Friday, 14:30-14:50

TRANSMEMBRANE DOMAIN-DEPENDENT PARTITIONING OF MEMBRANE PROTEINS WITHIN THE ENDOPLASMIC RETICULUM

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The Transmembrane domain (TMD) plays an important role in protein sorting, but the mechanisms underlaying this phenomenon are currently not understood. We have used two C-tail-anchored fluorescent proteins differing in the length of their membrane-spanning domain: one (named FP-17) has the short (17 aa) and moderately hydrophobic TMD of cytochrome b5; in the other (named FP-22) this TMD has been lengthened to 22 aa. We have previously demonstrated that the two constructs are sorted at the ER exit sites (ERES), where FP-22 is accumulated and exported to reach its final localization at the plasma membrane, while FP-17 is excluded from the CopII-coated budding vesicles.

We have now demonstrated, with the FRAP technique, that FP-22 moves into and out of ERES with a small amount accumulating there. In contrast, a protein known to be recruited to ERES by a specific signal, recovers very poorly at ERES after bleaching. Thus, the recruitment of FP-22 seems to be dependent on weak mechanisms such as protein-lipid interaction or the preference of membrane curvature and not on a high affinity interaction with a protein receptor. We have then compared the transport of FP-22 with the one of FP-VSVG and found that FP-22 takes a longer time to reach the plasma membrane, but that the two proteins travel together after export from the ER towards the Golgi, suggesting that the rate-limiting step for TMD-dependent traffic is the receptor-independent recruitment at ERES.

Moreover, before export, FP-17 and -22 appeared segregated between different ER domains, with the first one mainly concentrated in cisternae that excluded FP-22. Colocalization studies with the tubular ER marker Reticulon-4a confirmed the flat nature of FP-17 labeled structures. Thus, we have demonstrated that membrane curvature has an important role for protein sorting within the endoplasmic reticulum.

Taken together our data demonstrate that the physical-chemical characteristics of the TMD are important sorting determinants which can prevent proteins from diffusing into different ER subdomains (cisternae and/or ERES), due to the preference for a given lipid environment or to a particular membrane curvature.

Friday, 14:50-15:10

DIFFERENT REGIONS MEDIATE TARGETING OF TRIADIN TO THE JUNCTIONAL SARCOPLASMIC RETICULUM MEMBRANES

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Sarcoplasmic reticulum (SR) is a specialized form of endoplasmic reticulum characteristic of muscle cells. The SR can be divided in at least two compartments: the terminal cisternae and the longitudinal tubules, different for structure, function and protein composition. Terminal cisternae represents the junctional sites, where the SR and the plasma membrane come in close contact and where calcium is released in the cytoplasm through the mechanisms of EC-coupling. In contrast, the longitudinal tubules, represent the sites of calcium recovery from the cytoplasm into the lumen due to the activity of the SERCA pumps. Many functional proteins are located to the triads. However, the molecular mechanisms that drive these molecules to these SR domains are still unknown. In order to investigate whether specific targeting signals are present in the primary sequences of triadic proteins, we focused our attention to Triadin, an integral membrane protein localized to the junctional region of the sarcoplasmic reticulum.
Triadin is characterized by a short cytoplasm N-terminal portion that contains a site of interaction with the ryanodine receptor (RyR), a single alpha helix transmembrane domain and a long C-terminal luminal tail that contains a second RyR binding site overlapping with a binding site for calsequestrin (CSQ).
A series of GFP-tagged cDNAs, characterized by deletion of the Triadin sequence were expressed in primary myotubes and their localization to the triads was evaluated in comparison with that of the full-length protein. We were able to identify three regions involved in the localization of Triadin to the triads: one in the cytoplasmic portion and two in the luminal tail. Both the cytoplasmic region and one of the two luminal regions totally or partially overlap with the binding sites for RyR and CSQ, suggesting that interaction of Triadin with other triadic proteins may be relevant for its correct targeting. The degree of association of Triadin to the junctional SR has been evaluated by FRAP analysis. The results obtained showed that, among the triadic proteins analysed, Triadin displays the lowest mobility, indicating that it may be engaged in multi-protein complexes anchoring it to the triads. Interestingly, deletion of the C-terminal tail was found to increase protein mobility suggesting a role of this region in stably retaining Triadin at the junctional SR.

Friday, 15:10-15:30

ERO1 AND ERP44 COOPERATIVELY COUPLE REDOX AND CA2+ HOMEOSTASIS AND SIGNALLING AT THE ENDOPLASMIC RETICULUM-MITOCHONDRIA INTERFACE

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The endoplasmic reticulum (ER) is a multifunctional organelle integrating diverse physiological tasks. Efflux of Ca2+ via IP3R1 is fundamental for many signalling processes. Moreover, suitable redox conditions must be maintained for oxidative protein folding and signal generation and tuning. ERp44, a chaperone of the early secretory pathway, mediates the localization of Ero1α, the main source of ER oxidative power; ERp44 also regulates IP3R1 activity in a redox-dependent manner. By modulating the expression of either Ero1α or ERp44, we show that a dynamic interplay between the two molecules regulates ER Ca2+ homeostasis and efflux via IP3R1. Ero1alpha controls both the redox state of IP3R1 and its association with ERp44. All three molecules are enriched in mitochondria-associated membranes (MAM). Thus, Ero1α and ERp44 cooperatively link redox and Ca2+ homeostasis, integrating diverse signalling pathways at the ER-mitochondrial interface.

Friday, 15:30-15:50

REDUCTION OF ENDOPLASMIC RETICULUM CA2+ LEVELS FAVORS PLASMA MEMBRANE SURFACE EXPOSURE OF CALRETICULIN

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Anthracyclins, a class of chemotherapeutic agents that inhibit DNA and RNA synthesis, have been recently shown to be particularly efficient in inducing immunogenic cell death, correlating with the early, pre-apoptotic translocation of calreticulin (CRT) from inside the cell (endo-CRT) to the cell surface (ecto-CRT) of anthracyclin-treated tumor cells. The mechanism/s through which anthracyclins induce CRT exposure does/do not involve the nucleus, thereby suggesting a non-nuclear, cytoplasmic-mediated response. In this study we investigated the role of changes in cellular Ca2+ homeostasis on CRT exposure. We found that a human neuroblastoma cell line (SH-SY5Y) failed to expose CRT in response to anthracyclin treatment. This defect in CRT exposure could be overcome by overexpressing Reticulon-1C, a manipulation that led to a decrease in Ca2+ concentration within the sarco-endoplasmic reticulum lumen. The combination of Reticulon-1C expression and anthracyclin treatment yielded a more pronounced endoplasmic reticulum Ca2+ depletion than either the two manipulations alone. Chelation of intracellular (and endoplasmic reticulum) Ca2+, targeted expression of the ligand-binding domain of the IP3 receptor and inhibition of the sarco-endoplasmic reticulum Ca2+-ATPase pump reduced endoplasmic reticulum Ca2+ load and promoted pre-apoptotic CRT exposure on the cell surface, in SH-SY5Y and HeLa cells. These results provide evidence that ER Ca2+ levels control the translocation of CRT on the cell surface.

Friday, 15:50-16:10

A NOVEL HIGH DATA OUTPUT AND 3D CORRELATIVE MICROSCOPY METHOD

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Correlative light/electron microscopy allows the simultaneous observation of a given subcellular structure by fluorescence light microscopy and electron microscopy. The use of this approach is becoming increasingly frequent in cell biology. Here we report on a new high data output correlative light/electron microscopy method, based on the use of cryosections. We successfully applied the method to analyse the structure of rough and smooth Russell's bodies, used as model systems. The major advantages of our method are: (1) the possibility to correlate several hundreds of events at the same time; (2) the possibility to perform 3D correlation; (3) the possibility to immuno-label both endogenous and recombinantly expressed proteins, at the same time. We have identified and optimized critical steps in sample preparation, defined routines for sample analysis and re-tracing of regions of interest, developed a software for semi/fully-automatic 3D reconstruction, and defined preliminary conditions for an hybrid light/electron microscopy morphometry approach.

Friday, 16:40-17:00

A TRAFFIC-ACTIVATED GOLGI-BASED SIGNALLING CIRCUIT COORDINATES THE SECRETORY PATHWAY

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The Golgi complex is the central intracellular organelle for transport, maturation and sorting of proteins and lipids to their final destinations, a process in which other intracellular organelles participate, including the endoplasmic reticulum (ER) and endosomes. During this membrane trafficking, these organelles exchange proteins, membranes and lipids in a coordinated fashion. Our knowledge of the molecular machineries involved is extensive, although the regulatory systems remain poorly defined.

We have identified an endomembrane-based signalling cascade that is activated by incoming traffic from the ER to the cis-Golgi. Here, the signal for incoming traffic is provided by the ER chaperones that reach the Golgi complex within the traffic carriers leaving the ER. Within the Golgi complex, the chaperones bind to the KDEL receptor (a seven-transmembrane-domain protein known to cycle between the ER and the Golgi complex to retrieve the chaperones that escape from the ER), which initiates a signalling reaction that includes the activation of a pool of Src family kinases (SFKs) located on the Golgi complex. In turn, this activates intra-Golgi trafficking, allowing carriers to leave the Golgi complex for the plasma membrane. In an initial investigation of the mechanisms through which the KDEL receptor activates these SFKs, the receptor interacted with Src in a two-hybrid assay. These data indicated that analogous to some of the classic G-protein-coupled receptors, the KDEL receptor could activate SFKs through a direct interaction, although they do not exclude that this interaction could be part of a more complex mechanism. Indeed, we have evidence for the involvement of the phospholipase C/ Ca2+ signalling system. For the mechanism through which the SFKs regulate Golgi-to-plasma-membrane transport, at the moment we have identified dynamin II as the main target (see presentation of Capitani et al.).

We believe that one of the functions of this pathway is to balance the membrane input that arrives at the Golgi complex with the signal level generated, to maintain the dynamic equilibrium of the Golgi complex. Another possible function that has broader interest is that this traffic-induced Golgi-Src phosphorylation cascade could influence the other machineries that underlie different cellular functions.

Friday, 17:00-17:20

SRC KINASE REGULATES THE SECRETORY FUNCTION AND STRUCTURAL INTEGRITY OF THE GOLGI COMPLEX VIA ACTIVATION OF DYNAMIN 2

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The Golgi complex is a cytosplasmic organelle that constantly receives proteins and lipids from the endoplasmic reticulum that need to be correctly modified, packaged and delivered to their final destinations. Thus, this process requires the coordinated engagement of a series of organelles and machineries; how this coordination is accomplished remains to be determined.
This process is in part carried out by a Golgi-based signalling cascade that is activated by incoming traffic from the endoplasmic reticulum to the Golgi complex. The signal for this incoming traffic is provided by chaperones that reach the cis-Golgi in the cargo-containing transport carriers. At this level, the binding of the chaperones to the KDEL receptor triggers the activation of a Golgi pool of Src, the tyrosine kinase activity of which promotes Golgi-to-plasma-membrane transport (for details, see Sallese et al. abstract).
Here, we report on the dynamin 2 (Dyn2) as the first Src substrate we have identified in the control of Golgi trafficking and integrity. Dyn2, which localizes to the Golgi complex, is phosphorylated by Src in position Y231/597 and promotes post-Golgi-carrier formation.
Here we show that the arrival of a traffic pulse at the Golgi complex results in the activation of Src on the Golgi complex itself, triggering a further cascade of phosphorylation of a number of proteins, one of which is Dyn2. In determining whether Src-mediated Dyn2 phosphorylation is involved in Golgi-to-plasma-membrane transport, we saw that cells expressing the Y231/597F Dyn2 double mutant had delayed trafficking of VSV-G out of the trans-Golgi network, which resulted in Golgi cisternae filled with VSV-G. Under time-lapse microscopy, the cells expressing the Y231/597F Dyn2 double mutant also had tubules emerging from the Golgi complex, potentially indicating an impairment of the fissioning machinery. Further, the arrival of a traffic pulse at the Golgi complex also induced Golgi fragmentation. This process became dramatic upon the expression of an active Src kinase (SrcY530F), while it was significantly reduced in cells co-expressing this active Src kinase and the Y231/597F Dyn2 double mutant. Also of particular interest, tumour cell lines with an augmented Src kinase activity have a fragmented Golgi complex, a situation that can be reversed by the use of Src inhibitors. Together, these data indicate that Src-mediated Dyn2 activation is important for driving the fissioning of the Golgi membranes during carrier formation, and thereby has a role in maintaining Golgi homeostasis and integrity during membrane trafficking.

Friday, 17:20-17:40

THE SEPARATION OF THE GOLGI RIBBON INTO SEPARATE STACKS IN G2 IS REQUIRED TO ENTER INTO MITOSIS

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The Golgi ribbon is a complex structure composed of many stacks that are interconnected by tubules. During mitosis, this Golgi ribbon undergoes extensive fragmentation through a multistage process that allows its correct partitioning and inheritance by the daughter cells. Strikingly, this fragmentation process is required not only for inheritance, but also for entrance into mitosis itself, as its block results in the arrest of the cell cycle in G2. We have found that the severing of the Golgi ribbon into its constituent stacks during early G2 is the precise stage of Golgi fragmentation that controls entry into mitosis. The fissioning protein CtBP1-S/BARS (BARS) is essential for this, and is itself required for mitotic entry: a block in Golgi fragmentation results in cell-cycle arrest in G2, defining the “Golgi mitotic checkpoint”. Cells without a Golgi ribbon are independent of BARS for Golgi fragmentation and mitotic entrance. This identifies the precise stage of Golgi fragmentation and the role of BARS in the Golgi mitotic checkpoint, setting the stage for molecular analysis of this process.

Friday, 17:40-18:00

THE ROLE OF GOLGI COMPLEX FRAGMENTATION IN THE REGULATION OF G2/PROPHASE TRANSITION

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The Golgi complex is composed of individual stacks that are laterally connected by tubules to form a ribbon-like structure. During G2/mitosis, this Golgi ribbon undergoes extensive fragmentation prior to its correct partitioning into the daughter cells. The first step of Golgi fragmentation occurs in G2 and involves cleavage of the tubules that interconnect the stacks of the ribbon. This ribbon-cleavage stage is required for G2/M transition and is controlled by the fissioning protein CtBP1-S/BARS (BARS), thus defining the "Golgi mitotic checkpoint" (1, 2). We have recently found that the segregation of the ribbon in G2 is concomitant with the "maturation" and separation of the centrosomes. This separation of the duplicated centrosomes is required for formation of the mitotic spindle and is regulated by the "maturation" of the centrosome, which in turn is essential for entry into mitosis. This process consists of the sequential recruitment to the centrosomes of mitotic kinases and CDC25B phosphatase, which then activates the Cdk1/cyclinB complex (3). Our results indicate that inhibition of the severing of the Golgi ribbon in HeLa and NRK cells induced by the injection of a blocking anti-BARS antibody correlates with inhibition of centrosome maturation and separation. Collectively, our data suggest signalling crosstalk between the Golgi ribbon and the centrosome in the control of G2/mitosis transition.

1. Hidalgo Carcedo et al. Science (2004) 305: 93-6
2. Colanzi et al. EMBO J. (2007) 26(10):2465-76
3. Zhao et al. Mol. Cell (2005) 20: 237-49

Friday, 18:00-18:20

PICHIA PASTORIS AS A HOST FOR SECRETION OF SAPORIN-BASED CHIMERAS WITH ANTICANCER POTENTIAL

Maria Serena Fabbrini*,Alessio Lombardi*,Sara Bursomanno*, Teresa Lopardo*, Roberta Traini[+], Marco Colombatti[+], Rodolfo Ippoliti[#], Sopsarmon Flavell[§], David J. Flavell[§] and Aldo Ceriotti*

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Plant ribosome inactivating proteins (RIPs) are N-glycodidases that act by catalytically depurinating an Adenine residue (A4324 in rat) of a conserved stem-loop region in 23/26/28S large ribosomal RNA subunit. This causes an irreversible arrest in protein synthesis, leading to apoptotic cell death in mammalian cells. The prototype plant N-glycosidase is ricin AB dimer that enters mammalian cells by endocytosis and undergoes retrograde transport via the Golgi complex to the endoplasmic reticulum (ER), exploiting the ER-associated degradation (ERAD) pathway, normally used for the disposal of misfolded or unassembled host polypeptides. Ricin toxic A chain and monomeric Saporin from Saponaria officinalis, as well as other bacterial toxin domains (deriving from diphtheria toxin or Pseudomonas exotoxin A) are currently used to produce cytotoxic chimeras. These are able to specifically kill tumor cells by virtue of the targeting domain(s) being selected. Most of these domains belong to secretory proteins, such antibody fragments or growth factors that would normally fold in the ER.

To investigate the use of an eukaryotic host for chimera™ production, we have chosen Pichia pastoris being a microbial host that posses a tightly regulated Alchool-oxydase 1 promoter for growth on methanol as a sole carbon source, coupled to ER quality control system, allowing secretion-competent polypeptides reaching the extracellular medium to be easily purified. Inducible expression of “non optimized” saporin constructs resulted in lack of expression following methanol induction of a protease- deficient SMD1168 strain and host toxicity in GS115 (his4) cells which expressed low levels of secretory saporin. Expression levels were 10-fold higher using a mutant at the catalytic site, obtained by changing Glu176 to Lysine and Arg179 to Gln.

We deeply investigated several parameters affecting protein expression levels and correct polypeptide processing in order to produce active biomolecules for pre-clinical development.

Despite the presence of some host toxicity, Pichia cells could produce and secrete chimeras constituted by fusions to the NH2 terminus of Saporin of either a model humanized single chain antibody fragment or the Amino-terminal fragment of human urokinase (ATF). The ligand binding domain ATF directs this chimera against cells overexpressing the human urokinase receptor, a target receptor in myeloid leukaemias which is also involved in metastatic spread of different tumor cell types . ATF-saporin was not correctly folded during cytosolic expression in bacteria, leading to “homeopathic” amounts of this chimera being recovered. Secretory yeast saporin and ATF-saporin could be produced in much higher amounts than when expressed in E. coli and the polypeptides purified from Pichia culture supernatants showed expected RIP activity.The yeast secretory Saporin inhibits reporter translation with an IC50 of 15x10-12M in a cell-free assay, as seed-extracted protein. Secretory ATF-saporin chimera having an exahistidine tag also shows the expected killing activity against promyelocytic target cells, being almost 1000 times more potent than Saporin, with an IC50 of 6x 10-11 M on acid-washed U937 cells.

Saturday, 8:30-8:50

MOLECULAR MECHANISMS OF SYNAPSE ORGANIZATION: THE ROLE OF SYNAPSINS

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The synapsins (Syns) are evolutionarily conserved neuron-specific phosphoproteins that localize at the presynaptic level. They are implicated in interactions with synaptic vesicle (SV) proteins, phospholipids and actin playing multiple roles at the synapse. Their biological activity is tightly regulated through phosphorylation. Syn I is the best-characterized member of the family. It has been shown to control the availability of SV for exocytosis tethering the vesicles to each other and linking them to the actin cytoskeleton, thus regulating neurotransmitter release. It is known from literature that Syn I is involved in synaptogenesis and synaptic plasticity. The residue Ser 9, phosphorylated by cAMP-dependent protein kinase is involved in precocious stages of neuron development. We found that the overexpression of the Syn Ia non-phosphorylatable mutant in hippocampal neurons caused a decrease in the number of glutamatergic synapses, while the wild type or the pseudophosphorylated protein overexpression had no effect. Interestingly, any variation of Syn Ia concentration and phosphorylation caused a change in the number of puncta positive for the presynaptic scaffolding protein Bassoon but negative for presynaptic vesicle markers. Recent data indicate that the formation of synapses involves the delivery of “transport packets” containing several sets of proteins necessary for proper synaptic function. It has been hypothesised that as yet undefined molecules can sense the synaptic microdomain and stop the travelling transport packets leading to the rapid accumulation of vesicles at the sites of synapse formation. We have not clarified yet the nature of Bassoon positive puncta, however we hypothesize that they could be Bassoon transport packets and that Syn Ia could be involved in their targeting. In fact it has been shown recently at the ultrastructural level that Syn Ia can associate with these packets.

Saturday, 8:50-9:10

THE SYNAPTIC VESICLE CYCLE IS AFFECTED BY CHOLESTEROL DEPLETION

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In a previous study carried on in rat brain synaptosomes, we demonstrated that SNARE proteins and P/Q type calcium channels (Cav2.1) colocalize in membrane subdomains enriched in cholesterol. This localization seems to be important for the physical and functional coupling of the secretory machinery. We have recently investigated whether cholesterol and lipid microdomains may play a role in presynapses formation and activity. In order to investigate these aspects we used hippocampal neurons in culture. Firstly we demonstrated the distribution of aliquots of SNARE proteins and Cav2.1 channels in detergent resistant membranes (DRMs) isolated from hippocampal neurons by floatation on sucrose gradients. In addition, we showed that Cav2.1 and SNAP-25 patches detected by immunocytochemistry at presynaptic boutons have properties of cholesterol-enriched domains being insoluble to Triton-extraction but completely soluble after saponin pre-treatment.

To analyze whether lipid microdomains are involved in the stability of presynapses, hippocampal cultures at 14 DIV were treated with fumonisin B1, (inhibitor of sphingolipid synthesis) and mevastatin or squalestatin S1, two drugs know to affect the synthesis of cholesterol by inhibiting HMG-CoA reductase or squalene synthase. In untreated neurons, immunoreactivity for Cav2.1 and the synaptic vesicle marker synaptobrevin2 appeared as small puncta along the dendritic branches. In fumonisin or mevastatin treated cultures, the cholesterol levels are lowered to about 20% of control. In these neurons the density of puncta immunolabeled for presynaptic proteins was reduced to ~30% of control and the size of the remaining puncta appears increased (~1.5 fold compare to control). Similar results were obtained with 5mM squalestatin whereas higher concentrations of the drug (10mM) alter the distribution of synaptobrevin2 and largely increased the lost of presynaptic terminals. To analyze whether the morphological modifications observed with the drugs were associated with alteration of neurotransmitter release, the exo-endocytic recycling of synaptic vesicles was monitored by the selective uptake in synaptic vesicles of the the fluorescent styryl dye FM1-43. The results demonstrated that whereas fumonisin and mevastatin do not significantly inhibit the activity dependent uptake of FM1-43, 5 mM squalestatin induces a significant reduction of synaptic vesicle exo-endocytosis (~50% of control). Since alteration of cholesterol level may influence the binding of the lipophilic dye FM1-43 to the membranes, to detect synaptic activity we used a well characterized method based on the use of an antibody direct against the luminal epitope of the synaptic vesicle protein synaptotagmin which has been found to efficiently monitor the exo-endocitic traffic of synaptic vesicle. The results demonstated that squalestatin, but not fumonisin or mevastatin, efficiently reduces the uptake of the antibody (35% of control). Altogether these data suggest that lipid microdomains play an important role in maintaining the morphology and stability of presynapses and that cholesterol depletion may impair presynaptic function, by affecting the synaptic vesicle cycling.

Supported by Cariplo Foundation (2006.0882/10.4878).

Saturday, 9:10-9:30

A LYSOLECITHIN/FATTY ACID MIXTURE PROMOTES AND THEN BLOCKS NEUROTRANSMITTER RELEASE AT THE DROSOPHILA MELANOGASTER LARVAL NEUROMUSCULAR JUNCTION

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The study of the effect of snake presynaptic neurotoxins with phospholipase A2 activity on nerve terminals has recently unveiled the inhibitory action of a lysophosphatidylcholine (LysoPC)/fatty acid mixture. We report here that these neurotoxins have no activity on Drosophila melanogaster nerve terminals. However, a 1:1 mixture of LysoPC and oleic acid induces an early increase, followed by an inhibition of both evoked and spontaneous neurotransmitter release. This effect is also induced by LysoPC alone. The present findings provide an indirect evidence that the lipid hemifusion-to-pore transition is a key event in neuroexocytosis in Drosophila. Moreover, these findings substantiate the use of LysoPC as a general agonist of membrane fusion at nerve terminals. This novel tool could contribute to the unraveling of the molecular steps involved in neuroexocytosis, particularly in Dros.

Saturday, 9:30-9:50

MECHANISM OF ACTION OF PRESYNAPTIC SNAKE PLA2 NEUROTOXINS

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Presynaptic snake neurotoxins endowed with PLA2 activity (SPANs) are major components of the venom of four families of venomous snakes (Crotalidae, Elapidae, Hydrophiidae and Viperidae). These neurotoxins play a crucial role in envenomation of the prey by causing a persistent blockade of neurotransmitter release from nerve terminals with a peripheral paralysis through inhibition of the neuromuscular junction. We found that the phospholipids hydrolysis produced by the SPANs cause a massive exocytosis of the synaptic vesicles not followed by endocytosis and this results both from increased calcium influx and reduced efflux due to mitochondrial dysfunction with reduced ATP production. We also found that these toxins are able to penetrate spinal cord motoneurons and cerebellar granule neurons, and to selectively bind to mitochondria. As a result of this interaction, mitochondria depolarize and undergo a profound shape change from elongated, to round and swollen. These neurotoxins facilitate opening of the permeability transition pore (PTP), an inner membrane high-conductance channel. The relative potency of SPANs was the same for PTP opening and for PLA2 activity, suggesting a causal relationship. These findings contribute to define the cellular events that lead to SPAN intoxication of nerve terminals and suggest that mitochondrial dysfunction is an important determinant of their toxicity.ophila, where it is straightforward to combine it with electrophysiology and genetics.

Saturday, 10:20-10:40

A NOVEL RAB5-BASED SIGNALING PATHWAY PARTICIPATES IN CENTROSOME COHESION

Valentina Margaria[1], Sanne Jensen[2], Laura Virgili[1], Jiri Bartek[2], Federico Bussolino[1], Pier Paolo Di Fiore[1,2] and Letizia Lanzetti[1]

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The centrosome is made of two connected centrioles that replicate at the G1/S transition. Duplicated centrosomes are held together until G2, when they separate and migrate to the opposite poles of the cell. Here, we report the identification of a Rab5-dependent pathway participating in centrosome separation at late G2/mitosis. We found that Rab5, a critical GTPase controlling membrane trafficking, is associated with the centrosome in human cells and that both the Rab5 GTPase activating protein, RN-tre, and the Rab5 GDP/GTP exchange factor, Rabex-5, localize at the centrosome. Increased Rab5 activity caused loss of centrosome cohesion, suggesting a role for this GTPase in centrosome function. Indeed, reduction of the Rab5 activity, by simultaneous silencing of Rab5A, Rab5B and Rab5C, inhibited centrosome separation during G2 and reduced the distance between the spindle poles at mitosis. The molecular mechanisms downstream of Rab5 involve KIF3A, a kinesin motor protein that is also a component of the centrosome. We show that KIF3A binds preferentially to the active GTP-bound form of Rab5 and that it is required for proper centrosome separation. Finally, KIF3A is recruited to centrosomes in a Rab5-dependent manner. We therefore propose that Rab5 has a function in the separation of duplicated centrosomes at G2/M through a novel effector mechanism, relying on the recruitment of KIF3A.

Saturday, 10:40-11:00

THE ROLE OF MYOSIN VI IN INTRACELLULAR TRAFFICKING AND CELL MIGRATION

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The molecular motor Myosin VI (Myo6) belongs to the family of non-conventional myosins. Myo6 structure comprises an actin-binding region (i.e., motor/head) and a hinge domain (i.e., neck), which binds myosin light chains. The following C-terminal coiled-coil (i.e., tail domain) contains a ubiquitin binding domain (i.e., motif interacting with ubiquitin, MIU) and a terminal cargo-binding region (Buss et al., 2002). Myo6 moves towards the minus end of actin filaments, away from plasma membrane, thus Myo6 can work as a carrier for the intracellular trafficking of cargo proteins. Furthermore, Myo6 plays a role in clathrin-mediated endocytosis of specific membrane receptors (e.g., transferrin receptors) (Buss et al., 2002). Recent data from our lab have defined that endocytosis of the ligand-activated epidermal growth factor receptor (EGFR) occurs, as a function of ligand dose, through both clathrin-mediated and non clathrin-mediated internalization routes and that EGFR ubiquitination is required for the intracellular trafficking of the receptor via the non-clathrin pathway (Woelk et al., 2007). Whether Myo6 is involved in any step of EGFR endocytosis still remains unexplored. Remarkably, Myo6 is also involved in EGFR-mediated cell migration through a yet unidentified mechanism (Buss et al., 2002).
Here, we investigated the putative role of Myo6 MIU domain in both EGFR endocytosis and EGF-mediated cell migration. The characterization of the ubiquitin-derived features of the Myo6 MIU domain revealed that it can bind isolated lysine 48- and lysine 63-liked polyubiquitin chains as well as ubiquitinated proteins. Mutation of the critical alanine residue in the MIU domain strongly reduced its ability to interact with ubiquitin. Furthermore, the same point mutation interfered with the capacity of Myo6 to undergo coupled monoubiquitination. The role of Myo6 in EGFR endocytosis was evaluated in HeLa cells infected with leniviral-based constructs in which Myo6 expression levels were stably kocked down. In Myo6 knocked-down cells the initial rate of ligand-induced EGFR internalization, EGFR downmodulation as well as EGFR recycling were unaffected. Although no differences in the number of surface EGFR were detected in Myo6 knocked-down cells with respect to empty vector infected cells, an increase in the amount of total EGFR was scored in the absence of Myo6, thus suggesting a role for Myo6 in the biosynthetic pathway of EGFR. On the other hand, the role of Myo6 in EGFR-mediated cell migration was evaluated in the highly invasive breast cancer MDA-MB-231 cells. Stable depletion of Myo6 resulted in a significant reduction in cell proliferation and migration. Indeed, these cells displayed both impaired cell motility and directional migration as evaluated by scratch and Boyden chamber assays as well as time-lapse experiments. Finally, the role of the MIU domain of Myo6 in these Myo6-dependent phenotypes is under investigation through rescue experiments with constructs bearing wild type or MIU-devoid Myo6.

References

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Woelk et al., 2007; Cell Div 13:2-11.

Saturday, 11:00-11:20

PROTECTIVE AUTOPHAGY DURING TERMINAL PLASMA CELL DIFFERENTIATION

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Plasma cell (PC) differentiation entails drastic changes in gene expression, proteome and cellular morphology. All these changes are needed to reprogram the cell towards its new task: antibody secretion. To cope with new challenge differentiating PCs expand their secretory apparatus, by taking advantage of single branches of the unfolded protein response.
In an apparent paradox to the teleology of this cellular reprogramming we have previously found that both proteasomal subunits and proteasome activity are down regulated during plasma cell differentiation, leading us to foresee a link between proteasome stress and the very short life of these cells.
The unique combination of proteasome stress and high metabolic activity also directed our interests on macroautophagy (hereafter referred to as autophagy), the second most important catabolic process of the cell. Autophagy is a widely conserved process that encloses cytoplasmic contents and organelles in a double membrane vesicle, and delivers them to lysosomes. Using both differentiating murine lymphoma and primary B cells differentiated with LPS we found an overall increase in the acidic compartments and also induction of autophagy, as inferred by the increase in the conversion of the LC3 protein, an established autophagic marker, and immunofluorescence analyses. Inhibition of lysosome activity resulted in a further increase in the processed form, showing that autophagosomes are actively fusing with lysosomes. We also found that LC3 mRNA progressively increases during differentiation presumably to continuously fuel the autophagic compartment. Inhibiting autophagy with 3-methyl adenine increased plasma cell death, suggesting that autophagy protects antibody-secreting cells from apoptosis.
Our results show a new role of autophagy in adaptive immunity as a protective mechanism during plasma cell differentiation and provide also a challenging framework to the study of the interplays between protein stress, autophagy and the secretory pathway.

Saturday, 11:20-11:40

ANALYSIS OF MECHANISMS OF SORTING OF GPI-ANCHORED PROTEINS IN POLARIZED EPITHELIAL CELLS: ROLE OF THE GPI-ANCHOR AND ECTODOMAIN

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Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) are directly targeted to the apical domain of plasma membrane of the majority of epithelial polarized cells and are enriched in specific membrane domains called rafts or DRM (detergent resistant membrane) because of their resistance to detergent extraction. We have recently demonstrated that GPI-APs travel to the apical membrane of polarized epithelial cells in high molecular weight complexes and that impairment of their oligomerization blocks their apical sorting.
Nonetheless several questions are still open: What does promote oligomerization? Which are the roles of the ectodomain and/or of the GPI anchor in facilitating protein clustering and consequently apical sorting? To address these questions we used two different approaches. We first analyzed in MDCK stable clones the intracellular trafficking of proteins, which have the same ectodomain (GFP) and different GPI attachment signal (derived from an apical GPI-protein, FR, or from a basolateral one, PrP). We have analysed the behaviour of the resulting fusion proteins, GFP-FR and GFP-PrP, by analysing three parameters: DRM-association, oligomerization and apical sorting. Strikingly we report that different GPI-attachment signal modulate both the ability of the attached ectodomain (GFP) to oligomerize and to be apically sorted. This is likely to derive to differences in the GPI anchor and the surrounding lipid environment. Indeed addition of cholesterol is necessary and sufficient to revert the behaviour of the basolateral GFP-protein and to allow its oligomerization and consequent apical sorting.
In the alternative approach, we investigated the possible role of N- and O- glycosylation by analyzing the behaviour of two glycosylated GPI-anchored apical proteins, p75GPI and PLAP, and their glycosylation mutants. We found that both the N- and O- glycosylation mutants are apically sorted, associate to DRMs and are able to oligomerize, like the wild-type proteins, suggesting that glycosylation does not have a direct role in GPI-AP oligomerization and apical sorting. Interestingly, when cells are depleted of cholesterol and treated with tunicamycin, PLAP is not able to oligomerize and is missorted to the basolateral surface, thus supporting an indirect role of N-glycosylation, possibly mediated by a raft-associated glycosylated interactor.
Together, these data suggest that oligomerization is promoted by protein ectodomain, but it needs also a favourable lipid environment to occur and the GPI anchor could mediate this process. Hence both the protein ectodomain and the GPI anchor are involved together in determining apical sorting of GPI-APs.

Saturday, 11:40-12:00

ENDOCYTOSIS AND THE RAB5 EFFECTOR RABENOSYN-5 IN PLANAR CELL POLARITY

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In addition to apico-basal polarization, some epithelia also display polarity within the plane of the epithelium. To what extent polarized endocytosis plays a role in the establishment and maintenance of planar cell polarity (PCP) is at present unclear.
Here, we investigated the role of Rabenosyn-5 (Rbns-5), an evolutionarily conserved effector of the small GTPase Rab5, in the development of *Drosophila* wing epithelium. We found that Rbns-5 regulates endocytosis at the apical side of the wing epithelium and, surprisingly, further uncovered a novel function of this protein in PCP. Rbns-5 binds to the Exocyst component Sec5, which forms a complex with the PCP protein Fmi. During planar polarization, Rbns-5 is recruited along with Sec5 by Fmi at the apical cell boundaries and redistributes along the P/D axis. Loss of Rbns-5 causes intracellular accumulation of Fmi, which leads to typical PCP alterations such as defects in cell packing, polarized distribution of PCP proteins and hair orientation. Our results indicate that Rbns-5 regulates endocytic trafficking of Fmi at the apical cell boundaries required for establishment of planar polarity.

Saturday, 13:30-13:50

FGD1 (FACIOGENITAL DYSPLASIA PROTEIN) INVOLVED IN REGULATION

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Faciodenital dysplasia (Aarskog syndrom, FGDY) is an X-linked disease, which is accompaned by several defects of bones, urogenital abnomalites and mental retardations (Aarskog 1970). FGD1 gene (affected in FGDY patients) encodes a GEF that specificali activates the Rho GTPase Cdc42. In turn, Cdc42 is known to be an important regulator of membrane trafficking. Whether or not the secretion of proteins, which is a process essential for bone formation, is altered by mutations in FGD1 is of great interest. We have found that both FGD1 and its target, Cdc42, were preferentially associated with the TGN compartment of the Golgi complex suggesting that both proteins are involved in the export of cargo proteins from the trans side of the Golgi. Indeed, the expression of FGDY-related FGD1 mutant, as well as FGD1 silencing, resulted in reduction of post-Golgi transport of various cargoes (comprising bone specific proteins in osteoblasts). Live cell imaging revealed that formation of post-Golgi transport intermediates carrying cargo proteins to the cell surface appears to be affected in FGD1-deficient cells due to impairment of interaction between TGN membranes and microtubules. This effect on transport appears to depend on FGD1 ability to regulate Cdc42 activations as well as its association with the Golgi membranes and may contribute to FGDY pathogenesis.

Saturday, 13:50-14:10

IDENTIFICATION OF PRAF2 AS A NOVEL BCL-XL INTERACTING PROTEIN

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Bcl-xL is a commonly overexpressed oncogene with potent anti-apoptotic activity. To gain insights into the biochemical implications of Bcl-xL overexpression in tumour development, we performed an extensive screen for membrane proteins interacting with Bcl-xL using the Tandem Affinity Purification (TAP) technology. Using this approach we identified a group of proteins with a known or predicted role in cellular trafficking as novel Bcl-xL-interacting proteins. We focused our effort on the characterization of Praf2, a protein belonging to the PRA1 (Prenylated Rab Acceptor) protein family. The ability of Praf2 to interact with Bcl-xL was confirmed by co-immunoprecipitation. Deletion analysis showed that the C-terminal TM domain of Bcl-xL mediates the interaction with Praf2, while deletion of either the N-terminus or the C-terminus of Praf2 abolished the binding. Interestingly we found a general ability of members of the Bcl-2 protein family (Bcl-xL, Bcl-2, Bak and Bax) to interact with members of the PRA family (Praf2 and Praf3). Transient transfection of Praf2 results in a time dependent induction of apoptotic cell death, which is prevented by the co-transfection with Bcl-xL. Furthermore, knock-down of Praf2 expression by RNA interference decreases the sensitivity of HeLa cells to etoposide-induced cell death. Immuno-localisation of endogenous Praf2 showed that the protein is localised in small vesicles evenly distributed in the entire cytoplasmic space of the cell. We have evidences that Praf2 has a role in protein secretion. We found that Praf2 knocked down RPE cells have an increased secretion of Urokinase (uPA) in the cell culture medium. Furthermore, increased Praf2 expression reduces the targeting of multi membrane-spanning receptors to the plasma membrane. We propose that Praf2 could influence cellular viability by modulating the autocrine secretion of growth/survival factors and the amount of membrane receptors presented on the surface.

Saturday, 14:10-14:30

GLIADIN PEPTIDE P31-43 INTERFERES HRS (HEPATOCYTE GROWTH FACTOR-REGULATED TYROSINE KINASE SUBSTRATE) LOCALIZATION TO ENDOCYTIC VESICLES: IMPLICATIONS FOR CELIAC DISEASE

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Background and Aims
We previously observed that A-gliadin peptide P31-43 induces effects similar to Epidermal growth factor (EGF) both in cultured cell lines and in enterocytes from celiac disease (CD) patients. We also showed that the effect is mediated by delayed EGF degradation and prolonged EGF receptor (EGFR) activation in endocytic vesicles. Here we address the molecular mechanisms underlying gliadin peptide effects on trafficking and maturation of vesicles responsible for EGFR endocytosis.
Methods
A sequence similarity search revealed that P31-43 is strikingly similar to a region of Hrs, a key molecule involved in endocytic maturation. Western blot and immunofluorescence microscopy were used to determine Hrs localization to endocytic vesicles and cytosol. Pulse and chase labelling in time-lapse experiments allowed to follow uptake and sub-cellular localization of gliadin peptides in CaCo 2 cells and enterocytes from CD patients and controls.
Results
A-gliadin peptide P31-43 interferes with Hrs localization to early endosomes. Both P31-43 and the control P56-68 peptide enter CaCo 2 cells and interact with the endocytic compartment, but P31-43 is localized to vesicles carrying early endocytic markers at time points when P56-68-carrying vesicles mature into late endosomes. Dynamic analysis shows that P31-43 labelled vesicles are slowed down in time-lapse experiments. The effect is independent of the cargo they carry: dextran containing vesicles, behave similarly to EGFR containing ones. Trasferrin receptors and Lamp, markers of recycling pathway, are increased on the surface of P31-43 treated cells.
Conclusions
P31-43 delays vesicle trafficking by interfering with Hrs mediated maturation to late endosomes and also promotes the recycling pathway. As a consequence, in P31-43 treated cells, EGFR activation is extended and more transferrin receptor finds its way to the surface. This may help explaining the role played by gliadin peptides in CD pathogenesis.

Saturday, 14:30-14:50

TRASTUZUMAB INDUCES ERBB2 SIGNALLING AND ENDOCYTIC EVENTS IN A BREAST CANCER CELL LINE

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The ligand-less tyrosine kinase receptor ErbB2 is overexpressed in approximately 30% of invasive breast cancers. Its overexpression occurs primarily through amplification of the wild type *her-2* gene, and it is associated with poor disease-free survival. Patients with ErbB2 over-expressing breast cancer have substantially lower survival rates and shorter time to relapse then patients without the overexpression. Due to its importance in breast cancer, ErbB2 has become an important therapeutic target.
Clinical trials have shown the efficacy of the humanized monoclonal antibody, direct against the extracellular domain of ErbB2, called Trastuzumab (Herceptin®) in metastatic breast cancer patient. Trastuzumab is actually used in therapy; nevertheless, 70% of patients are unresponsive from start of treatment and nearly all become unresponsive during treatment. However, the understanding of the molecular mechanisms underlying this resistance is impaired by the poor knowledge of the precise mechanism of action of Trastuzumab.
Here we report two apparently distinct effects of Trastuzumab on ErbB2 fate in ErbB2 over-expressed SKBR3 breast cancer cell line.
We found an early effect of Trastuzumab on ErbB2, consisting in the induction of ErbB2/ErbB1 heterodimerization, the specific phosphorylation of ErbB2-Y1248 and ErbB1-Y1173, and recruitment in lipid RAFTs domains. Moreover, Trastuzumab induces the activation of specific intracellular signals determining the de-phosphorylation of Akt, and the activation of Erk1/2. Our data indicates that the Akt de-phosporylation is Erk1/2-phosphorylation dependent.
These events are associated to the induction of internalization, followed by recycling of a sizable fraction of ErbB2, occurring within 20 minutes from start of treatment. In addition, no ubiquitination of the receptor heterodimer is observed within this time scale.
At later time points, we observed a slow degradation of the receptor, starting after 72 hours of treatment, and the induction of a G_1 growth arrest after 24 hours of treatment.
Our data suggest that Trastuzumab may be responsible of early events leading to ErbB2/ErbB1 heterodimer activation and signalling, and later events leading to growth arrest and receptor degradation. In particular, our data indicates that internalization and recycling events are needed for the activation of Erk1/2, responsible for the de-phosphorylation of Akt.
Our model purpose the internalization/recycling events as the mechanism for the activation of the signal cascade that induces Akt down-regulation in early times, in order to lead the following induction of G_1 arrest in late time points.

Saturday, 14:50-15:10

OA1 SIGNALING PATHWAY: ROLE IN MELANOSOME BIOGENESIS AND MELANOMA PROGRESSION

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OA1 is a pigment cell specific membrane glycoprotein, whose misfunction is responsible for Ocular Albinism type 1, an X-linked disease involved in the visual system. The characteristic phenotype of the disease is the presence of giant melanosomes (macromelanosomes) in RPE (Retinal Pigmented Epithelium) and skin, suggesting that OA1 plays a role in melanosome biogenesis.
The OA1 protein belongs to the GPCR (G protein Coupled Receptors) superfamily, but unlike all others known GPCR, it localizes on endolysosomal and melanosomal membranes.
In spite of this knowledge, the physiological function of OA1 is still unclear.
Our results show that OA1 regulates MITF (MIcrophthalmia-associated Transcription Factor), a transcription factor located in the center of multiple signaling pathways, controlling the differentiation, morphology, proliferation, and survival of the melanocyte lineage (i.e. melanoblasts, melanocytes and melanoma). In differentiation, Mitf plays a major role in melanogenesis, inducing the expression of key enzymes. In proliferation, Mitf plays a double role as inducer/repressor of cellular proliferation. Furthermore, changes in Mitf activity are implicated in melanoma progression.
We demonstrate that OA1 regulates Mitf at transcriptional level in two distinct cell systems: the rate of production of MITF is sustained by the presence of OA1. In cells depleted for OA1, the mRNA and protein levels of MITF show a sizable decrease.
In light of these results, our experiments have been directed to understand the OA1 signaling pathways involved in MITF regulation.
Moreover, we performed WB assays in human melanoma cell lines, finding a tight correlation between OA1 and MITF expression. This way can lead to elucidate a putative role of OA1 in melanoma progression.