. Nevertheless, by affecting PtdIns(3)P directly either by masking or by acute treatment with synthetic PtdIns(3)P strongly inhibits or increases mTORC1 activity, respectively. Surprisingly, pharmacological inhibition of the PtdIns(3,5)P2-synthesizing enzyme PIKfyve decreases mTORC1 signaling that is paralleled by diminished late endosomal localization of mTOR. However, when mTORC1 is artificially docked to late endosomes mTORC1 activity becomes independent of PtdIns(3)P and PtdIns(3,5)P2.
Our data confirm a positive role of PtdIns(3)P in the mTORC1 pathway and demonstrate a novel role for PIKfyve and its phospholipid PtdIns(3,5)P2 in the regulation of mTORC1 signaling in response to amino acids.

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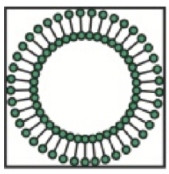

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