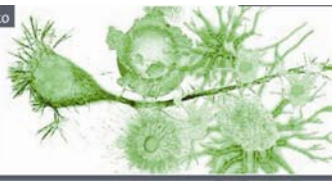
Associazione di Biologia Cellulare e del Differenziamento

ABCD



ABCD Meeting

"Membrane Trafficking and Organelle Biogenesis"

Certosa di Pontignano, 16-17 April 2010

Programme & Abstracts

Friday, 16 April

13:00-14:00 *lunch*

14:25 **Conference opening**

14:30-16:30 Session I: **Endoplasmic Reticulum: biogenesis, trafficking and beyond**
***chairs**: P. Remondelli (Salerno), R. Sitia (Milano)*

14:30-14:50 P. Cassella (Milano) "Role of folding of the C-terminal domain in unassisted translocation across phospholipid bilayers"
14:50-15:10 M. Fossati (Milano) "Role of membrane curvature in transmembrane domain-dependent partitioning of membrane proteins whithin the endoplasmic reticulum"
15:10-15:30 C. Bencini (Siena) "Molecular determinants of triadin localization and mobility at the junctional domain of the sarcoplasmic reticulum"
15:30-15:50 Margherita Cortini (Milano) "Coupling efficiency and fidelity in the IgM factory"
15:50-16:10 M. Bertolotti (Milano) "B to plasma cell terminal differentiation entails oxidative stress and profound reshaping of the antioxidant responses"

16:20-17:00 *Coffee break*

17:00-17:40 Session II: **In and through the Golgi Complex**
***chair**: S. Paladino (Napoli)*

17:00-17:20 R. Venditti (Napoli) "Sedlin is a novel Sar1 effector in the control of procollagen trafficking"
17:20-17:40 R. Rizzo (Napoli) "Polymerization of a resident Golgi protein to understand how the proteins are transported through the Golgi"

17:40-18:20 Session III: **GTPase in membrane trafficking and cell polarity**
***chair**: F. Filippini (Padova)*

17:40-18:00 M. Egorov (Napoli) "FGD1 (faciogenital dysplasia protein) regulates post-Golgi transport via CDC42 and its down-stream targets"
18:00-18:20 M. Santoriello (Napoli) "Rac1 and the control of cell polarity in FRT thyroid epithelial cells"

18:30-19:30 **Guest lecture:**
Matthias Weiss (German Cancer Research Centre and Institute for Modelling and Simulation in the Biosciences, Heidelberg, Germany)
"Towards a quantitative understanding of membrane and protein traffic"

19:30-20:00 General discussion: election of Vice-Chair for next year

20:00-21:00 Dinner

21:00 Wine, cheese & billiard

Saturday, 17 April

9:00-9:40 Session IV: **Membrane traffic at synapses**
***chair**: B. Chini (Milano)*

9:00-9:20 A. Fratangeli (Milano) "Role of cholesterol in synaptic vesicle exo-endocytosis"
9:20-9:40 M. Zordan (Padova) "Synaptic and muscular alterations in larvae of *Drosophila melanogaster* sphingosine-1-phosphate lyase mutants"

9:40-10:40 Session V: **Regulation of membrane trafficking**
***chair**: C. Bucci (Lecce)*

9:40-10:00 L. Paolini (Brescia) "Adaptor protein complex AP-4 phosphorylation: a functional, tightly, modulated event"
10:00-10:20 N. De Franceschi (Padova) "Ten years of Longin domain: from SNARE regulator to building block and central player in subcellular trafficking"
10:20-10:40 L. Albania (Padova) "Subcellular targeting can be regulated by splicing-dependent modulation of domain architecture: the example of non-Longin and non-SNARE variants of TI-VAMP/VAMP7"

10:40-11:20 Coffee break

11:20-12:40 Session VI: **Membrane trafficking and diseases**
***chair**: C. Zurzolo (Napoli)*

11:20-11:40 Romeo Betto (Padova) "Folding, assembly and degradation of skeletal muscle sarcoglycan complex"
11:40-12:00 Céline Schaeffer (Milano) "GPI-anchoring loss plays a role in the formation of uromodulin intracellular aggregates"

12:00-12:20 Paola Rusmini (Milano) "The involvement of Small Heath Shock Protein B8 in the degradation of mutant proteins in motor neuronal disease"
12:20-12:40 M.V. Barone (Napoli) "Endocytosis is constitutively altered in celiac disease (CD)"

13:00 Lunch and departure

ABSTRACTS

Subcellular targeting can be regulated by splicing-dependent modulation of domain architecture: the example of non-Longin and non-SNARE variants of TI-VAMP/VAMP7

Lara Albania[1], Valeria Rossi[1], Marcella Vacca[2], Andrea Carpi[1], Floriana Della Ragione[2], Nicola De Franceschi[1], Maria Strazzullo[2], Ornella Rossetto[3], Maurizio D'Esposito[2], Francesco Filippini[1]
[1]Dipartimento di Biologia, Università di Padova
[2]IGB-CNR, Napoli
[3]Dipartimento di Scienze Biomediche, Università di Padova ed Istituto di Neuroscienze, CNR

Longin R-SNAREs are conserved in all Eukaryotes and they are prototyped by Ykt6, Sec22b and TI-VAMP/VAMP7. Ykt6 and Sec22b are involved in trafficking steps at the ER and Golgi compartments and in most organisms they are present as single genes; instead, VAMP7 shows a higher variation in gene number, ranging from a single copy in human and animals to 10-15 and even more in land plants. In Arabidopsis, eleven VAMP7 proteins are involved in trafficking pathways at different subcellular localizations. In animals, different VAMP7 proteins can be produced by alternative splicing (AS) of single VAMP7 genes. Splicing variants of human TI-VAMP/VAMP7 show an intriguing modulation of the domain architecture. In addition to the already characterized non-longin variant TI-VAMP/VAMP7c, we report here on the non-SNARE variant TI-VAMP/VAMP7b, which shares with the main isoform both a complete longin domain and the N-terminal part of the SNARE domain. However, because of exon 6 skipping and subsequent coding frameshift, the C-terminal part of the SNARE domain, the TMD and the intravesicular tail are replaced by a unique, unknown function region. The expression of isoforms a, b and c of TI-VAMP/VAMP7 was investigated in different tissues and cell lines and the relative RNA levels were assessed by quantitative real-time RT-PCR. Oligoclonal antibodies specific to the unique domain of TI-VAMP/VAMP7b were designed, purified and assayed vs. the recombinant domain expressed in E. coli. This allowed to obtain immunological evidence of the TI-VAMP/VAMP7b protein in human cell extracts. Finally, a comparative analysis between the main isoform and the b variant was performed by structural modeling, two-hybrid assay in yeast and fluorescence/confocal microscopy to check intramolecular binding and subcellular localization.

Endoplasmic reticulum stress reduces the export from ER and alters the architecture of post-ER compartments

Giuseppina Amodio[1,2], Maurizio Renna[1,2],Simona Paladino[3], Consuelo Venturi[4], Carlo Tacchetti[4], Ornella Moltedo[1], Silvia Franceschelli[1], Massimo Mallardo[2], Stefano Bonatti[2], Paolo Remondelli[1]
[1]Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Fisciano-Salerno, Italy
[2]Dip. di Biochimica e Biotecnologie Mediche, Univ. degli Studi di Napoli "Federico II", Naples, Italy
[3]Dip. di Biologia e Patologia Cellulare e Molecolare, Univ. degli Studi di Napoli "Federico II", Naples, Italy
[4]MicroSCoBiO Research Center and IFOM Center of Cell Oncology and Ultrastructure, Dip. di Medicina Sperimentale, Univ. di Genova, Italy

In eukaryotic cells several physiologic and pathologic conditions generate the accumulation of unfolded proteins in the endoplasmic reticulum (ER), leading to ER stress. To restore normal function, some ER transmembrane proteins sense the ER stress and activate coordinated signalling pathways collectively called the Unfolded Protein Response (UPR). Little is known on how the UPR relates to post-ER compartments and to the export from the ER of newly synthesized proteins. Our findings show that the ER stress response induced by either thapsigargin or nitric oxide modifies the dynamics of the intracellular distribution of ERGIC-53 and GM130, two markers of the ER-Golgi Intermediate Compartment and of the cis-Golgi, respectively. In addition, induction of ER stress alters the morphology of the ERGIC and the Golgi complex and interferes with the reformation of both compartments. Moreover, ER stress rapidly reduces the transport to the Golgi complex of the temperature sensitive mutant of the Vesicular Stomatitis Virus G glycoprotein (VSV-G) fused with the Green Fluorescent Protein (ts045G). Incubation with TG did not alter the oligomerization of the reporter protein, thus suggesting that the reduced rate of transport was not due to a rapid decrease of the amount of the cargo protein competent for the export step. Interestingly, we observed a parallel rapid reduction on the ER membranes of the number of fluorescent puncta labelled by the COPII components Sec31 and Sec23. Instead, in the same experimental conditions the number of the sites labelled by Sec16, which defines the ER Exit Sites (ERES) remained unchanged. Our results, suggest that the ER stress alters the ER export and the dynamic of post-ER compartments by rapidly targeting the formation and/or the stability of the COPII-coated transport intermediates.

Endocytosis is constitutively altered in celiac disease (CD)

Sara Santagata, Giuliana Lania, Merlin Nanayakkara, Raffaele Troiano, Virginia Vitale, Roberta Kosova, Valentina Discepolo, M. Vittoria Barone
Dept Pediatrics and ELFID, University Federico II, Naples, Italy

Celiac Disease CD is frequent disease (1:100) and an interesting model of disease induced by food. The disease is triggered by gluten and glutenins contained in wheat and other cereals. It consists in an immunogenic reaction that happens in a specific genetic background only partially understood. We have, previously investigated the early events of the disease, and in particular the interaction between gliadin, and epithelial intestinal cells. We found that gliadin and the so-called gliadin toxic peptide (P31-43) induce EGF dependent proliferation and actin rearrangements, on several cell lines and intestinal crypts enterocytes of CD patients. We also found that gliadin and P31-43 are not ligand for EGFR, but they can delay the activated receptor decay by interfering with the endocytic pathway.

Why these effects of gliadin peptides on the endocytic compartment are so disruptive on the CD mucosa? To understand whether any alteration of this pathway could represent a predisposing condition to gliadin effects we have investigated the endocytic pathway in mucosa from CD patients, both in the active phase of the disease and on gluten free diet (GFD), and from potential CD respect to controls. Potential CD are relatives of CD patients at gluten containing diet, with predisposing HLA, positive for auto-antibodies in the serum, but without intestinal alterations. The analysis of the endocytic compartment in CD mucosa showed that more EEA1 positive vesicles are present in CD enterocytes respect to controls and mainly in the active CD. In CD patients on GFD the amount of EEA1 vesicles was intermediate between active CD and controls. Interestingly the enterocytes from potential CD have many EEA1 positive vesicles comparable to the active CD. Indicating that alteration of the endocytic compartment could be one of the first signs of damage in the CD mucosa preceding intestinal atrophy and a possible marker of the disease. We also show that the endocytic compartment in CD biopsies is malfunctioning as peptide P31-43 is delayed in EEA1 positive vesicles in biopsies from CD patients, but not in controls. Moreover, fluorocrome tagged-EGF, remains in the vesicular compartment of celiac enterocytes (both in the acute and in the remission phase of the disease) longer than in controls.

In conclusion we show that the endocytic pathway, the same pathway gliadin peptides can interfere with, is constitutively altered in CD mucosa, this explaining the specificity of gliadin peptides effects in CD. These data also imply that the interaction between common alimentary proteins and epithelial intestinal cells is not completly understood and needs to be farther studied and clarified.

Molecular determinants of triadin localization and mobility at the junctional domain of the sarcoplasmic reticulum

Cristina Bencini, Daniela Rossi, Francesca Benini, Stefania Lorenzini, Marina Maritati, Vincenzo Sorrentino

Sarcoplasmic reticulum (SR) is a specialized form of endoplasmic reticulum composed by a complex network of tubules and cisternae that surrounds the myofibrils. It can be structurally divided in two regions: the longitudinal sarcoplasmic reticulum (lSR) and the junctional sarcoplasmic reticulum (jSR), characterized by a different protein composition. The main protein of the longitudinal tubules is SERCA pump involved in the uptake of calcium from the cytoplasm. In the junctional reticulum the macromolecular complex of calcium release, composed by the ryanodine receptor (RyR), triadin, calsequestrin and junctin is localized.
However the mechanisms that drive these molecules to these specific SR domains are still unknown. It can be speculated that either targeting signals or interaction with other proteins could be involved in protein localization.
In order to investigate this point, studies on the localization and the mobility of jSR proteins have been performed in our laboratory. In particular, we focused our attention to triadin, an integral SR membrane protein that has been proposed to interact with RyR and calsequestrin. Triadin is characterized by a short cytoplasm N-terminal portion that contains a site of interaction with RyR, a single alpha helix transmembrane domain and a long C-terminal luminal tail that contains a binding site for calsequestrin overlapping with a second RyR binding site.
Studies performed in our laboratory identified three regions important for triadin localization at the jSR. Interestingly, FRAP analysis of the mobility revealed that triadin shows the lower mobility among other jSR proteins. Deletion of the C-terminal portion (aa 264-729) resulted in an increase of mobility.
To further investigate the role of the three identified regions in mediating either the localization or the mobility of triadin we performed knocked down experiments and GST pull down experiments both on skeletal muscle tissue and cellular lines.

B to plasma cell terminal differentiation entails oxidative stress and profound reshaping of the antioxidant responses

Milena Bertolotti[1], Sun Hee Yim[2], Silvia Masciarelli[1], Yoo-Jin Kim[2], Jose M. Garcia-Manteiga[1], Roberta Vené[3], Anna Rubartelli[3], Sue Goo Rhee[2], Roberto Sitia[1,4]
[1]Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milano, Italy
[2]Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea
[3]Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy
[4]Università Vita-Salute San Raffaele, Milano, Italy

Whilst limited amounts of reactive oxygen species (ROS) are necessary for cell survival and signaling, their excess causes oxidative stress. H_2O_2 and other ROS are formed as byproducts of several metabolic pathways, possibly including Ero1-dependent oxidative protein folding in the endoplasmic reticulum. B to plasma cell differentiation is characterized by a massive expansion of the endoplasmic reticulum, finalized to sustain abundant Ig synthesis and secretion. Thus, the increased production of disulfide-rich Ig might cause oxidative stress that could serve signaling roles in the differentiation and lifespan control of antibody-secreting cells. Here we show that terminal B cell differentiation entails redox stress, Nrf2 activation and reshaping of the antioxidant responses. To evaluate the role of the latter, we analyzed LPS-stimulated splenocytes obtained from mice lacking different antioxidant systems (Prxs, GPxs, Nrf2). Our results suggest that the early intracellular production of H_2O_2 facilitates B cell proliferation, and reveal a role for the Nrf2-pathway in the differentiation and function of IgM secreting cells. Novel ratiometric and organelle-targeted reporters are being developed to monitor the local production and fate of H_2O_2.

Folding, assembly and degradation of skeletal muscle sarcoglycan complex

Elisa Bianchini[1], Dorianna Sandonà[1], Stefano Gastaldello[1,2], Maurizio Molinari[3], Ruediger Rudolf[4], Chistoph Kaether[5], Romeo Betto[6]
Dept Biomedical Sciences, University of Padova, Italy
[2]Dept. of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden
[3]Institute for Research in Biomedicine, Bellinzona, Switzerland
[4]Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, Germany
[5]Leibniz Institut für Altersforschung-Fritz Lipmann Institut, Jena, Germany
[6]CNR Institute of Neuroscience, Padova, Italy

Multi-subunit membrane protein complexes undergo chaperone-assisted assembly in the endoplasmic reticulum (ER). We are investigating the molecular events controlling the folding, assembly and degradation of sarcoglycan complex, a four-subunit (α, β, γ, and δ) membrane complex critical for skeletal muscle integrity. The sarcoglycan complex is assembled within the ER and probably undergoes chaperone-assisted oligomerization control before passing on to the plasma membrane. Preliminary data indicate that the β, γ, and δ subunits, which are type II membrane proteins, form a sub-complex that is retained in the ER until α-sarcoglycan, a type I membrane protein, is recruited. The tetramer is eventually transported to the plasma membrane. Present research is investigating the function of i) retention motifs in β, γ, and δ subunits, ii) chaperone-mediated retrieval of subunits, and iii) α-sarcoglycan in masking retention/retrieval signals of β, γ, and δ sub-complex.
Mutations of subunits or chaperone malfunction can severely affect complex processing, inducing lack of complex assembly, enhanced subunit degradation and ER stress. Defects in sarcoglycan genes cause muscular dystrophy. Analysis of muscle of patients shows that mutations in any of the four genes leads to instability and thus to the reduction/absence of the entire complex from the muscle membrane. We have recently demonstrated that misfolded and unassembled sarcoglycans are routed to degradation by ERAD. Moreover, controlling degradation of disease-causing but still "functional" mutants produces positive retrograde effects that permit the formation of a complex correctly localized in the cell membrane. This observation might have important implications for the cure of sarcoglycanopathy.

Role of folding of the C-terminal domain in unassisted translocation across phospholipid bilayers

Patrizia Cassella[1], Anna Luciani[1], Sara Colombo[1], Nica Borgese[1,2]
[1]Consiglio Nazionale delle Ricerche Institute of Neuroscience and Dept of Pharmacology Università degli Studi di Milano, Milano, Italy
[2]Dept of Pharmacobiological Science, Univ. di Catanzaro "Magna Graecia"

Although transmembrane proteins generally require membrane-embedded machinery for integration, a few can insert spontaneously into liposomes. For example some tail anchored (TA) proteins, such as the electron carrier cytochrome b5 and the protein tyrosine phosphatase PTP-1B, are able to translocate into pure lipid vesicles without assistance from membrane proteins or cytosolic chaperones. Access to, or exclusion from the unassisted pathway is determined by the trasmembrane domaine (TMD of the TA) substrate. Moderately hydrophobic and short TMDs can insert in an unassisted manner, whereas more hydrophobic ones require assistance.
Our previous investigations revealed, quite unexpectedly, that the capacity for unassisted translocation of b5 is maintained, also when the C-terminus is elongated with as many as 85 residues. We are presently investigating the role of folding of the C-terminal domain in this spontaneous translocation phenomenon. To this aim we constructed a b5 variant, called b5-VSVGcys, in which a VSVG tag and a unique cysteine were appended to the C-terminus of Cyt b5. We used Sulfo-SMCC as crosslinker to link Bovine Pancreatic Trypsin inhibitor (BPTI) to the C-terminal Cys residue of b5-VSVGcys. BPTI is very small (we cannot exceed the limit of 85aa at C- term) and can be reversibly unfolded in vitro, simply by reducing its 3 disulfide bonds. The crosslinked protein in a folded or in an unfolded state was incubated with pure phosphatidylcholine vesicles. A protease protection assay, previously developed in our laboratory, was applied to detect the properly inserted C-terminal tail of our constructs. In this assay, post-translational translocation of the C-terminus across the membrane of a closed vesicle results in protease protection of a peptide. The protected fragment is detected by western blotting with antibodies against the C-terminal tag. The results of these experiments demonstrated that, although both the unfolded and folded protein bind to liposomes, only the unfolded protein efficiently translocates its C-terminus. We hypothesize that this unassisted translocation occurs by a sliding mechanism. We are presently extending our observations to another small protein HiPIP (High Potential Iron-Sulfur Protein) that can be reversibly unfolded in vitro by removing the Fe_2S_4 cluster.

Coupling efficiency and fidelity in the IgM factory

Margherita Cortini[1,2], Roberto Sitia[1,2]
[1]Università Vita-Salute San Raffaele, Division of Genetics and Cell Biology
[2]San Raffaele Scientific Institute, Milano

Antibody secreting cells (ASC) can release up to thousands of immunoglobulins (Ig) per second. They face a difficult task in producing polymeric IgM, planar molecules consisting of 21 or 24 subunits, depending on whether they are secreted as J-chain containing 'pentamers' [(μ2L2)5-J] or 'hexamers'.Owing to the quality control mechanisms operating in the early secretory compartment, only native proteins are secreted. Despite the difficulties in assembling planar IgM polymers, antibody secreting cells can release thousands of IgM per second. We recently demonstrated that ERGIC-53 a lectin transporter that cycles in the early secretory compartment and binds to selected glycoproteins in a Ca++ dependent-fashion, binds secretory Ig-μ (μs) chains and interacts with ERp44. The finding that secretory μ (μs) chains bind to ERGIC-53, suggested that ERGIC-53 hexamers could provide a polymerization platform. Since ERp44 also binds IgM subunits, these tripartite interactions could be important in IgM biogenesis. Indeed, the latter occurs sequentially in the early secretory compartment: μ2L2 assembly is rapid and is assisted by ER-resident chaperones, primarily BiP and PDI. μ2L2 'monomers' are then assembled via disulfide bonds involving Cys575 in the highly conserved, C-terminal μs tailpiece (μstp). Cys575 acts as a three-way switch, mediating assembly, retention and degradation of unpolymerized subunits. Based on these observations, we proposed a concerted action of ERGIC-53 and ERp44 in IgM polymerization. In our model, ERGIC-53 hexamers provide a platform for the assembly of planar polymers, receiving μ2L2 subunits that have already passed the BiP-dependent checkpoint. ERp44, abundant also in cisGolgi stacks, ensures that unpolymerized subunits are retrieved into the ER. To confirm and extend the model, here we investigate which of the five N-glycans present in μs chains mediates binding to ERGIC-53. We focused our attention on the two most C-terminal oligosaccharides, N-linked to Asn402 and Asn563. In secreted IgM, in fact, these are found in an endoglycosidase-H (Endo-H) sensitive, high mannose configuration, suggesting that polymerization makes them poorly accessible to the Golgi-resident enzymes. We also analyze the binding mechanisms of IgM subunits to ERp44.We have shown that conserved elements in the μstp, the Asn563 N-glycan and Cys575, bind ERGIC-53 and ERp44, respectively. The tripartite interaction between the μstp, an hexameric cargo receptor (ERGIC-53) and a retrieving chaperone (ERp44) facilitates IgM polymerization.

Ten years of Longin domain: from SNARE regulator to building block and central player in subcellular trafficking

Nicola De Franceschi, Andrea Zanardo, Marco Vedovato, Rosario Dioguardi, Paolo Bolognese, Lara Albania, Francesco Filippini
Dipartimento di Biologia, Università di Padova

Almost a decade ago, the Longin domain (LD) was identified bioinformatically as a conserved N-terminal region characterizing a subfamily of R-SNAREs, named the longins to be distinguished from the well-known "short" R-SNAREs or brevins. The suggested capacity of the LD to inhibit SNARE bundle formation by intramolecular binding to the R-SNARE domain was confirmed; however, next years investigations demonstrated that the LD is quite far from being only a SNARE regulatory domain. The LD is a building block and a central player in subcellular trafficking, as suggested by several lines of evidence: (i) longins are conserved in all Eukaryotes, whereas brevins are restricted to Opistokonta and Amoebozoa; (ii) the LD is a dominant signal for subcellular localization; (iii) an entire family of non-SNARE longins is conserved in land plants and non-SNARE longin genes e.g. Sec22a/c are present also in animals; (iv) the LD fold is conserved in important, non-SNARE trafficking proteins such as subunits of the AP, TRAPP and SRP complexes. Genome/proteome mining by homology search and pattern scanning, structure comparison and modeling and preliminary evolutionary analyses allowed us to infer conserved and special features of the LD. Its central block is conserved in several trafficking single- or multi-domain proteins and the alpha 1 helix shows a conserved helix-binging capacity, resulting in either homo/hetero-dimerization or in inter- and intramolecular binding. This latter was originally observed between LD and SNARE; however, intermolecular binding to Hrb and our preliminary data suggest alpha 1 may bind further non-SNARE regions. Special insertions modulate LD interaction properties, as shown by example proteins. In addition to insertions and variation of local surface patches, likely (or known to be) involved in interactions, functional modulation can be mediated by alternative splicing. Last but not least, LD genes and variants are involved in a number of human genetic diseases.

FGD1 (faciogenital dysplasia protein) regulates post-Golgi transport via CDC42 and its down-stream targets

Mikhail V. Egorov[1], Maria Grazia Capestrano2, Jerome L. Gorski[3], Alberto Luini[1], Roman S. Polishchuk[1]
[1]Tigem (Telethon Institute of Genetics and Medicine), Napoli, Italy
[2]Consorzio Mario Negri Sud, Santa Maria Imbaro, Chieti, Italy
[3]Division of Medical Genetics, Department of Child Health, University of Missouri-Columbia School of Medicine, Columbia, MO, USA

Faciogenital dysplasia (Aarskog-Skott syndrom, FGDY) is a rare X-linked disease, which is accompanied by different defects of bones, urogenital abnor-malities and mental retardations (Aarskog, 1970). FGD1 gene (altered in FGDY patients) encodes a GEF that specifically activates the Rho GTPase Cdc42. In turn, Cdc42 is known to be an important regulator of membrane trafficking. It seems to be that phenotype of patients is resulted of ability of FGD1 to regulate activity of CDC42 at the Golgi. FGD1 and its target, Cdc42, were preferentially associated with the TGN of the Golgi complex suggesting that both proteins are involved in the export of cargo proteins from the trans side of the Golgi. We have been shown that FGD1-CDC42 signaling is important component of TGN-plasma membrane transport. The expression of FGDY-related Fgd1 mutant, as well as CDC42 dominant negative mutant, caused a reduction of post-Golgi transport of main component of the bone such as procollagen-I. This effect on transport appears to depend on FGD1 ability to regulate association of cy-toskeleton with the Golgi membranes via activity of Cdc42. We have also as-sessed the down-stream targets of CDC42, which may be involved in regulation of cytoskeleton dynamics at of the Golgi such as N-WASP, PAK1 and IQGAP1. SiRNA screening evidently showed that N-WASP does not impaired TGN-plasma membrane traffic of VSVG. In turn, silencing of PAK1 and IQGAP1 strongly inhibited transport of VSVG in HeLa (versus control cells). We have noted that Golgi complex was segregated during traffic of VSVG in all cases. Aberration of transduction of signal of GEF activity FGD1 upon CDC42 and its down-stream targets during traffic of bone related proteins may contribute to understanding of FGDY pathogenesis.

Role of membrane curvature in transmembrane domain-dependent partitioning of membrane proteins within the endoplasmic reticulum

Matteo Fossati[1,2], Jean-Baptiste Manneville[3], Paolo Ronchi[4], Bruno Goud[3], Nica Borgese[1,2,5]
[1]Consiglio Nazionale delle Ricerche, Institute of Neuroscience, Milan, Italy
[2]Dept of Pharmacology, Univ. degli Studi di Milano, Milan, Italy
[3]Laboratoire Mécanismes moléculaires du transport intracellulaire, Institut Curie, CNRS UMR, Paris, France
[4]Cell Biology and Cell Biophysics Unit, European Molecular Biology Laboratory, Heidelberg, Germany
[5]Dept of Pharmacobiological Science, Univ. of Catanzaro, Catanzaro, Italy

Our group recently characterized a novel sorting mechanism within the ER that is independent from specific sorting signal and depends on transmembrane domain (TMD) length/hydrophobicity. More specifically, two fluorescent tail-anchored (TA) proteins differing only in TMD length (FP-22 and FP-17, with TMDs of 22 and 17 residues respectively) are segregated from each other at short time after expression: FP-22 is recruited to ER exit sites (ERES) and partitions into ER tubules, while FP-17 is excluded from ERES and has access to both ER tubules and sheets. Since FP-22's TMD is predicted to be longer than the thin ER bilayer (positive mismatch), we hypothesized that this hydrophobic mismatch may affect FP-22's sorting behaviour: one way of obtaining a more favourable interaction between the TMD and the acyl chains could be by segregating the longer TMD in curved ER domains, such as tubules. To investigate the role of membrane curvature in TMD-dependent sorting, purified FP-17 and FP-22 were initially reconstituted in small unilamellar vesicles (SUVs) of uniform lipid composition (POPC, palmitoyl-oleyl-phosphatidylcholine) and analyzed by negative staining. EM analysis didn't reveal any difference in the distribution of vesicle sizes between FP-17 and FP-22 proteoliposomes, however a biconcave disk morphology was more frequently seen in the case of proteoliposomes containing FP-22. To further investigate this potential mechanism of TMD-dependent partitioning, the two purified proteins were incorporated in giant unilamellar vesicles (GUVs), grown by the electroformation method. Since GUV sizes range between 10 and 100 μm, they allow analysis through optical microscopy. By inducing tubulation of the GUVs with molecular motors (Bruno Goud group, Institute Curie, Paris) we investigated whether FP-22 preferentially partitions into the pulled tubules. By comparing the normalized protein signal (using fluorescent lipids) of each protein in vesicles and tubes and analyzing the signal ratio of FP-22 to FP-17 in GUVs containing the two proteins together, no preferential segregation of FP-22 in tubes was observed. Rather, the two proteins seem to be homogenously distributed in both flat and curved domains. Taken together, these preliminary results suggest that membrane curvature alone is not sufficient to drive the TMD-dependent partitioning of FP-22 into ER tubules. We are presently carrying out more experiments to obtain a larger number of analyzable images and to investigate sorting in GUVs made with lipid mixtures.

Role of cholesterol in synaptic vesicle exo-endocytosis

Alessandra Fratangeli, Pamela Valnegri, Maura Francolini, Michela Matteoli, Maria Passafaro, Patrizia Rosa
CNR Institute of Neuroscience, Dept of Pharmacology, Milan

Previous studies using rat brain synaptosomes, 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 in cultured neurons whether cholesterol-enriched microdomains may play a role in presynapse formation and activity. Firstly, we studied the distribution of SNARE proteins and Cav2.1 channels in detergent resistant membranes isolated from cultured neurons. To analyze the role of lipid microdomains and cholesterol in presynapses activity, we treated neurons at 14 DIV with fumonisin B1 (inhibitor of sphingolipid synthesis) and mevastatin or squalestatin S1, two drugs known to affect the synthesis of cholesterol by inhibiting HMG-CoA reductase or squalene synthase. Drug treatment does not alter protein synthesis but modifies the cellular level of either GM1 or cholestrol. Moreover the use of these drugs do not have any effect on vesicle ultrastructure. To analyze whether sphingolipid or cholesterol depletion altered neurotransmitter release, the exo-endocytic recycling of synaptic vesicles was monitored in two ways: i) the selective uptake in synaptic vesicles of the fluorescent styryl dye FM1-43 and ii) the internalization of an antibody direct against the luminal epitope of synaptotagmin. The results demonstrated that squalestatin, but not fumonisin and mevastatin, induces a reduction of the uptake of both FM1-43 and synaptotagmin antibodies in glutamatergic- and gabaergic-positive synapses. Inhibition of anti-synaptotagmin antibodies uptake was observed after depolarization but also after incubation with hypertonic sucrose solution. Interestingly, anti-synaptotagmin uptake was restored in squalestatin treated neurons after cholesterol reloading. Taken together these results suggest a role of cholesterol in the exo-endocityc cycle of synaptic vesicle. To make a distintion between the involvment of the lipid in these two steps of vesicle cycling we monitored the exocytosis of synaptic vesicles with synaptopHluorin. After depolarisation we observed a decrease of synaptopHluorin fluorescence intensity in the synaptic boutons. Our data indicate that squalestatin treatment impairs synaptic vesicle exocytosis, thus suggesting that cholesterol may play a role in this process.

Adaptor protein complex AP-4 phosphorylation: a functional, tightly, modulated event

Lucia Paolini, Radeghieri Annalisa, Putzu Anna, Caimi Luigi, Ricotta Doris
Dipartimento di Scienze Biomediche e Biotecnologie, Università degli Studi di Brescia, Brescia, Italy

AP-4 is a heterotetrameric adaptor protein complex composed by 4 subunits like AP-1, AP-2 and AP-3 (Epsilon, beta-4, mu-4 and sigma-4). The AP-4 complex is predominantly localized at the Trans Golgi Network and throughout the citosol. No cytosolic binding partners have been discovered yet and so far only a mu-4 interaction with chanonical sorting signals has been demonstrated. Some new data, concerning the ability of mu-4 subunit to interact with a novel sorting signal contained in the Amyloid Precursor Protein, the major trigger of the neuronal Alzheimer's disease, will be published soon. In the present study we show that the cellular distribution of the heterotetrameric complex AP-4 is regulated by phosphorylation / dephosphorylation events, indeed this cycle is controlled by the PP2A like protein phosphatases family and by staurosporine sensitive kinases. We could also show that phosphorylation at different serine/threonine sites of the hinge / ear region is critical for molecular charge modifications and this process needs more than one actively phosphorylated site. A critical region, encompassing aa 839 to aa 871 is involved in the regulation of AP-4 / membranes interactions and finally we characterized three different phosphorylation sites in this region that act cooperatively on the epsilon ear molecular charge modification.

Polymerization of a resident Golgi protein to understand how the proteins are transported through the Golgi

Riccardo Rizzo, Seetharaman Parashuraman, Alberto Luini
TIGEM - Telethon Institute of Genetics and Medicine, Napoli, Italy

Golgi apparatus is the central station in the secretory pathway that receives the newly synthesised secretory proteins from the Endoplasmic Reticulum, processes them (mainly glycosylation) and then sorts them to their correct destinations. It consists of a stack of cisterna that each contain a distinct set of glycosylation enzymes. One of the main question that remain still open is how the proteins are transported through the Golgi : Is anterograde (i.e., cis to trans) transport of cargo mediated by carriers, as tubules or vesicles? or, is anterograde transport mainly due to the en-bloc movement of entire cisternae (i.e., cisternal progression) through the Golgi stack instead? (Rothman and Wieland 1996; Glick and Malhotra 1998; Pelham 1998). To search for an answer to these questions, I'm studying the dynamics of the Golgi-resident enzymes to determine how these are coupled to the dynamics of the cargoes that are transported through the Golgi. First, to investigate the dynamics of the Golgi enzymes, I have engineered a Golgi-resident glycosylation enzyme that can be polymerised in a regulated way, to obtain a polymer that is large enough not to enter retrograde carriers. I'm also studying if and how this polymerization affects the localisation of the Golgi-resident proteins and the functioning of the Golgi apparatus. I find that the polymerisation does affect the localization of the golgi resident and also the transport through the Golgi apparatus thus adding credence to the cisternal maturation model of intra-Golgi transport.

The involvement of Small Heath Shock Protein B8 in the degradation of mutant proteins in motor neuronal disease

Paola Rusmini, Valeria Crippa, Francesca Simonini, Elena Bolzoni, Angelo Poletti
Dipartimento di Endocrinologia, Fisiopatologia e Biologia Applicata, Centro di Eccellenza sulle Malattie Neurodegenerative, Università degli Studi di Milano, Milano,Italy

Autophagy is a catabolic process in which cell components are delivered to lysosome compartment for degradation. Lysosomes are able to degrade both functional proteins and damaged proteins that are no longer functional, to avoid their accumulation inside cells. Most of the mutant misfolded proteins involved in neurodegenerative disorders organize into irreversible oligomers and aggregates which has to be cleaved from the cells. We study two motorneuron disorders, the Spinal and Bulbar muscular atrophy (SBMA) and a familial form of Amyotrophic Lateral Sclerosis (fALS). SBMA is caused by an polyglutamine tract expansion (polyQ) in the Androgen Receptor (AR) proteins, while fALS is often associated to mutations in Superoxide Dismutase 1 (SOD1). Mutant AR (ARpolyQ) and SOD1 do not share structural or functional domains , but are unstable and form aggregate.
We produced immortalized motorneuron, NSC34, expressing mutant form of SOD1 (SOD1G93A) or ARpolyQ and found that contained intracellular aggregates and have reduced proteasome activity.
We have observed that the overexpression of a small heat shock protein (HspB8) decreased the levels of both mutant proteins and desaturated the proteasome activity. Inhibition of proteasome functions did not block the HspB8 chaperone activity suggesting the HspB8 does not require a functional proteasome to remove the misfolded proteins. HspB8 might facilitate the mutant proteins degradation using the autophagic pathway. In SBMA and fALS cellular models, HspB8 overexpression stimulated the formation of LC3-II, a key molecule involved in macroautophagy, and it increased the number of autophagosomes. These results suggest an important role of HspB8 in the degradation of SOD1G93A and ARpolyQ trough macroautophagy. It appears that HspB8 might bind the mutant misfolded proteins and act in a chaperone-complex with Bag3 stimulating the autophagic pathway.
GRANTS: Telethon - Italy (GGP06063 and GGP07063); Italian Ministry of Health (2007-36 and 2008-15) Convenzione Fondazione Mondino/UNIMI); Universita' degli Studi di Milano; Fondazione CARIPLO (2008-2307).

Rac1 and the control of cell polarity in FRT thyroid epithelial cells

M. Santoriello[1],Corteggio A.[1],Nitti M.[1] ,Mascia A.[2], Calì G.[2], Paladino S.[1],Montella R.[1], Nitsch L.[1], Garbi C.[1]
[1]Dip. Biologia e Patologia Cellulare e Molecolare di "L. Califano", Univ. di Napoli "FEDERICO II", Italia
[2]IEOS-CNR Napoli, Italia

The acquisition of cell polarity, which includes the establishment of the tight junction barrier, polarized assembly of the cytoskeleton and appropriate organization of membrane traffic, requires external cues, that in epithelial cells are represented by the interaction of cells with their neighbors and with the extracellular matrix. The Rho family of small GTPases, regulate many biological processes including cell cycle progression, apoptosis, migration and intercellular adhesion. We have focused on the analysis of the role of Rac1 protein in the acquisition and mantainance of the polarized phenotype in the FRT rat thyroid epithelial cell line. The FRT cells exhibit a fully polarized epithelial phenotype, manifest high transepithelial electrical resistance and express apical and basolateral marker proteins. They also form organized tridimensional follicular structures in suspension culture. The Rac1 subcellular distribution in FRT cells was initially investigated. Subsequentely, in order to analyze the role of Rac1 in the control of cell polarization in the FRT cells, the cultures were treated with NSC23766, a molecule that does not allow Rac1 specific GEFs , such as Tiam1 and Trio, to bind to Rac1 and therefore acts as an inhibitor of Rac1 activation. Several aspects including directional migration, TER acquisition, cell aggregation and formation of polarized follicles were investigated and found to be affected by the pharmacological inhibition of Rac1 activity. Moreover in this type of experiment, changes in subcellular localization of the E-cadherin molecule were analyzed in parallel with those regarding the Rac1 molecule. Preliminary data also suggest that Rac1 may be involved in the control of the Golgi apparatus integrity and therefore in the polarized intracellular traffic of proteins. This experimental appoach allowed us to establish that Rac1 is a major regulator of the polarization process in FRT cells.
In the FRT-β1B cells, derived from FRT parental cells after transfection of the dominant-negative β1B integrin, an impairment of the polarized phenotype have been described To test the hypothesis that this may be due to a reduced Rac1 activity, subclones derived from FRT-β1B cells were obtained that stably express a Rac1 construct that is constitutively activated upon 4-OH-tamoxifen treatment. However it was not possibile to rescue the normal phenotype in these cells by this experimental approach. Furthermore also FRT parental cells expressing the same construct appeared to be hampered in the expression of certain properties of the polarized epithelium, indicating that sustained but not regulated acivation of Rac1 impairs the acquisition of cell polarity.

GPI-anchoring loss plays a role in the formation of uromodulin intracellular aggregates

Céline Schaeffer[1], Simone Perucca[1], Ilenia Bernascone[1], Matteo Trudu[1], Elena Polishchuk[2], Giorgio Casari[3], Luca Rampoldi[1]
[1]Dulbecco Telethon Institute c/o Molecular Genetics of Renal Disorders Unit, Division of Genetics and Cell biology, San Raffaele Scientific Institute, Italy
[2]Dept of Cell Biology and Oncology, Istituto di Ricerche Farmacologiche Mario Negri Sud, Santa Maria Imbaro, Italy
[3]Neurogenomics Unit, Center for Genomics, Bioinformatics and Biostatistics, San Raffaele Scientific Institute, Italy

Medullary Cystic Kidney Disease (MCKD) and Familial Juvenile Hyperuricemic Nephropathy (FJHN) are autosomal dominant diseases characterised by alteration of urinary concentrating ability, tubulo-interstitial fibrosis, hyperuricemia and gout and progressive kidney failure frequently leading to end-stage renal disease and dialysis. They are caused by mutations in the UMOD gene, encoding uromodulin. It is a GPI-anchored protein that is specifically produced by epithelial cells lining the thick ascending limb of Henle's loop (TAL) from which it is proteolytically released into the urine, where it represents the most abundant protein. Our studies in transfected cells have shown that uromodulin mutants are defective in export to the plasma membrane due to retention in the endoplasmic reticulum (ER), however without eliciting the unfolded protein response (UPR) or cell death. Noteworthy in the cellular models studied so far, mutant uromodulin does not aggregate in the ER, as seen in patient biopsies. We hypothesised that GPI-anchoring could play a key role in protein aggregation and generated MDCK, HEK, CHO and Huh7 cells stably expressing soluble uromodulin, truncated before the GPI-anchoring site. While soluble wild-type protein was efficiently secreted, soluble isoforms carrying patient mutations C150S and N128S were mainly retained in the ER where they form aggregates via the formation of intermolecular disulfide bonds. Interestingly, the number of cells showing aggregates increased when cells were kept confluent. Moreover, part of wild-type GPI-anchored protein was trapped in aggregates formed by mutant soluble isoforms when they were co-expressed, suggesting a dominant-negative effect. We are currently assessing the consequences of aggregation on cellular viability and proliferation as well as on the activation of ER stress pathways. Noteworthy, we recently generated a transgenic mouse model expressing mutant uromodulin that recapitulates all the hallmarks of the disease, including ER aggregation of the protein and ER membrane hyperplasia. It will thus provide us with a unique in vivo model where to test our hypothesis on the role of GPI anchoring in uromodulin aggregation and where to analyse in depth the mechanisms of uromodulin ER retention and proteotoxicity.

Sedlin is a novel Sar1 effector in the control of procollagen trafficking

Rossella Venditti[1], Tiziana Scanu[2], Giuseppe Di Tullio[3], Michele Santoro[1], Renato Gaibisso[4], Alexander Spaar, Alexander A. Mironov[3], Galina V. Beznoussenko[3], Cathal Wilson[3], Maria Antonietta De Matteis[1,3]
[1]Telethon Institute of Genetics and Medicine (TIGEM) Naples, Italy
[2]The Netherlands Cancer Institute, Amsterdam, The Netherlands
[3]Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro (CH), Italy
[4]ETH, Zurich, Switzerland

The small GTPase Sar1 and its effector, the COPII coat, have key roles in the packaging of neosynthesised cargo into transport carriers directed to the Golgi complex from the endoplasmic reticulum (ER). Also, the multimolecular complex TRAPP is believed to have a role in docking of these carriers to the Golgi complex. A defect in Sar1b is the cause of chylomicron retention disease, or Anderson disease, which is characterised by impaired transport of lipid particles (an exceptionally large secretory cargo); defects in Sec23a are responsible for cranio-lenticulo-sutural dysplasia, which is characterized by severe reduction in secretion of cartilage extracellular matrix components; and finally, genetic defects in sedlin are responsible for recessive X-linked spondyloepiphyseal dysplasia tarda (SEDT), which is characterized by short stature, flattening of the vertebrae, premature osteoarthritis, and an absence of systemic complications. Although the molecular mechanisms underlying SEDT remain to be defined, they are believed to involve the altered processing (and possibly trafficking) of extracellular matrix components. Our aim is to specifically study the role of sedlin, and in general, of the TRAPP complex, in the trafficking of extracellular matrix components, such as procollagen. Using RNA interference, we have knocked-down sedlin expression in different cell lines, including procollagen secreting cells. Here we report that the transport of procollagen, but not of small soluble cargo or membrane cargo, is selectively inhibited, and that the COPII components remain more stably associated with ER membranes in sedlin-knocked-down cells. Moreover, we show that sedlin binds directly to Sar1, suggesting a new regulatory role for TRAPP in the COPII cycle.

Synaptic and muscular alterations in larvae of *Drosophila melanogaster sphingosine-1-phosphate lyase* mutants

Mauro A. Zordan[1], Anna Magnabosco[1], Michele Scorzeto[1], Greg Harris[2], Uwe Rose[3], Aram Megighian[1]
[1]University of Padova Fly Neurobiology Lab, Italy
[2]San Diego State University, CA, USA
[3]Ulm University, Germany

Sphingolipids are increasingly found to be involved as major players in a number of aspects of cell and organismal biology. Homologs of key enzymes belonging to the sphingolipid metabolic pathway have been discovered in *Drosophila melanogaster*, making this an ideal model to study the effects of sphingolipid metabolism dysregulation. We are currently studying *sphingosine-1-phosphate lyase* (*sply)* null mutants. These animals, in the adult stages, show a profound reduction in flight performance which appears to be related to degeneration of flight muscle fibers. Larvae also show locomotor impairments. In this respect we had previously reported that neuromuscular junctions (NMJ) show significant pre- and post-synaptic morphological alterations, while functional imaging, using the lipophilic strylyl dye FM1-43 and electrophysiological analyses suggest anomalous synaptic vesicle recycling. We have extended our analyses to the larval muscular system. While the number, position and general morphology of muscles appears normal following staining with rhodamine-phalloidin, electron-microscopic analyses of muscle fibers reveals profound alterations at the sub-sarcolemmal level, with striking accumulations of glycogen-like granules, leading to a displacement, and reduction in the number, of mitochondria. Furthermore, functional experiments suggest that *sply* mutant muscles develop significantly less force in relation to muscle fiber membrane clamp potential. It would thus appear that the observed locomotor defects are the result of the combined impairments at the synaptic and muscular levels.