Instituto Juan March
de Estudios e Investigaciones

67

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Membrane Fusion

Organized by

V. Malhotra and A. Velasco

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INTRODUCTION

Vivek Malhotra

Membrane fusion is essential for the formation of myoblast, cell division, organelle assembly, protein transport in vesicle fission and fusion. An understanding of this process is therefore of fundamental importance. At the recent meeting on "membrane fusion" it became clear that significant progress has been made in our understanding of the process but a lot is still unknown.

A variety of approaches have led to the identification of important components of the fusion machinery. The major known components such as NSF, SNAPs, SNAREs, Rabs, proteins involved in phosphoinositide metabolism, and other accessory proteins were discussed at the meeting. Some of the more recent surprises regarding the role of these components are: NSF can act prior to docking of the fusing membrane, v - and t-SNAREs align in a parallel fashion on the opposing fusing membranes, not all v-SNAREs are essential for survival, the overall structures of NSF, P97 and hsp 104 revealed by electron microscopy are very similar and some fusion events do not require NSF and SNAPs. A suggestion was made to consider the paired SNAREs equivalent to the haemagglutinin (HA) molecules that are anchored in both the viral and the cellular membrane. In both cases, a ring of these membrane-anchored molecules appears to form a fusion pore. It was concluded that the SNAREs play a central role in membrane fusion, but their role in docking/targeting is less clear. This is a general problem because assays to distinguish clearly between the docking and the fusion steps are not presently available.

Lipids and lipid metabolites which are essential components of the fusion machinery are also being revealed, although like their partners (i.e., proteins) the exact role is far from clear. It is also not obvious whether all the protein components involved in the terminal stages of the fusion process have been identified or if there are many unknowns after the t-SNAREs. One should also remember that no SNAREs, or their close cousins, have been found on mitochondria and peroxisomes, which also rely on fusion for growth and division. The events regulating vesicle fission (during the budding process) are also likely to involve a different mechanism and therefore will continue to generate new twists in the ongoing quest.

The electrophysiologists are developing very clever assays to obtain a kinetic analysis of the terminal stages of the docking and fusion events. Mice defective in specific proteins should not only reveal their significance in the fusion event but also help determine the physiological role of these molecules in the development of synaptic junctions and the nervous system.

Surprisingly, there are a number of new members identified by genetic trickeries using the simple organism yeast for which an exact role in the terminal stages of the secretory pathway is presently not known. The requirement for G-proteins and GTP-hydrolysis is clear, but the exact functions which are being regulated is still rather controversial.

In other words, as concluded by some "that we are more or less there" may be a bit premature. It is however clear that the enthusiasm to address this problem at the molecular level is only going to escalate, and therefore the next meeting in a decade or so on this topic will have (hopefully) all the details.

Session 1. Known components of the vesicle docking/fusion machinery

Chairman: Ira Mellman

The Exocytotic Machinery of Yeast.

Peter Novick, Christiane Walch-Solimena, Ruth Collins, Barry Elkind, Daniel TerBush*, Wei Guo, Dagmar Roth, Fern Finger and Eric Grote. *Department of Cell Biology, Yale University School of Medicine, New Haven Connecticut*

We have been focusing on a set of genes whose products are required specifically for the final stages of the secretory pathway in yeast. We can now define three systems that together mediate polarized vesicle delivery, docking and fusion. The first system includes Sec4 and Sec2. Sec4 functions as a nucleotide dependent switch that resides on the surface of secretory vesicles. Mutations in *SEC2*, itself an essential gene acting at the same stage of the secretory pathway, cause Sec4p to mislocalize as a result of a random, rather than polarized accumulation of vesicles. Sec2p and Sec4p directly interact, with the nucleotide-free conformation of Sec4p being the preferred state for interaction with Sec2p. Sec2p functions as an exchange protein, catalyzing the dissociation of GDP from Sec4 and promoting the binding of GTP. We propose that Sec2p functions to couple the activation of Sec4p to the polarized delivery of vesicles to the site of exocytosis. These regions are marked by a large complex of proteins, that we have termed the Exocyst, that is peripherally associated with the plasma membrane. This complex contains one copy of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15 and a new gene product termed Exo70. Once vesicles have been properly delivered, the interaction with the plasma membrane is mediated by an integral membrane protein on the vesicle (Snc1&2) and an integral membrane protein on the target membrane (Sso1&2). This protein-protein interaction is regulated by Sec9 and Sec1 in response to Sec4 function. [Supported by NIH grants GM-35370 and CA46128].

* Present address: Department of Biochemistry, University of the Health Sciences, Uniformed Services, Bethesda, Maryland

Mechanisms of Synaptic Transmission

Richard H. Scheller, Ph.D., Investigator

When the action potential travels down the nerve and enters a release zone, changes in the membrane potential open channels which allow calcium to enter the cell. The calcium promotes transmitter release and membrane fusion. The membrane then recycles forming new vesicles which are then replenished with chemical transmitter. This cycle might be considered the fundamental process that underlies nervous system function, yet little is known about the molecular mechanisms involved. In an attempt to define the molecular mechanisms which regulate membrane flow in the nerve, we have begun to characterize the proteins associated with the critical organelle in the process, the synaptic vesicle.

Studies of these molecules have led to a working hypothesis for synaptic vesicle docking and fusion. They propose that the plasma membrane protein syntaxin is associated with a soluble factor, n-sec1, on the plasma membrane. As the vesicle docks, a 7S particle is formed as n-sec1 is displaced from the complex. The 7S particle is comprised of two vesicle-associated proteins, VAMP and synaptotagmin, and two components of the plasma membrane, SNAP-25 and syntaxin. At least part of the specificity of vesicle docking may be determined by interactions of the components of this 7S particle. Experiments *in vitro* demonstrated that as the soluble factor α-SNAP is added to the complex synaptotagmin is displaced. Only after α-SNAP is associated with the complex, can NSF bind forming an approximately 20S particle. Upon ATP hydrolysis by NSF, the particle dissociates leading to intermediates that precede the fusion of the lipid bilayers.

The steps of this pathway, with the exception of those involving synaptotagmin, are proposed to occur in all vesicular docking and fusing reactions and are mediated by molecules which are members of gene families defined by the neural isoforms described above. We propose that in a nerve terminal the constitutive vesicle docking and fusion mechanism is used for synaptic transmission, however, a series of regulatory steps leading to the exquisite properties of the synapse are superimposed upon this machinery. One of the nervous system regulatory molecules may be synaptotagmin, however the mechanism of synaptotagmin action is not yet known.

Many of the molecules discussed in the above model are structurally and functionally homologous to proteins which have been genetically identified in yeast to function in the secretory process. In yeast there are 15 genes which have been demonstrated to be required for the fusion of vesicles derived from the Golgi with the plasma membrane. Of these 15 genes only 7 have known homologues in mammalian systems. This suggests at least two possibilities. First, only a part of the pathway of secretion may be conserved from yeast to the mammalian nervous system or second, perhaps the homologous proteins have not yet been identified in mammals. In order to begin to differentiate between the two hypotheses, we have identified the first mammalian homologues of two of these yeast genes. The genes are homologues of the yeast proteins sec6 and sec8 and the rat homologues are referred to as rsec6 and rsec8. Both rsec6 and rsec8 are present in a 750 kDa complex comprised of 8 proteins. Amino acid sequencing of peptides derived from each of the bands reveals that each of the molecules are novel, previously undescribed proteins. The peptide sequence information is being used to isolate the corresponding cDNA clones. The rsec6/8 complex is present in nerve terminals and is largely associated with the plasma membrane. Further biochemical and structural studies are aimed at understanding the specific roles of this large complex of proteins in secretion.

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The role of cholesterol in apical transport

K. Simons, P. Keller, and P. Scheiffele
EMBL Meyerhofstr. 1, 69117 Heidelberg, Germany

MDCK cells employ two different biosynthetic routes from the trans Golgi network to the polarized cell surface. The basolateral route employs cytosolic sorting signals and a Rab8/NSF/SNAP/SNARE mechanism for correct delivery. The apical route, on the other hand, makes use of a different set of sorting determinants (GPI-anchors, transmembrane protein domains, and N-glycans). Also the apical delivery machinery is of a novel type involving annexin 13b and VIPs. The key element in apical transport is the involvement of sphingolipid-cholesterol rafts as moving platforms onto which apical proteins specifically attach. The incorporation of proteins into rafts can be monitored by detergent-insolubility. For instance, the apical marker proteins, influenza hemagglutinin (HA) becomes insoluble in the detergents Triton X-100 and CHAPS at 4°C after arrival to the Golgi complex. We have analyzed the requirements for inclusion into the complex and found that cholesterol is an essential component. Recent results also demonstrate that cholesterol plays an essential role in apical transport. Depletion of cellular cholesterol by lovastatin and methyl-beta-cyclodextrin treatment of MDCK cells results in inhibition of apical transport while basolateral delivery is not affected. Further results suggest that VIP21/Caveolin-1 which is a cholesterol-binding protein plays a role in facilitating apical transport.

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Yukiko Goda

Exocytosis at central synapse: regulation of a late step by rab3A

The rab family of low MW GTP-binding proteins, an example of the yeast-to-man conservation mentioned above, has been generally implicated in guiding membrane fusion between a transport vesicle and the target membrane. The docking and fusion steps of vesicles comprise a complex multistep reaction, and the precise point at which any rab protein acts in the fusion scheme is not known. Previously, mice lacking rab3A - a rab protein that in brain is specific to synaptic vesicles - were created in order to evaluate its proposed function in synaptic vesicle exocytosis (1). Rab3A deficient mice exhibited greatly reduced levels of rabphilin but no other biochemical or morphological changes were observed. The physiological properties of the mutant synapses were unremarkable, consistent with a non-essential role for rab3A in various steps involved in synaptic vesicle formation, docking, and fusion. In the current most popular model, rab proteins are thought to facilitate docking of vesicles to the target membrane, possibly determining the specificity of docking. We have re-investigated transmitter release properties of rab3A knock-out mice with a variety of electrophysiological methods to resolve the precise point at which rab3A acts in the sequence of the synaptic vesicle docking and fusion steps that constitute synaptic transmission. We find that the mutant phenotype is expressed when the first exocytotic event is triggered such that the absence of rab3A leads to multiple vesicle fusions upon initiation of exocytosis.

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IDENTIFICATION OF THREE PUTATIVE NEGATIVE REGULATORS OF SNARE COMPLEX FORMATION AND EXOCYTOSIS IN YEAST ((J.E. Gerst, D. David, V. Lustgarten, and S. Sundarababu)) Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel.

Yeast possess two homologs of the synaptobrevin/VAMP family of proteins which function as v-SNAREs on secretory vesicles. These homologs, Snc1 and Snc2, are acylated membrane proteins which are required for normal constitutive exocytosis. Snc proteins appear to promote vesicle fusion by interacting with partner t-SNAREs (e.g., Sec9 and Sso1,2) from the plasma membrane to form a prefusion SNARE complex, as has been proposed for synaptobrevin/VAMP.

We are continuing to use yeast as a genetic model in which to study the actions of Snc-synaptobrevin proteins. Recent studies have identified three genes which appear to regulate vesicle docking and exocytosis. One gene (*VSM1*) encodes a novel 428 amino acid protein which was identified as a Snc-binding protein in the two-hybrid assay. Vsm1 localizes to both types of exocytic vesicles in yeast and, based upon genetic evidence, inhibits Snc protein functions. We have designated this negative regulator as a putative "SNARE-master", due to its ability to specifically downregulate v-SNARE functions.

Two other genes, *VBM1* and *VBM2*, were isolated as chromosomal suppressors for the loss of *SNC* gene expression in yeast. Recessive mutations in either gene restore v-SNARE-independent secretion. Interestingly, each mutation selectively re-couples the parallel exocytic paths found in yeast cells. Thus, while the Snc v-SNAREs may act promiscuously to confer docking and fusion on either path, the Vbm proteins appear to act as gatekeepers, each specifically allowing only one type of vesicle to dock and fuse. Cloning of *VBM1* shows that it encodes a five transmembrane domain-containing protein that is presumed to be vesicle-associated. Whether the mutant Vbm proteins confer v-SNARE-like functions themselves; allow other proteins to act as v-SNAREs; or act in a v- and t-SNARE-independent fashion, altogether, to confer docking and fusion is under investigation, and will be discussed.

Session 2. Small molecular weight GTP-binding proteins regulating membrane fusion.
Chairman: Graham Warren

The GTPase switch in the regulation of endocytic membrane dynamics

M. Zerial
EMBL, Meyerhofstrasse 1, 69017 Heidelberg, Germany.

Small GTPases of the Rab family act as specific regulators of membrane traffic. In the endocytic pathway, Rab5 regulates tranport from the plasma membrane to the early endosomes as well as homotypic endosome fusion. Recycling from the early endosomes to the plasma membrane is regulated by Rab4 and through the recycling endosome by Rab11. In vitro studies have revealed several properties of the GTPase switch of Rab5. This protein uses GTP hydrolysis as a timer that determines the frequency of membrane docking/fusion events. On the membrane, nucleotide exchange converts Rab5 into the GTP-bound, active form. We have recently identified a 100kD cytosolic protein, Rabaptin-5 that acts as a Rab5 effector. Rabaptin-5 is recruited by Rab5:GTP on the endosome membrane and is essential for Rab5 function. Chromatographic fractionation of cytosol and immunoprecipitation experiments indicate that Rabaptin-5 is complexed to another 60kD cytosolic protein. While the inhibition of early endosome fusion caused by immunodepletion of Rabaptin-5 from cytosol cannot be rescued by adding recombinant Rabaptin-5 alone, addition of a fraction of purified Rabaptin-5-p60 complex not only restores but also potently stimulates the fusion reaction. More recently we have found that p60 acts as a potent exchange factor for Rab5. These results suggest that Rabaptin-5 and p60 constitute a complex required for Rab5-dependent endosome docking/fusion.
Rab proteins are not the only GTPases implicated in the regulation of endosome docking and fusion. We have recently identified a novel member of the Rho family of small GTPases, RhoD, which regulates both the actin cytoskeleton and the motility and distribution of the early endosomes. An activated mutant of RhoD inhibits the Rab5-stimulated fusion of early endosomes *in vivo*. RhoD is therefore a candidate regulatory factor for the interaction between endosomal membrane and cytoskeleton.

MOLECULAR ANALYSIS OF NEUROTRANSMITTER RELEASE, Thomas C. Südhof, Howard Hughes Medical Institute and Dept. of Molecular Genetics, The University of Texas Southwestern Medical Ctr., Dallas, TX 75235-9050 USA.

Molecular studies on synaptic vesicles have led to a detailed description of their components. Recent studies using three approaches have now led to insights into mechanisms involved in their synaptic vesicle traffic: **1.** Biochemically, binding activities of a number of vesicle proteins have led to hypotheses regarding their roles in vesicle traffic. For example, the Ca^{2+}-binding properties of synaptotagmin suggest a role in the Ca^{2+}-dependent regulation of synaptic vesicle exocytosis. **2.** Studies on neurotoxins that either stimulate synaptic vesicle exocytosis (α-latrotoxin) or inhibit it (tetanus and botulinum toxins) have identified target proteins that are presumably directly or indirectly involved in synaptic vesicle traffic. **3.** Genetic experiments in mice, Drosophila, and C. elegans have allowed in vivo tests of the functions of identified vesicle proteins and their interacting partners. For example, this approach has revealed that synapsins have discrete functions in short term synaptic plasticity. In my talk, I will try to summarize implications from the three complementary approaches to studying synapse function for an understanding of synaptic vesicle exocytosis as it is emerging in the most recent work coming out of our laboratory and other laboratories in the field. Although we are still far from a detailed understanding of the molecular basis of neurotransmitter release, specific models can now be formulated that are testable, for example regarding the protein-protein interactions of synaptotagmins in the release reaction.

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Small GTPases and interacting components involved in membrane transport and fusion.

D.Gallwitz, Max-Planck-Institute for Biophysical Chemistry, Department of Molecular Genetics, D-37070 Göttingen, Germany.

Vesicular protein transport between distinct membrane-enclosed compartments is regulated by monomeric GTPases. In yeast, Ypt GTPases involved in secretion (Ypt1p, Ypt31/32p and Sec4p) are essential for cell viability, GTPases acting in post-Golgi vacuolar protein transport and in endocytosis are not. Using biochemical strategies and the two-hybrid system, we have identified several new proteins which act in the Ypt1p- and Ypt31/32p-regulated ER-to-Golgi and intra-Golgi membrane traffic. Some of them will be discussed. Genetic interaction studies also show that Ypt1p and Ypt31/32p may act in consecutive transport steps. Evidence for a role of Ypt31/32 GTPases in Golgi transport is suggested by a severe secretion defect and a massive accumulation of Golgi-like cisternae in *ypt31/32* mutant cells. Ypt6p, in contrast, appears to function in protein transport between the late Golgi and a post-Golgi compartment. We have isolated the genes encoding the GTPase-activating proteins (GAPs) for Ypt1p, Ypt31/32p and Ypt6p. Deletion of these genes did not significantly affect vesicular protein transport, most likely because of overlapping substrate specificity of the different GAPs.

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RECRUITMENT OF RAB4 AND OTHER GTPASES TO MEMBRANES: ISOLATION & REGULATION OF THE RAB4 RECEPTOR, ENDOCYTOSIS, AND CELL POLARITY

Ira Mellman, Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, PO Box 208002, New Haven, Connecticut 06520

Although the precise role of rab proteins in membrane fusion remains uncertain, it seems likely that they play an important role in ensuring the specificity of certain fusion events. This specificity in function is implicit from the fact that each member of the rab protein family has a characteristic intracellular distribution. Little is known, however, concerning the mechanisms by which rab proteins are recruited to their respective organelles.

We have examined the recruitment of rab4 to early endosomes in vitro. Rab4 plays an important role in the pathway of constitutive receptor recycling, but also controls specialized events such as the delivery of receptor-bound antigen to sites where functional antigen processing and antigenic peptide loading can occur in antigen presenting cells of the immune system. Rab4 is one of two rab proteins known to be substrates for phosphorylation by activated cdc2 kinase. In mitotic cells, rab4-GDI complexes accumulate in the cytosol where they can presumably no longer mediate rab4-dependent transport steps perhaps in part explaining the transient arrest of endocytosis during cell division.

We have found that the failure of rab4 to remain associated with endosome membranes in mitotic cells reflects the inability of phosphorylated rab4 to bind to a specific membrane receptor. The putative rab4 receptor is physically distinct from receptors for other early endosome rab proteins (e.g., rab5) and is also distinct from rab4 guanine nucleotide exchange activity. Although an integral membrane protein, an active soluble fragment of the presumptive rab4 receptor can be released by limited proteolysis. This fragment has been purified and characterized as a ~60 kD protein which is currently being sequenced.

Investigation of the distribution of rab4 and other rab proteins has indicated that distinct subpopulations of early endosomes exist. We have found that these subpopulations represent physically and functionally distinct compartments which play different roles in receptor recycling and transcytosis in polarized epithelial cells. In addition, the ordered but highly dynamic recruitment of other GTPases (e.g., cdc42) to specific intracellular sites, may also play a role in establishing and/or maintaining polarity. Such events may be of particular relevance to establishing an actin-stabilized molecular fence that prevents the randomization of membrane proteins whose polarity was produced by the asymmetric fusion of transport vesicles with the distinct membrane domains found in epithelia, neurons, and other polarized cells.

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Specific requirements for the ER to Golgi transport of GPI-anchored proteins in yeast.

Christine Sütterlin, Tamara L. Doering and Howard Riezman
Biozentrum of the University, CH-4054 Basel, Switzerland, Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley CA, 94720, USA.

GPI-anchored proteins are attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor whose carbohydrate core is conserved in all eukaryotes. Apart from membrane attachment, the precise role of the GPI-anchor is not known, but it has been proposed to play a role in protein sorting. We have investigated the transport of the yeast GPI-anchored protein Gas1p. We identified two mutant strains involved in very different cellular processes that are blocked selectively in the transport of GPI-anchored proteins before arrival to the Golgi. The *lcb1-100* mutant is defective in ceramide synthesis. *In vitro* data suggest a requirement for ceramides after the exit from the ER. We therefore propose that ceramides might function in the fusion of a GPI-containing vesicle with the Golgi, but we cannot exclude a role in the ER. The second mutant that blocks the transport of GPI-anchored proteins to the Golgi is *ret1-1*, a mutant in the α-subunit of coatomer. In both mutants, GPI-anchor attachment is normal and in *ret1-1* cells, the GPI-anchors are remodelled with ceramide to the same extend as in wild type cells. We propose that coatomer is involved in the retrieval of transport factors from the Golgi that are required specifically for the ER to Golgi transport of GPI-anchored proteins.

REGULATION OF ARF1 RECRUITMENT ONTO GOLGI MEMBRANES BY PROTEIN KINASE A

Angel Velasco. Department of Cell Biology. Faculty of Biology. University of Seville Spain

The ADP-ribosylation factor (ARF) family of small GTP-binding proteins are involved in vesicle formation at different steps of intracellular traffic. ARF1, for instance, is a cytosolic factor that upon binding to the membranes of the Golgi complex catalyzes coat assembly and ultimately vesicle budding. We have studied the role played by protein kinase A (PKA) in the regulation of the constitutive transport of the integral membrane protein vesicular stomatitis virus G glycoprotein (VSV-G) along the secretory pathway. Transfer of VSV-G from the *trans*-Golgi network (TGN) to the cell surface was shown to be dependent on PKA activity. Thus, transport *in vivo* was stimulated by PKA activators such as IBMX and forskolin while it was inhibited by H-89 and myristoylated PKI which are selective PKA inhibitors. Data from both *in vivo* and *in vitro* studies indicated that vesicle release from the TGN was regulated by PKA activity. This control was exerted through the mechanism that detemines the association/dissociation of ARF1 to the TGN. Treatment of intact cells with PKA modulators affected ARF1 binding to the Golgi complex. Furthermore, PKA activity modified the kinetics of interaction of ARF1 with purified Golgi membranes. Taken together these results indicate a regulatory role for PKA activity in the production of constitutive secretory vesicles at the TGN.

Structural Analysis of Ca^{2+}-Dependent Binding of Synaptotagmin to Syntaxin

X. Shao[1], C. Li[2,3], I. Fernandez[1], X. Zhang[2,3], T. C. Südhof[2,3] and J. Rizo[1]. Departments of Biochemistry[1] and Molecular Genetics[2], and Howard Hughes Medical Institute[3], U.T. Southwestern Medical Center, Dallas, TX 75235.

Many components of the protein machinery that regulates neurotransmitter release have been identified but the mechanism of release is still poorly understood. Two key proteins are synaptotagmin I, which is believed to act as the Ca^{2+} receptor in fast neurotransmitter release, and, syntaxin 1, which interacts with eight other components of the exocytotic apparatus. Ca^{2+}-dependent binding of the first C_2-domain of synaptotagmin I (C_2A-domain) to syntaxin 1 may be a crucial step to initiate membrane fusion. We are studying the mechanism of this Ca^{2+}-dependent interaction through analysis of the structure of the C_2A-domain, of syntaxin 1, and of the C_2A-domain/syntaxin 1 complex by NMR spectroscopy. We have shown that the C_2A-domain contains a novel bipartite Ca^{2+}-binding motif involving five conserved aspartate residues. Ca^{2+}-binding to the C_2A-domain only causes small structural changes but the two Ca^{2+} ions change dramatically the electrostatic potential of the C_2A-domain. Binding to syntaxin 1 is caused by this change in electrostatic potential and is mediated by a ring of basic residues surrounding the Ca^{2+}-binding sites. Based on these observations, we propose that synaptotagmin acts as an electrostatic switch in neurotransmitter release. The complete solution structure of Ca^{2+}-bound C_2A-domain and initial structural studies on syntaxin 1 will also be presented.

Session 3. Membrane fusion in organelle assembly

Chairman: Richard H. Scheller

THE ROLE OF *CDC48*/p97 IN MEMBRANE FUSION

Martin Latterich, Nicole Levine, Sheetal Patel, Fred Indig, Lien Tran, Juneth Joaquin. The Salk Institute, La Jolla, CA 92037, USA. e-mail: mlatterich@aim.salk.edu

Intracellular membranes of eukaryotes require specific AAA ATPases for membrane fusion. The fusion of endoplasmic reticulum (ER) and nuclear envelope membranes in yeast require Cdc48p (p97/VCP/TERA) as determined in an *in vitro* membrane fusion assay that reconstitutes ER membrane fusion. Since Sec18p (NSF) is not required for ER membrane fusion *in vivo* and *in vitro*, it is possible that the Sec18p homolog Cdc48p carries out a related function by associating with other proteins that may play a role in membrane docking and fusion.

We will present evidence that Cdc48p associates to the endoplasmic reticulum membrane via a soluble adapter protein which, in part, is homologous to the Sec17 protein (α-SNAP). This protein is not essential for membrane fusion, but enhances the association of Cdc48p with the membrane. Once Cdc48p has been recruited to the ER membrane, it then interacts with a syntaxin homolog (t-SNARE) which is required for ER membrane fusion. These important findings suggest that SNAP-like proteins and syntaxin homologs can act both in vesicle mediated fusion events catalyzed by Sec18p (NSF) as well as in organelle or homotypic membrane fusion events catalyzed by Cdc48p, and that the choice of AAA protein reflects whether membranes fuse heterotypically or homotypically.

Mitotic disassembly and reassembly of the Golgi apparatus

Graham Warren, Timothy Levine, Nobuhiro Nakamura, Martin Lowe, Catherine Rabouille and Birte Sönnichsen, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX

When rat liver Golgi stacks are incubated with mitotic cytosol, fragmentation occurs by two overlapping pathways [1]. The first involves the continued budding of COP I-coated transport vesicles which uncoat and accumulate because fusion with the target membrane is inhibited [2]. Fusion appears to be inhibited because the vesicle docking protein, p115, can no longer bind to its receptor, GM130 [3, 4]. The other is a COP I-independent pathway that appears to involve tubular networks as intermediates [5]. These appear at early times during fragmentation and then disappear as they break down into smaller fragments.

Re-isolation and re-incubation of these fragments with interphase cytosol triggers the reassembly process [6]. Cisternal remnants appear to grow by the rapid fusion of vesicles which results in extensive tubular networks emanating from the cisternal rims. Cisternae stack and continue to grow in part by lateral fusion of pre-existing stacks. The tubular networks become flattened and more cisternal-like. The final product is a cup-shaped stack of 4-5 cisternae surrounding an electron-lucent space that is largely devoid of membranes. Stacking is enhanced by GTPγS and inhibited by microcystin. Cisternal re-growth requires NSF, α- and γ-SNAPs and p115 as well as an NSF-like protein, p97 [7]. The components involved in stacking are currently being characterised.

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Vivek Malhotra: Membrane fusion during Golgi stack formation.
Biology Department, University of California San Diego, La Jolla, Calif. 92093-0347

Membrane fusion, be it between transport vesicles and the target membrane or during organelle biogenesis is a fascinating process of fundamental importance. This exceedingly complex and tightly regulated process guides events as diverse as synaptic transmission, hormone release, cellularization and development. While some components of the fusion machinery have recently been identified, many key components remain unknown. We are interested in membrane fusion mainly during the process of Golgi stack formation.

We have taken a unique approach to first dismantle Golgi stacks into small vesicles (VGMs for vesiculated Golgi membranes) of 60-90 nm diameter by treatment of cells with a novel compound ilimaquinone (IQ) . Stack formation from VGMs is then reconstituted in permeabilized cell preparations. This assay has allowed us to identity novel proteins involved in membrane fusion and in shaping these membranes into a very unique structure, i.e., stacks of cisternae. The role of proteins such as NSF, SNAPs, p97, rabs and SNAREs will be discussed. In addition, we will discuss the role of a novel protein of 120kD mol.wt, which is required for the clustering of VGMs prior to fusion in the process of stack formation.

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SNAREs and the organisation of the secretory pathway in yeast.

Hugh Pelham, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

Fusion of transport vesicles with organellar membranes is mediated, at least in part, by the interaction of v-SNAREs (largely active on vesicles) and t-SNAREs (largely active on target membranes). These are C-terminally anchored integral membrane proteins which interact via coiled-coil motifs; in addition, SNARE complexes may contain other components, some of which are peripheral or lipid-linked membrane proteins.

The most universal and conserved family of SNAREs are members of the syntaxin family of tSNAREs - so far, all fusion events mediated by the soluble factors NSF and SNAPs (Sec18p and Sec17p in yeast) have been found to involve a syntaxin-like protein. From the genome sequence, we know of eight members of this family in yeast. The v-SNAREs are more varied in sequence, but ten good candidates are known from genetic and biochemical studies.

We have identified typical v- and t-SNAREs on the yeast vacuolar membrane, and find that they can interact with each other. In collaboration with the group of William Wickner we have shown that these proteins are involved in the in vitro fusion of vacuoles with each other. Although both are normally present on each membrane, it suffices to have the v-SNARE present on one vacuole and the t-SNARE on the other; at least one is required on each of the fusing membranes. Regardless of the combination, NSF/Sec18p is required only for a priming step, not for the actual docking and fusion reactions. This supports previous suggestions that NSF and SNAPs act primarily to dissociate SNARE complexes and get the SNAREs into a state where they are capable of binding to each other.

In other experiments, we have investigated the SNARE complex involved in retrograde transport to the ER. These studies identify a novel complex containing the syntaxin-like t-SNARE Ufe1p, another integral membrane protein, Sec20p, a peripheral membrane protein Tip20p and a v-SNARE, Sec22p, previously implicated in forward traffic to the Golgi complex. Our results imply that SNARE complexes at different pojnts on the secretory pathway differ significantly from each other. They also show that a single SNARE can be involved in transport in two directions, and thus that SNARE content alone is unlikely to be the sole determinant of a vesicle's destination.

A more global survey suggests that the principle of bidirectional movement of v-SNAREs may be more general. In addition, we have localised the remaining syntaxin family members and studied the effects of their deletion. Surprisingly, we find only one Golgi t-SNARE that is essential for growth. The implications for our understanding of the structure and function of the Golgi apparatus will be discussed.

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Studies of vacuolar membrane scission: A requirement for normal vacuole inheritance and morphology. L. S. Weisman, N. L. Catlett and C. Bonangelino. Department of Biochemistry, University of Iowa, Iowa City, IA 52242. T. 319-335-8581. lois-weisman@uiowa.edu

Vacuolar membrane scission, the fusion of the internal faces of the vacuole membrane, is essential for normal vacuole inheritance and morphology. In wild-type *Saccharomyces cerevisiae*, the mother vacuole donates 50% of its vacuolar membrane and contents to the bud. In a search for mutants defective in vacuole inheritance (*vac*), we have identified a set of mutants that are defective in vacuole membrane scission. Instead of a multi-lobed vacuole, these mutants contain a single, large vacuole. A portion of the population contains a vacuole that spans through both the mother and the bud. Even in the narrow confines of the yeast neck, there is a clear gap between the opposing membranes due to a lack of membrane scission. Most yeast mutants that have enlarged vacuoles do not form these "open figure eights". Likewise artificially swelling the vacuole does not produce these structures. Finally in studies mating a membrane scission mutant with wild-type cells, there is a very rapid formation of vacuole lobes concomittent with cell fusion. To date three mutants of this type have been identified, *vac7-1*, *vac14-1* and *fab1-2*. The *VAC7* gene has been identified and partially characterized. It encodes a potential open-reading frame with no signficant homology with known proteins. Immunofluorescence localization indicates that Vac7p resides on the vacuole membrane. *FAB1*, was identified and characterized in a screen for aploid and binucleate cells.[1] Fab1p shares significant homology with a mammalian PI4,(5)P kinase. Isolation of *VAC14* is in progess. Because phosphatidylinositol polyphosphates have been implicated in other membrane fusion events, one may postulate a requirement for phosphatidylinositol (4,5)P_2 in vacuolar periplasmic membrane fusion. Studies are currently in progress in our laboratory in order to test this hypothesis.

Membrane scission occurs in all forms of vesicle traffic. The last step in forming a vesicle is periplasmic membrane fusion/membrane scission. For clathrin-coated vesicles this step is mediated by dynamin. However for other forms of vesicle traffic how this event is catalyzed or if it is catalyzed is less clear. In *S. cerevisiae* there are many membrane trafficking pathways, but only three dynamin homologues. Dnm1p is involved in endocytosis, functioning after the initial internalization step.[2] Vps1p is involved in the formation of Golgi vesicles that are destined for the vacuole (reviewed in (3). Mgm1p plays a role in maintanence of the mitochondrial genome [4]. Thus, it is clear that for most membrane trafficking pathways in yeast the molecular basis of membrane scission remains to be established. It is our hope that by studying *vac* mutants defective in membrane scission, the molecules involved in this event will be identified and characterized.

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Calphostin C induces selective disassembly of the Golgi complex

Josefina Hidalgo and Angel Velasco

Department of Cell Biology, Faculty of Biology, University of Seville, 41012-Seville, Spain.

SUMMARY

Intact cells incubated with calphostin C, an inhibitor of the regulatory domain of protein kinase C (PKC), showed fragmentation and dispersal of the Golgi complex by a light-dependent mechanism. At the ultrastructural level Golgi stacks were replaced by clusters of vesicles and short tubules that resembled the Golgi remnants present in control mitotic cells. Vesicle-mediated transport processes along both the exocytic and endocytic routes were also inhibited by calphostin C treatment. Golgi disassembly, however, was not due to PKC inhibition since several inhibitors of the catalytic domain did not cause a similar effect. In contrast, pretreatment with phorbol 12-myristate 13-acetate (PMA) partly protected the Golgi complex from disassembly by calphostin C. The *in vitro* effect was shown to be reversible, required both cytosol and ATP, and it was inhibited by pretreatment of the Golgi membranes with trypsin but not with high salt. These results suggest the interaction of calphostin C with a structural Golgi protein containing a phorbol ester binding domain and necessary for the stability of this organelle during interphase.

Docking and homotypic fusion of yeast vacuoles

Andreas Mayer*, Zuoyu Xu[#], Albert Haas[§] and William Wickner[#]

Vacuole fusion is part of a reaction sequence mediating the ordered transmission of this low copy organelle into daughter cells. Vacuole fusion can be reconstituted in vitro and be utilized as a model system for an NSF-dependent reaction of priming, docking and fusion. We have developed biochemical and microscopic assays for the docking step of in vitro vacuole fusion and characterized its requirements. The vacuoles must be primed for docking by the action of Sec17p (alpha-SNAP) and Sec18p (NSF). Priming is necessary for both fusion partners and produces a labile state. This labile state can only lead productively to fusion if 1) docking occurs rapidly and 2) LMA1, a heterodimer of thioredoxin and proteinase B inhibitor 2, is present. In addition to Sec17p/Sec18p, docking requires the activity of the Ras-like GTPase Ypt7p and of phosphatidylinositol phosphates (PIPs). Unlike Sec17p/Sec18p, which must act prior to docking, Ypt7p and PIPs are involved in the docking process itself.
Vacuole fusion also depends on SNAREs. Despite its homotypic nature, vacuole fusion requires cognate pairs of a typical v-SNARE (Nyv1p) and a typical t-SNARE (Vam3p). Thus, homotypic vacuole fusion is based on the same biochemical mechanisms as other, heterotypic fusion reactions.

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*Present address: Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37-39, 72076 Tübingen, Germany
§ Present address: Biozentum, Am Hubland, 97074 Würzburg, Germany

Session 4. Lipids and phosphoinositides in membrane fusion

Chairman: Wolfhard Almers

Late stages of dense core granule (DCV) exocytosis. T.F.J. Martin, Department of Biochemistry, University of Wisconsin, Madison, WI, USA 53706.

To elucidate stages in the dense core granule exocytotic pathway and the biochemical mechanisms involved in membrane fusion, we have studied ATP-dependent, Ca^{2+}-activated norepinephrine secretion in mechanically-permeabilized PC12 neuroendocrine cells. In spite of the fact that the majority of the DCVs are anchored close to the plasma membrane and possibly docked, an ATP-dependent step is required to prime DCVs in preparation for Ca^{2+}-activated fusion. Two distinct ATP-dependent priming steps have been identified.

One priming mechanism requires the cytosolic factors PI transfer protein and PI4P 5-kinase to catalyze $PI(4,5)P_2$ synthesis. $PI(4,5)P_2$ synthesis on DCVs during priming was detected with $PI(4,5)P_2$-specific antibodies (from K. Fukami). Immunocytochemistry using epifluorescence and immunogold microscopy demonstrated that the $PI(4,5)P_2$ formation was ATP-dependent, restricted to DCVs, and closely associated with the plasma membrane. At high antibody dilutions, only membrane $PI(4,5)P_2$ concentrations exceeding 10% were detected, indicating that the majority of PI in DCVs undergo conversion to $PI(4,5)P_2$ during priming. High concentrations of negatively charged phospholipids would alter the DCV membrane, which could impede fusion. $PI(4,5)P_2$-binding proteins may be required to sequester acidic phospholipids to enable fusion to proceed. The CAPS protein was discovered as a factor that is required after priming for Ca^{2+}-triggered fusion. CAPS has been found to be a phosphoinositide binding protein that exhibits specific interactions with $PI(4,5)P_2$. Efforts to determine the $PI(4,5)P_2$-binding site on CAPS to assess the relevance of phospholipid-binding to the role of CAPS at a late step in DCV fusion are in progress.

A second priming mechanism employs NSF and SNAP proteins. Detergent-soluble "docking" complexes containing VAMP, syntaxin, SNAP-25, NSF and α/β-SNAP were found to dissociate during ATP-dependent priming in permeable cells. Disassembly was blocked by NEM and ATPγS, and restored following NEM inhibition by recombinant NSF and α-SNAP. NSF and SNAP proteins associated with permeable cells were adequate to support ATP-dependent priming, but depletion of these proteins by a cycle of priming/de-priming demonstrated that NSF and α-SNAP were co-required for ATP-dependent priming. The simplest interpretation is that NSF is required for a priming step that follows DCV docking and precedes Ca^{2+}-triggered fusion. NSF may activate SNARE proteins in preparation for the Ca^{2+}-triggered fusion step. Indeed, Ca^{2+}-triggered fusion in ATP-primed permeable cells can be entirely blocked by botulinum neurotoxin B, C1 and E, indicating a post-priming role for SNARE proteins. Botulinum neurotoxin A treatment, in contrast, was partially inhibitory in spite of complete proteolysis of SNAP-25, suggesting that the C-terminus of SNAP-25 between the A and E toxin cleavage sites is required for a late step in DCV exocytosis. Recent studies on a late-stage role for the C-terminus of SNAP-25 will be discussed.

MECHANISMS OF MEMBRANE COALESCENCE AND PORE FORMATION DURING FUSION: VIRAL PROTEINS AND LIPID HEMIFUSION

JOSHUA ZIMMERBERG. *Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1855*

Fusion pore opening (an aqueous connection between fusing compartments) is detected prior to lipid mixing in biological membrane fusion. Following fusion protein activation and conformational change, progress towards bilayer merger and aqueous continuity in exocytosis and viral fusion depends upon the lipid composition of the membrane in ways that suggest that proteins induce a non-bilayer intermediate to fusion -- hemifusion. Hemifusion is the contact of hydrocarbon tails of distal leaflets of fusing membranes. Accordingly, such lipids inhibit spontaneous hemifusion between purely lipid membranes, and the hemifusion triggered by membrane-anchored HA which lacks its transmembrane domain and tail (GPI-HA). Recently we found transient fusion pores between purely phospholipid membranes after their hemifusion. Thus protein is not needed for fusion pore formation per se. However, in phospholipid bilayers, hemifusion is stable: these pores do not expand and lead to full fusion unless there is significant tension on both membranes.

Influenza hemagglutinin (HA) is the best characterized fusion pore-forming protein. We find transient pores in hemifusion mediated by GPI-HA. Thus the transmembrane domain is not necessary for fusion pore formation in biological membranes. Furthermore, at reduced membrane density and mobility, wild-type HA caused hemifusion and small, flickering pores which did not lead to complete fusion. So the HA ectodomain retains its hemifusion activity, even at relatively low concentrations. As with GPI-HA, this hemifusion was stable. If no pores were open prior to

onset of lipid mixing, pores would not open at all. Thus HA, when it runs normal fusion, needs to prevent premature formation of this irreversible stage, and provide tension to expand the fusion pore. Our working model is that the macromolecular complex of HA trimers form a ring-like fence around a small patch of lipid, to allow HA's hemifusion activity within the fence to lead to a restricted, local lipidic connection, but to prevent lipid diffusion into stable hemifusion. This complex of HA and lipid can also provide tension to expand any fusion pore which forms within it.

Using low temperature, we have now isolated a second intermediate along the fusion pathway, downstream of the stage sensitive to the lipid composition of the contacting leaflets. To test the above model (the idea that the role of protein at this stage is to prevent lipid diffusion), we treated membranes arrested in this low-temperature stage with proteinase-K, which hydrolyses HA's fusogenic form. Proteinase-K treatment led to stable hemifusion, consistent with our model. Finally, kinetic analyses suggest that a complex of about 6 viral fusion protein trimers mediate fusion pore formation and widening.

Fusion and confusion in exocytosis

Reinhard Jahn, Dirk Fasshauer, Phyllis I. Hanson, and Henning Otto.

Howard Hughes Medical Institute and Departments of Pharmacology and Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

There is compelling evidence that the three membrane proteins synaptobrevin (also referred to as VAMP), syntaxin, and SNAP-25 are essential for neuronal exocytosis but their mechanism of action is not yet clear. Rothman and coworkers have recently extablished that in detergent extracts these three proteins form a complex that is dissociated by the ATPase NSF with the aid of SNAP-proteins (Söllner et al. 1993. Cell 75:418). In collaboration with the laboratory of A. Brünger (HHMI, Yale University), we have investigated structural changes associated with the assembly of the ternary complex using recombinant proteins lacking their respective membrane anchors. Complex formation is associated with large increases in a-helical content an in thermal stability. A defined, but less stable binary complex is formed between syntaxin and SNAP-25 whereas other binary combinations did not result in stable associations. Furthermore, the stoichiometry and size of these complexes was characterized with the aid of biophysical techniques.

Complex formation and disassembly was also studied by quick-freeze, deep-etch electron microscopy, in close collaboration with J. Heuser (Washington University). The results show that NSF undergoes dramatic conformational changes during its catalytic cycle and that the ternary complex binds to one side of the barrel-shaped molecule. Furthermore, we have investigated formation and disassembly of the ternary complex in intact membranes. We found that the complex can assemble and disassemble in the membranes of purified and monodisperse synaptic vesicles suggesting that NSF acts upon the proteins when they are residing as neighbors in the same membrane. These findings agree with structural studies demonstrating that syntaxin and synaptobrevin are oriented in a parallel manner within the ternary complex. Together, the data suggest that NSF operates at a priming step in exocytosis that may precede vesicle docking.

Regulation and function of the ARF and PtdInsP$_2$-dependent binding of spectrin to the Golgi complex

Anna Godi*, Ivana Santone*, Paolo Pertile*, Prasad Devarajan°°, Paul R. Stabach°, Jon S. Morrow°, Giuseppe Di Tullio*, Roman Polishchuk*, Tamara C. Petrucci§, Alberto Luini*, and Maria Antonietta De Matteis*

*Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti), Italy
Departments of °Pathology and °°Pediatrics, Yale University School of Medicine, New Haven, Connecticut, USA
§Laboratory of Cell Biology, Istituto Superiore di Sanità, Rome, Italy

Despite the early recognition of a key role for the small G protein ADP-ribosylation factor (ARF) in controlling the dynamic architecture of the Golgi apparatus, and recent advances in identifying structural components of this organelle, the mechanisms by which such control is effected remain obscure. Recently, homologues of two major components of the well-characterized erythrocyte plasma-membrane-skeleton, ankyrin (Ank$_{G119}$) and βIΣ* spectrin, (a still uncharacterized putative spliceform of βI spectrin, hence the designation Σ*), have been identified in the Golgi complex. We find that the activation of ARF is required for the association of βIΣ*spectrin, Ank$_{G119}$, and actin with Golgi membranes and that the Golgi βIΣ*spectrin is recognized by an antibody specific for βIΣ*spectrin C-terminus which contains a pleckstrin homology (PH) domain. ARF recruits Golgi βIΣ* spectrin by stimulating the synthesis of phosphatidylinositol 4,5-bisphosphate (PtdInsP2). This lipid, known to bind PH domains, acts as a regulated docking site for Golgi spectrin. ARF recruits spectrin to the Golgi apparatus independently of its known abilities to recruit the cytosolic COPI-coat protein complex to the same membranes and to stimulate phospholipase D (PLD). Agents that block spectrin's binding to Golgi membranes inhibit the transport of secretory proteins from endoplasmic reticulum (ER) to the medial-compartment of the Golgi complex, suggesting that the spectrin skeleton plays a major role in regulating the structure and function of this organelle.

Isolation Of Novel Cytosolic Factors Involved In Intra-Golgi Traffic

Zvulun Elazar, Dept. Membrane Research & Biophysics

The Weizmann Institute of Science, Rehovot 76100, Israel

Despite the fact that many cytosolic proteins involved in intracellular vesicular transport have been isolated and cloned in recent years, it is still impossible to replace crude cytosol with pure components in vitro. We have developed a purification scheme that allows the identification and isolation of novel cytosolic factors involved in cell-free, intra-Golgi transport. We found that in addition to NSF, SNAP, and p115, three other cytosolic factors are required to fulfill the cytosolic requirements of intra-Golgi transport in vitro. Each of these factors was purified to apparent homogeneity: the first pool contains a 16 kD polypeptide (p16); the second pool contain a 42 kD polypeptide; and the third pool contains 58 and 62 kD polypeptide bands. The 42 and 58/62 kD proteins are currently being sequenced. However, amino acid sequences obtained from tryptic peptides of p16 were used to construct degenerate oligonucleotides which were then used to amplify a PCR fragment from bovine brain cDNA. Cloning the full length cDNA of p16, revealed that it is a novel protein. Recombinant p16 exhibited transport activity similar to that of the endogenous protein. Polyclonal antibodies raised against recombinant p16 specifically inhibit intra-Golgi transport in vitro. Together, these data strongly indicate that p16 is involved in intra-Golgi transport. Immuno-electron microscopy studies performed with these antibodies indicate that p16 is localized along the secretory pathway, i.e. in the endolpasmic reticulum, the Golgi apparatus, multivesicular bodies, and in the plasma membrane.

Session 5. New developments in aspects of membrane fusion

Chairman: Hugh Pelham

Transport, docking and exoyctosis of secretory granules observed in live neuroendocrine cells. JUERGEN STEYER, THORSTEN LANG, I. WACKER, HEINZ HORSTMANN and WOLFHARD ALMERS, *Department. Molekulare Zellforschung, Max-Planck-Institut für medizinische Forschung, Heidelberg, Germany.*

In chromaffin cells (Heinemann et al., 1994; Biophys. J. 67, 2546-2557) and melanotrophs (Thomas et al., 1993; Neuron 11, 93-104) the exocytic response to a step rise in cytosolic [Ca^{2+}] shows at least four kinetic components. These are thought to represent four pools of secretory granules parked at the last four stations of the secretory pathway. Three require no MgATP and hence result from "primed" granules. In particular a "readily releasable pool" of 20 to 200 granules is released rapidly enough to respond to action potentials, and is thought to be parked at the last station that is stable in the absence of Ca^{2+}. Do any of these pools represent docked granules? By quick-freeze electron microscopy, we have identified a population of secretory granules that appear bound to the plasmalemma, i.e. docked. It is about as large as the pool of granules that can be released in the absence of MgATP, and tenfold larger than the "readily releasable pool" (Parsons et al., 1995; Neuron 15, 1085-1096). It is selectively depleted when chromaffin cells are stimulated to secrete.

To study interactions of secretory vesicles with the plasmalemma *in vivo*, we have viewed cells attached to glass coverslips, and selectively excited fluorescence in the 300 nm thick layer of cytosol next to the coverslip. This was done with the evanescent wave set up by a laser beam as it suffered total reflection at the interface between coverslip and the cytosol or bathing medium. Dense core granules were stained with acridine orange, a dye that partitions into acidic compartments (chromaffin cells), or with green fluorscent protein that was directed to dense-core secretory granules by recombinant fusion to neuropeptide-Y or chromogranin. Single subplasmalemmal granules were readily observed; about half of them were morphologically docked. Most were stationary except that they dithered around a resting position as if tethered by a 50 nm leash, or imprisoned in a cage 50 nm larger than the granule. Stimulation with elevated [K^+] caused granules to disappear abruptly due to exocytosis, leaving behind empty patches of plasmalemma

depleted of granules. In time, the plasmalemma was repopulated with fresh granules traveling towards the plasmalemma at 22 nm/s and taking an average of 6 min to arrive. While a few returned to the cytosol, most remained, exploring the plasmalemma in lateral motions. In cells stimulated for a second time, some later performed exocytosis. We conclude that a large pool of docked vesicles turns over slowly, that docking is reversible, that a vesicle spends at least 1 min at the plasmalemma before it can perform exocytosis, and that the "readily releasable pool" in electrophysiologic measurements is a small subset of docked vesicles.

RELEASE OF NEUROTRANSMITTERS THROUGH THE EXOCYTOTIC FUSION PORE. Guillermo Alvarez de Toledo*, Lucía Tabares*, Eva Alés* and Manfred Lindau[!]. Dept. Physiology and Biophysics. University of Seville. E-41009, Seville. Spain. [!]Dept. Molecular Cell Research, MPI f. Medical Research, D-69120, Heidelberg, Germany.

The activity of fusion pores during exocytosis was investigated by combined cell membrane capacitance measurements and electrochemical determination of secretory products. Capacitance measurements were performed in whole cell or cell attached configurations of the patch clamp technique. Typical whole cell recordings were used to resolve fusion pore activities of large secretory vesicles (> 700 nm diameter); for smaller vesicles we used cell attached capacitance measurements. Electrochemical detection of secretory products in cell attached were obtained by placing a carbon fiber electrode into the patch pipette. The dynamics of fusion pores measured in cell attached were comparable to the ones measured in whole cell, suggesting that exocytosis could develop normally in the membrane patch. In mast cells, in addition to the fusion of regular secretory granules, two types of events were obtained spontaneously and clearly distinguished. Small step increases in capacitance (0.17 ± 0.11 fF, mean ± s.d.) and relatively large capacitance events (15-60 fF). Small membrane fusion events were not accompanied by a detectable amperometric transient, probably indicating the fusion of small clear core vesicles. Some of these events were intermixed with back steps of the same amplitude that could correspond to endocytosis. The large events showed a typical amperometric spike due to the release of serotonin, indicating the fusion of mast cell granules with the plasma membrane. The fusion pore conductance could be calculated while developing the amperometric transient. The correlation between fusion pore conductance and amperometric current was linear during the upstroke of the spike, indicating that during this phase the pore is limiting for release. After the spike has reached its maximum amplitude the pore continues to grow. At that stage the kinetics for release seems to be governed by the binding properties of serotonin to the granule matrix.

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Analysis of the role of α-SNAP in exocytosis in adrenal chromaffin cells

Robert D. Burgoyne, Alan Morgan, A. Vicki Kibble and Richard J. O. Barnard, The Physiological Laboratory, University of Liverpool, Liverpool, L69 3BX.

Chromaffin cells are a useful model cell type in which to study the role of identified proteins in Ca^{2+}-regulated exocytosis. Exogenous proteins can be introduced either after cell permeabilisation or during patch-clamp recording allowing analysis of their effects on exocytosis. We have examined the role of α-SNAP in exocytosis using both approaches and combined this with mutagenesis of α-SNAP . Our data show that α-SNAP has a major role in priming the exocytotic machinery and that the ability of α-SNAP to stimulate exocytosis is critically dependent on its ability to activate the ATPase activity of NSF.

Introduction of exogenous α-SNAP into chromaffin cells after digitonin-permeabilisation resulted in a marked Ca^{2+}-dependent increase in adrenaline release. This stimulation could be prevented by botulinum toxin treatment [1]. Using stage-specific assays we demonstrated that α-SNAP acted during an early ATP-dependent priming reaction to increase release [2]. Inclusion of α-SNAP in the patch pipette during whole cell patch-clamp recording led to an increase in the extent of capacitance increase (exocytosis) in response to depolarisation. A truncated mutant of α-SNAP unable to bind to syntaxin was ineffective [3]. The effect on capacitance would be consistent with an increase in the size of the releaseable pool of granules due to the effect on priming. Since chromaffin cells possess few docked granules, the most likely interpretation of these data is that α-SNAP acts on undocked granules to prepare (prime) the docking/fusion machinery [4].

In in vitro experiments, α-SNAP was found to stimulate that ATPase activity of NSF [5]. In order to determine the physiological significance of this phenomenon, a series of deletion mutants of α-SNAP were generated to map the domains of α-SNAP required for NSF ATPase activation [6]. These studies resulted in the development of a single mutation within the C-terminus of α-SNAP that impaired NSF ATPase activation. This

mutant protein was otherwise normal in binding to the SNARE complex and recruiting NSF but did not support SNARE complex disassembly. In addition, it was unable to stimulate exocytosis in permeabilised chromaffin cells. These results suggest that NSF ATPase activation is crucially important for SNARE complex disassembly and exocytosis. This mutant protein inhibited the stimulation due to exogenous wild-type α-SNAP and may thus act as a dominant negative inhibitor of SNAP/NSF function.

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POSTERS

Dual inhibitory effect of Gangliosides on Phospholipase C-Promoted Fusion of Lipidic Vesicles.

Gorka Basáñez, Gerardo D. Fidelio, Félix M. Goñi, Bruno Maggio and Alicia Alonso

Grupo Biomembranas (Unidad asociada al C.S.I.C.), Departamento de Bioquímica y Biología Molecular, Universidad del País Vasco/Euskal Herriko Unibertsitatea, Aptdo. 644, E-48080 Bilbao, España.

The effect of a variety of gangliosides has been tested on the phospholipase C-induced fusion of large unilamellar vesicles. Bilayer composition was phosphatidylcholine: phosphatidylethanolamine:cholesterol (2:1:1 mole ratio) plus the appropriate amounts of glycosphingolipids. Enzyme phosphohydrolase activity, vesicle aggregation, mixing of bilayer lipids and mixing of liposomal aqueous contents were separately assayed. Small amounts (< 1 mol %) of gangliosides in the lipid bilayer produce a significant inhibition of the above processes. The inhibitory effect of gangliosides increases with the size of the oligosaccharide chain in the polar headgroup. Inhibition depends in a nonlinear manner on the ganglioside proportion, and is complete at ~5 mol %. Inhibition is not due to ganglioside-dependent changes in vesicle curvature or size. Ganglioside inhibition of vesicle fusion is due to two different effects: inhibition of phospholipase C activity and stabilization of the lipid lamellar phase. Enzyme inhibition leads to a parallel decrease of vesicle aggregation and lipid mixing rates. Mixing of aqueous contents, though, is depressed beyond the enzyme inhibition levels. This is explained in terms of the fusion pore requiring a local destabilization of the lipid bilayer, the lamellar structure being stabilized by gangliosides. ^{31}P-NMR and DSC experiments confirm the inhibitory effect of gangliosides in various lamellar-to-nonlamellar transitions.

Caveolin and MAL, Two Protein Components of Detergent-Insoluble Membranes, are in Distinct Lipid Microenvironments

Alonso, M. A., Puertollano, R., Martín-Belmonte, F., and Millán, J.

Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma, Cantoblanco, 28049-Madrid, Spain

Proteolipids are a heterogeneous group of proteins that share an unusually high solubility in the organic solvents commonly used to extract cell lipids (1). Although some members of the proteolipid group have been extensively characterized, the function of many of them remained elusive. The cDNA encoding the MAL proteolipid was initially identified during a search for genes differentially expressed during T-cell development (2). More recently, MAL expression has also been detected in myelin-forming cells (3), and in polarized epithelial cells (4). Moreover, the MAL protein has been identified in all these cell types in the fraction of glycolipid-enriched membranes resistant to detergent solubilization at low temperatures (3, 4, 5). A current model proposes the existence of a pathway for protein transport involving glycolipid-enriched membrane "rafts" or microdomains as platforms to generate vesicular carriers containing specific cargo molecules (6). To be operative as a transport pathway, it was postulated the existence of a protein machinery responsible for the specific recruitment of cargo proteins, vesicle formation, and targeting and fusion to the appropriate membrane. The presence of MAL in glycolipid-enriched membrane microdomains and its predominance in apical transport vesicles in MDCK cells have led to the proposal of MAL as a candidate component of the protein machinery for the glycolipid-mediated pathway. Caveolin, a protein initially identified as a component of caveolar architecture (7), is also present in glycolipid-enriched membranes (8). We have addressed the study of the glycolipid-enriched membranes in cells expressing endogenously only either MAL (Jurkat T cells) or caveolin (A498 cells), and in polarized MDCK cells which express both proteins simultaneously. Subcellular fractionation by centrifugation to equilibrium in sucrose density gradients of Triton X-100 cell extracts from Jurkat and A498 cells revealed that MAL and caveolin are incorporated in detergent-insoluble buoyant membranes independently of the expression of each other, and indicated the existence in these cells of insoluble membrane microdomains with either only MAL or caveolin. Immunofluorescence analysis in MDCK cells indicated that both MAL and caveolin were located in the Golgi region, whereas caveolin was found in addition at the cell surface. Biochemical analysis in these cells revealed the existence of distinct membrane microenvironments differentially susceptible to detergent solubilization containing either MAL or caveolin. The observed heterogeneity within the internal glycolipid-enriched membrane fraction suggests the existence of distinct specialized lipid microenvironments in MDCK cells.

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Effect of SV5 Fusion Protein Surface Density on the Extent and Rate of Cell-Cell Fusion

Rebecca Ellis Dutch and Robert A. Lamb
Department of Biochemistry, Molecular Biology and Cell Biology/
Howard Hughes Medical Institute
Northwestern University
2153 North Campus Drive
Evanston, IL 60208-3500 USA

The fusion protein (F protein) of the paramyxovirus SV5 serves as an excellent paradigm for membrane fusion promoted by a viral protein at neutral pH. The SV5 F protein resembles the influenza hemaglutinin (HA) in several important ways, as it contains a fusion peptide region, heptad repeat regions, and is processed from the precursor form F_0 to a disulfide linked heterodimer, F_1 and F_2, which exist in a trimeric form. Unlike HA, the major receptor binding function for the SV5 virus exists on a separate molecule, the SV5 hemaglutinin-neuraminidase (HN), allowing the separation of receptor binding and fusion functions during analysis. SV5 F protein is also the sole paramyxovirus fusion protein to promote membrane fusion in the absence of its homotypic HN protein, eliminating the need for examination of protein-protein interactions between F and HN during the course of membrane fusion.

In order to more fully characterize the membrane fusion reaction promoted by the SV5 F protein, we have employed the vaccinia-T7 transfection system with differing amounts of plasmid DNA to generate cell populations with F protein surface densities ranging from 15 to 130% of that seen in SV5 infected cells. Extent of fusion, as judged by the β-galactosidase fusion assay, increases with rising surface density, with a maximum seen when F protein is present at 100% of the surface density seen in SV5 infected cells. Surface densities higher than this level result in decreased extent of fusion, suggesting that high levels of the F protein are inhibitory to the membrane fusion reaction. This inhibition is not a result of uncleaved F_0 protein at the cell surface, as treatment with exogenous trypsin prior to the fusion reaction, though stimulating fusion at all points tested, does not change the relative decrease in fusion seen at high surface densities of SV5 F protein. As the SV5 HN protein is used in the β-galactosidase fusion assay to provide binding to the target cells, decreasing amounts of HN protein were employed. Extent of fusion was unaffected by changes in HN protein surface density of up to 15-fold, confirming that the SV5 HN protein is unlikely to be playing a direct role in the fusion reaction. Finally, fusion of R18-labeled red blood cells to cells expressing differing surface densities of SV5 F protein was examined. Relative extents of fusion were found to be similar to that seen in the β-galactosidase fusion assay and initial rates of the membrane fusion reaction were found to be increased with increasing F protein surface densities.

Ras-INDUCED DISRUPTION OF GOLGI COMPLEX ARCHITECTURE. INVOLVEMENT OF ACTIN CYTOSKELETON AND PHOSPHOLIPASE A_2

Gustavo Egea[1], Teresa Babià[1], Ferran Valderrama[1], Inmaculada Ayala[1], Eugènia Mato[1,2] Marta Bosch[1], Jan Willem Kok[3], Juan F. Santarén[4], and Timothy M. Thomson[5].

[1]Dept. Biologia Cel.lular i Anatomia Patològica. Facultat de Medicina. Universitat de Barcelona. Barcelona (Spain); [2]Servei d'Endocrinologia. Hospital de Sant Pau, Barcelona. [3] Dept. Physiological Chemistry. University of Groningen Medical School. Groningen (The Netherlands); [4]Centro de Biología Molecular Severo Ochoa. Facultad de Ciencias. Universidad Autónoma de Madrid-CSIC. Madrid (Spain); [5]Instituto de Biología del Cáncer. IMIM-CSIC. Barcelona.

Aberrant glycosylation of proteins and lipids is a common feature of many tumor cell types, often accompanied by alterations in membrane traffic and an anomalous localization of Golgi resident proteins and glycans (Egea et al., 1993, J. Cell Scie. 105:819-830; Francí et al., 1996, Biochem. J. 314:33-40). These observations suggest that Golgi complex is a key organelle for at least some of the functional changes associated with malignant transformation. To gain insight into this possibility, we have undertaken a systematic analysis of alterations in the Golgi structure and function induced by the expression of the transforming protein N-Ras(K61). A cell line (KT8) was generated from normal rat kidney (NRK) fibroblasts for the conditional expression of mouse N-*ras* and induction of a transformed phenotype. A remarkable and specific effect associated with Ras-induced transformation of KT8 cells was a conspicuous rearrangement of the Golgi complex from its relaxed and reticular morphology into a collapsed juxtanuclear localization. Ultrastructurally, the Golgi stack of transformed KT8 cells was extensively fragmented. The Ras-induced collapase of Golgi complex required intact microtubules and was accompanied by the disassembly of actin microfilaments. In addition, Golgi collapse was prevented by the phospholipase A_2 inhibitor 4-bromophenylacyl bromide. Functionally, the striking disruption of Golgi complex architecture induced by N-Ras(K61) produced an alteration in the lipid transport from the *trans*-Golgi network to the plasma membrane. We propose that the morphological integrity and cellular positioning of the Golgi complex depends on the actin microfilaments in conjunction with the Raf signalling pathway that is linked to the activation of phospholipase A_2. However, transformant N-Ras alone is not sufficient to induce the appearance of aberrant glycosylation in cancer cells.

SCAMPS: A LARGE FAMILY OF MEMBRANE TRAFFICKING PROTEINS

Rafael Fernández-Chacón, Robert H. Hammer and Thomas C. Südhof. Howard Hughes Medical Institute, U.T. Southwestern Medical Center, Dallas TX, 75235-9050. USA

Secretory carrier membrane protein (SCAMP 37) is an integral membrane protein with four transmembrane regions present in synaptic vesicles and secretory granules of exocrine and endocrine glands. A monoclonal antibody generated against secretory granules recognizes SCAMP 37 and crossreacts with other proteins, suggesting the existence of isoforms (Brand and Castle, 1993). We have now cloned multiple SCAMP isoforms from a rat brain cDNA library. Sequence analysis indicates that SCAMPs constitute a multigene family. Sequence alignment against EST (Expressed Sequence Tags) databases shows that SCAMPs are evolutionary conserved proteins present in the nematode *C. elegans*, mouse, rat and human. Northern blot analysis of mRNA from various rat tissues reveals that in general SCAMPs are ubiquitously distributed, but one of them is clearly enriched in brain. This finding suggests that this brain isoform could have an specialized role in membrane trafficking at the synaptic terminals. In order to study the physiological function of SCAMPs *in vivo* we have performed gene targeting experiments in mice. We partially cloned the gene of SCAMP 37, and constructed a knockout vector that deletes one exon coding for 33 aminoacids at the N-terminal cytosolic part of the protein and replaces it with the neomycin resistant gene. The vector was successfully used for mutagenizing embryonic stem (ES) cells by homologous recombination. Mutant ES cells were injected into mouse blastocysts and the resulting chimaeric animals gave germ line transmission. Interbreeding of heterozygous mice then led to the generation of homozygous null mutant mice which showed a total lack of the protein SCAMP 37, as revealed by inmunoblot analysis. The null mutant is viable, and a homozygous line could be established. These mice are currently being analyzed and results of this analysis will be discussed.

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ANALYZING THE RELATIONSHIP BETWEEN TRANSITIONAL ER AND GOLGI STRUCTURES IN BUDDING YEASTS. O. Rossanese, A. Hammond, I.B. Sears, and B.S. Glick. Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637.

Golgi stacks in many cell types are found next to transitional ER sites. Our working hypothesis is that the Golgi is a specialized outgrowth of the ER: new Golgi cisternae assemble near transitional ER sites and then mature as they move through the stack. To test this notion we are studying two budding yeasts, *Saccharomyces cerevisiae* and *Pichia pastoris*. Electron microscopy had suggested that unlike *S. cerevisiae*, *P. pastoris* contains a small number of coherent Golgi stacks situated next to specialized regions of the ER. Our immunofluorescence studies confirmed that in *P. pastoris*, as in mammalian cells, COPII proteins are concentrated in discrete transitional ER sites adjacent to Golgi stacks. This pattern contrasts strikingly with the dispersed localization of COPII components in *S. cerevisiae*. By comparing these two related yeasts, we hope to uncover the molecular mechanisms that generate organized transitional ER and Golgi structures.

This problem is being approached in three ways. [1] We suspect that Sec12p is the "master regulator" that localizes downstream COPII components to the transitional ER. The localization mechanism of *P. pastoris* Sec12p is being analyzed by gene fusion studies and by using affinity methods to identify putative partner proteins. [2] In complementary studies, we have initiated genetic screens to identify *P. pastoris* mutants with altered Golgi organization, as well as *S. cerevisiae* mutants defective in Golgi inheritance. The relationship between Golgi and ER elements will be examined in the mutant cells. For these genetic experiments the Golgi has been labeled with Green Fluorescent Protein (GFP). [3] Golgi proliferation is being examined in *P. pastoris* using video fluorescence microscopy. By observing GFP-labeled Golgi stacks, we will determine whether new Golgi stacks form by the fission of preexisting stacks, or whether Golgi organelles form *de novo*.

Morphological changes induced by phospholipase C and by sphigomyelinase on large unilamellar vesicles. A cryo-transmission electron microscopy study of liposome fusion.

Gorka Basáñez[1], M. Begoña Ruiz-Argüello[1], Alicia Alonso[1], Félix M. Goñi[1], Göran Karlsson[2] and Katarina Edwards[2]

[1]Grupo Biomembranas (Unidad asociada al C.S.I.C.), Departamento de Bioquímica y Biología Molecular, Universidad del País Vasco/Euskal Herriko Unibertsitatea, Aptdo. 644, E-48080 Bilbao, España.
[2]Department of Physical Chemistry, Uppsala University, Box 532, S-751 21 Uppsala, Sweden.

Cryo-transmission electron microscopy has been applied to the study of the changes induced by phospholipase C on large unilamellar vesicles containing phosphatidylcholine, as well as to the action of sphingomyelinase on vesicles containing sphingomyelin. In both cases vesicle aggregation occurs as the earliest detectable phenomenon; later, each system behaves differently. Phospholipase C induces vesicle fusion through an intermediate consisting of aggregated and closely packed vesicles (the "honeycomb structure") that finally transforms into large spherical vesicles. The same honeycomb structure is also observed in the absence of enzyme when diacylglycerols are mixed with the other lipids in organic solution, before hydration. In this case the sample then evolves towards a cubic phase. The fact that the same honeycomb intermediate can lead to vesicle fusion (with enzyme-generated diacylglycerol) or to a cubic phase (when diacylglycerol is pre-mixed with the lipids) is taken in support of the hypothesis according to which a highly-curved lipid structure ("stalk") would act as a structural intermediate in membrane fusion. Sphingomyelinase produces complete leakage of vesicle aqueous contents, and increase in size of about one third of the vesicles. A mechanism of vesicle opening and reassembling is proposed in this case.

A PEPTIDE MIMICKING THE C-TERMINAL DOMAIN OF SNAP-25 BLOCKS VESICLE "DOCKING" IN PERMEABIZED BOVINE CHROMAFFIN CELLS.

Luis M. Gutierrez[1], Antonio V. Ferrer-Montiel[2], Jaume M. Cánaves[2], Joaquin Rueda[3], Anabel Gil[1], Mauricio Montal[2] and Salvador Viniegra[1]. Deptos de Neuroquímica (1) e Histologia (3), Instituto de Neurociencias, Universidad de Alicante and Dept. of Biology, University of California at San Diego (2)

SNAP-25 (synaptosomal associated membrane protein of 25 kDa) participates in the process of vesicle-plasma membrane fusion that results in the exocytotic release of active substances in neuronal and neuroendocrine cells. A 20-mer synthetic peptide representing the sequence of the C-terminal domain of SNAP-25 (ESUP-A) and containing the cleavage sequence for botulinum neurotoxin A (BoNT A) blocks Ca^{2+}-dependent catecholamine release in digitonin-permeabilized bovine chromaffin cells with an IC50 = 20 mM. The inhibitory activity of the peptide was sequence-specific as evidenced by the inertness of a control peptide with the same amino acid composition but random order (1). In addition, ESUP-A abrogates the slow, ATP-dependent, temperature-sensitive component of the exocytotic release found in permeabilized chromaffin cells, without affecting the fast, ATP-independent, Ca^{2+}-mediated fusion event. Ultrastructural analysis indicates that ESUP-A induces a drastic accumulation of dense-core vesicles near the plasma membrane, mimicking the effect of botulinum neurotoxin A (2).

Together, these findings demonstrate that ESUP-A blocks ATP-primed exocytosis by preventing vesicle docking at the active sites and that the identification of blocking peptides that mimic sequences that bind to complementary partner domains on interacting proteins of the exocytotic machinery provides new pharmacological tools to dissect the molecular and mechanistic details of neurosecretion.

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GLUTAMATE RELEASE AT NEUROMUSCULAR JUNCTIONS IN WILD TYPE AND CYSTEINE STRING PROTEIN NULL MUTANT *DROSOPHILA*

M. Heckmann, H. Adelsberger and J. Dudel

Among the synaptic vesicle proteins thought to be involved in depolarization-release coupling are some containing an unusual cysteine motif (in *Drosophila*: $C_2X_5C_{11}X_2C_2$; C is cysteine and X is any amino acid) and a „J-domain", called cysteine string proteins. Deletion of the cysteine string protein (CSP) gene in *Drosophila* causes a temperature-sensitive block of synaptic transmission (Zinsmaier et al., Science *263* 977-980, 1994) that was proposed to be due to a disruption of depolarization-release coupling. At 30 °C evoked transmitter release declines over minutes and finally fails in CSP null mutant larvae but recovers when the temperature is returned to 22 °C (Umbach et al., Neuron *13* 899-907, 1994). If CSPs are involved in depolarization-release coupling the time course of release might be disturbed in CSP null mutant *Drosophila*. To test this we used an extracellular macro-patch-clamp electrode to depolarize nerve terminals and record excitatory postsynaptic currents (EPSCs) at neuromuscular junctions of wild type and CSP null mutant larvae at 16-18 °C. The amplitude of average EPSCs was reduced and the time constant of the exponential fit of the current decay was increased in CSP null mutant compared to wild type larvae. The number of quanta released per pulse was, however, at several levels of depolarization not different in CSP null mutant and wild type larvae. Facilitation in response to twin-pulse stimulation was slightly increased and the decay of the probability of evoked quantal release was delayed in CSP null mutant larvae. The results suggest that CSPs are involved in depolarization-release coupling at nerve terminals and help synchronize evoked neurotransmitter release possibly by activating molecular chaperones or through an interaction with presynaptic Ca^{2+} channels.

Physiologisches Institut der Technischen Universität München, Biedersteiner Straße 29, D-80802 München

ROLE OF SYNTAXIN-1 AND ITS H3 REGION ON CALCIUM AND GTP-γ-S-INDUCED INSULIN SECRETION IN MOUSE PANCREATIC BETA CELLS.

F. Martín*, E. Salinas+, J. Vazquez#, F. Moya¶, J. A. Reig+ and B. Soria*. *Department of Physiology, ¶Department of Microbiology and +Department of Neurochemistry, Institute of Neurosciences, University of Alicante, Aptdo. 374, 03080 Alicante; #Center of Molecular Biology, School of Sciences, University Autonoma of Madrid, Cantoblanco, 28049 Madrid, Spain.

Recent studies show that pancreatic endocrine β-cells are positive for several synaptic proteins involved in processes of vesicle docking, activation and fusion, which take part in the exocytotic phenomena. In the present study, we have examined the presence and functional role of syntaxin-1 and one of its regions (H3) in Ca^{2+}- and GTP-γ-S-stimulated insulin release. Immunoblotting studies showed that monoclonal antibodies HPC-1 and Mab44D5 recognized a single major band of 35 kDa in the fraction containing islet-cells membrane proteins and in the fraction containing total pancreatic islet-cells proteins. No positive labelling for syntaxin-1 was observed in the pancreatic islet-cell cytosolic fraction. Using an immunocytochemical approach we also confirmed that pancreatic islet-cells were positively stained with these antibodies. Functional studies using digitonin-permeabilized pancreatic islet-cells showed that whereas basal insulin release was not affected by the presence of the anti-syntaxin monoclonal antibodies, both antibodies provoked an inhibition of Ca^{2+}-dependent insulin secretion. the inhibitory effect was of 54% ($p< 0.05$) with clone HPC-1 and 26% with Mab44D5. When anti-syntaxin monoclonal antibodies where denaturalized any effect was observed on Ca^{2+}-dependent insulin secretion. In adittion, using two synthetic peptides (Syn-1 and Syn-2) corresponding to two not overlapping segments of 23 residues of the H3 region (amino acids 191-265) at the C-terminal domain of the syntaxin-1. Peptides Syn-1 and Syn-2 provoked a dose-dependent inhibition of Ca^{2+}-dependent insulin release (IC_{50} ~ 46 and 32 μmmol/l for Syn-1 and Syn-2, respectively). For both peptides, maximum inhibitory effect was achieved at 200 μmmol/l (Syn-1= 73% and Syn-2= 84%). Control peptides (Syn-1C and Syn-2C, with the same amino acid composition but in random sequence) had not inhibitory effect at 200 μmmol/l. By contrast, 200 μmmol/l of Syn-1 or Syn-2 did not prevent 100 μmmol/l GTP-γ-S-stimulated insulin secretion. These results demonstrate that syntaxin-1 through specific structural determinants of its H3 region selectively control Ca^{2+}-mediated insulin secretion but is not required for GTP-γ-S-stimulated insulin release. Thus, in pancreatic endocrine cells syntaxin-1 is involved in the control of exocytosis of the insulin-containing granules.

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DELETION OF THE FIVE CARBOXYL-TERMINAL AMINO ACIDS ABOLISHED THE RETENTION OF GLUT4 IN THE PERINUCLEAR STORE COMPARTMENT

SONIA MARTÍNEZ ARCA
Centro de Biología Molecular "Severo Ochoa"
Lab. 302, Fac. de Ciencias – Univ. Autónoma – Cantoblanco
28049 Madrid (Spain)

The insulin-sensitive glucose transporter GLUT4, which in the absence of the hormone is found in tubulo-vesicular elements associated with the Golgi complex, has two major sorting determinants: a dileucine-based signal in its COOH-cytoplasmic tail and a canonic FQQI internalization signal in its NH2-cytoplasmlc tail. Whereas these motifs might explain its sorting in the Golgi complex and surface internalization, there is no information about the motifs involved in its retencion in the Golgi and return from the surface to this organelle upon removal of insulin.

In the present study we have examined the retention of the transporter in the Golgi complex by comparing the distribution of wild-type GLUT4 with mutants bearing COOH-tail deletions, both in transiently transfected COS cells and in stably transfected 3T3-L1 cells. The retention of wild-type GLUT4 in VAMP2 positive elements within the Golgi area is in contrast with the transport of Glut4Δ5, a truncated mutant lacking the five carboxy-terminal aminoacids, to vesicular structures containing the late endosomal marker Rab7, but which exclude the cation-dependent mannose 6-phosphate receptor (CD-M6PR) . The truncated protein appears to be tranported directly from the Golgi to these vesicular structures. The distribution and abscence of GLUT4Δ5 from the plasma membrane is in contrast with the transport of the truncated mutant Glut4Δ17 to the cell surface. Furthermore, the COOH-cytoplasmic tail of GLUT4 confers the lysosomal membrane protein LIMPII a distribution indistiguishable of wild-GLUT4 and truncation of the last five amino acids in this chimera results in its delivery to the GLUT4Δ5 compartment. The putative retention motif does not reside in the last five amino acids, as shown by mutagenesis studies that included en-block and point alanine substitutions . It is concluded that deletion of the last five amino acids abolishes the retention of GLUT4 in the intracellular store.

In VITRO FUSION EVENTS INVOLVING YEAST PLASMA MEMBRANE VESICLES

Lorena Arrastua, Amaia Menéndez and Unai Ugalde

Unidad de Bioquímica. Facultad de Ciencias Químicas.Apartado 1072. 20080 San Sebastián. Spain

The last fusion step in cooperative exocytosis was studied "in vitro" after the purification and characterization of sealed inside-out plasma membranes, secretory vesicles and from the yeast *Saccaromyces cerevisiae.*

Preliminary results based on measurement of absorbance changes show a clear ATP-dependent membrane agregation. Coalescence of the vesicles was verified through lipid mixing of the two compartments. A self-quenching probe (R18, octadecylrhodamine B chloride) was included in secretory vesicles and the increased fluorescence resulting from lipidic dilution of the probe was interpreted as a measure of fusion.

The contents mixing will be assayed as the transference of the enzyme invertase tightly packed on secretory vesicles to plasma membranes. For this reason inside-out plasma membrane vesicles have been immunoisolated using magnetic beads.

The results using these methods are presented and discussed.

Different calcium channels mediate fast versus slow synaptic transmission in *Drosophila* neuromuscular junction

M Morales, I martinez, A Ferrus
Instituto Cajal. C.S.I.C. Madrid. Spain

Transmitter release from synaptic terminals is triggered by a large and rapid increase of calcium concentration restricted to the vicinity of the release machinery. By contrast, slow exocytosis, as it occurs in peptide release, appears to require a more generalized and persistent calcium increase within the presynaptic terminal. There is some evidence that the calcium channels involved in fast neurotransmission are different from those mediating peptide release. *Drosophila* larval muscle fibers are innervated by both glutamatergic and peptidergic nerve fibers, and the latter are readily accessible to direct electrophysiological recordings. We have taken advantage of these features to analyze which types of voltage operated calcium channels (VOCCs) are involved in fast versus slow synaptic transmission.

The ventral longitudinal abdominal muscles of *Drosophila* larvae are contacted by several types of synaptic terminals. Type I terminals contain glutamate and mediate fast synaptic transmission, whereas types II and III terminals appear to be of a peptidergic nature, as they contain dense-core vesicles and present immunoreactivity to insulin-like peptide, proctolin and octopamin. We have investigated whether specific types of calcium channels may be linked to either type of transmitter release.

Because type I terminals are not amenable to direct recordings, we have addressed the issue of which type of channel is involved in fast release, by assaying the effect of a number of calcium-channel blockers on the excitatory postsynaptic current (EPC) produced by nerve stimulation. Thus, fast synaptic transmission is reduced to 40% of its control by 1mM sFTX, a spider toxin which is specific for P-type calcium channels and completely blocked by 0.1μM PLTX a toxin from Plectreurys spider venom which is specific for insect neuronal calcium-channels. Conversely, synaptic transmission was not affected by classical L-type channel blockers (Dihydropyridines-DHPs, polyamines-PA, etc...), N-type channel blockers (ω-conotoxin) or other P-type channel blockers (ω-agatoxins). The fact that blockade by sFTX is only partial, suggests that in addition to P-type channels, some other PLTX-sensitive type of calcium channel may contribute to synaptic transmission in *Drosophila* neuromuscular junction.

We have developed a preparation that allows direct current and voltage-clamp recordings from type III peptidergic boutons. In contrast to type I, type III terminals appear to contain a single high-threshold (activation starts at -30mV), slowly inactivating calcium current that is DHP-sensitive. Almost complete blockade of the current is attained by 20μM concentration of nifedipine, suggesting that the calcium influx in these terminals occurs through L-like calcium channels. These currents exhibit properties similar to those of the calcium current recorded in the muscle fibers, which is also sensitive to DHPs.

In summary we identify a P-like calcium channel for type I terminals and a L-like for type III boutons.

INTERACTION OF RECOMBINANT SYNTAXIN-1A WITH MEMBRANES.

J.H.Villanueva, J.M.González-Ros, F.Moya
Instituto de Neurociencias. Universidad de Alicante. Spain

Syntaxin 1a has been described as a membrane protein involved in the regulatory release of neurotransmitters. Biochemical and physiological studies in neurosecretory systems suggest that syntaxin-1a participates actively in the fusion process of the secretory vesicles to the presinaptic membrane.

Using a cDNA clone that code Syntaxin 1a of Drosophila , we have obtained a recombinant syntaxin 1a (**Stx 119-292**), that contains the C-terminal portion of the protein. This domain of the molecule contains the potential transmembrane segment and the H3 segment which have been shown to interact with other proteins of the secretory complex. *E.coli* expression of this recombinant protein results in a mixture of monomeric and dimeric forms.

We are reporting on the interaction between the monomeric form of Stx 119-292 with phospholipid vesicles and native membranes. Protein reconstitution experiments were performed to establish the best conditions for protein-lipid association. The protein was labeled by cysteine alkylation with NBD-Iodoacetamide and it was assayed for binding to lipids.

Using distinct fluorophore-labeled syntaxin, we were able to carry out Fluorescence energy transfer (FRET) and pyrene excimers formation assays to establish whether recombinant syntaxin self-associate within a phospholipid bilayer.

By Differential Scanning Calorimetry (DSC), we have used Stx 119-292 monomeric forms and large multilamellar vesicles (LMV), to study the insertion of this protein into the membrane.

Finally, using Fourier Transform Infrared Spectroscopy (FTIR), we were have studied the adoption of secondary structure of syntaxin-1a in the presence of lipids.

Different Effects of Enzyme-generated Ceramides and Diacylglycerols in Phospholipid Membrane Fusion and Leakage.

M. Begoña Ruiz-Argüello, Gorka Basáñez, Félix M. Goñi and Alicia Alonso

Grupo Biomembranas (Unidad asociada al C.S.I.C.), Departamento de Bioquímica y Biología Molecular, Universidad del País Vasco/Euskal Herriko Unibertsitatea, Aptdo. 644, E-48080 Bilbao, España.

When large unilamellar vesicles consisting of sphingomyelin:phosphatidylethanolamine :cholesterol (2:1:1 molar ratio) are treated with sphingomyelinase, production of ceramides in the bilayer is accompanied by leakage of vesicle aqueous contents and by vesicle aggregation in the absence of lipid mixing or vesicle fusion. This is in contrast to the situation of phosphatidylcholine:phosphatidylethanolamine:cholesterol (2:1:1 molar ratio) liposomes when treated with phospholipase C. In that case, *in situ* generation of diacylglycerol leads to vesicle aggregation followed by vesicle fusion in the absence of leakage. Moreover, when ceramides (5-10 mol %) are included in the formulation of the phosphatidylcholine-containing vesicles, they reduce the lag time of phospholipase C-induced fusion, although they are less active than diacylglycerols in this respect. ^{31}P-NMR studies of aqueous lipid dispersions show that diacylglycerols as well as ceramides induce a thermotropic lamellar to non-lamellar phase transition in both phospholipd.cholesterol mixtures under study although sphingomyelin-containing bilayers are more stable than those containing phosphatidylcholine, and ceramide is less active than diacylglycerol in promoting non-lamellar phase formation- These observations are relevant to both the physiological role of ceramides and the current views on the mechanism of membrane fusion.

CONDITIONAL ACETYL-COA CARBOXYLASE MUTANTS LINK FATTY ACID CHAIN ELONGATION TO STRUCTURE AND FUNCTION OF THE NUCLEAR ENVELOPE / NUCLEAR PORE COMPLEX

R. Schneiter and S. D. Kohlwein, Dept. of Biochemistry, Technical University Graz, Petersgasse 12, A-8010 Graz

Acetyl-CoA carboxylase (*ACC1*) catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in *de novo* synthesis of fatty acids. Yeast mutants in acetyl-CoA carboxylase were previously isolated in screens for fatty acid auxotrophic cells. However, loss-of-function alleles of *ACC1* and a novel class of conditional *acc1* alleles are not rescued by long-chain fatty acid supplementation, indicating that malonyl-CoA is required for a vital function that is independent of long-chain fatty acid synthesis. Strikingly, conditional *acc1* alleles display an alteration of the nuclear envelope and are impaired in nucleocytoplasmic transport of mRNA. We find that the synthesis of very long-chain fatty acids (C_{26}) is affected in these mutants and propose a requirement for very long-chain fatty acid substituted lipids in stabilizing the nuclear membrane/pore complex interface. A novel C_{26} substituted lipid that may fulfill this stabilizing function has been identified in isolated nuclear membranes.

Emp47p as a marker to screen for Golgi to ER transport mutants.

Alexei Matiach, Howard Riezman* and Stephan Schröder-Köhne

Department of Molecular Genetics, Max-Planck-Institute for Biophysical Chemistry, P.O. Box 2841, 37018 Göttingen, Germany. *Biozentrum der Universität Basel, Klingelbergstr. 70, 4056 Basel, Switzerland.

In yeast the transport of proteins with a cytoplasmic di-lysine signal from the Golgi-complex back to the ER (retrograde transport) depends on coatomer, the coat complex found on COP I coated vesicles. Additional factors required are the t-SNARE Ufe1p and the ER-protein complex Sec20p/Tip20p (1, 2, 3, 4). The yeast Golgi-transmembrane protein Emp47p contains a di-lysine-signal in its cytoplasmic tail. Emp47p cycles between the Golgi-complex and the ER and requires its di-lysine signal for Golgi localisation (5). Destruction of the di-lysine signal leads to the mislocalisation of Emp47p to the vacuole, where it is rapidly degraded. The same effect is observed in mutants affected in the genes coding for Ufe1p (3), the Sec20p/Tip20p complex (3, 4) and the β'-, γ-, δ-, and ζ-COP subunits of coatomer. The Golgi-localisation of Emp47p is however not affected by the *ret1-1* allele of the gene coding for α-COP (5). In this respect Emp47p behaves differently from the di-lysine-reporter protein Ste2-Wbp1p, which was originally used to identify *ret1-1* (1). The differential behaviour of Emp47p and Ste2-Wbp1p as well as the fact that the screen of Letourneur *et al.* (1) has so far exclusively revealed coatomer subunits, prompted us to initiate a screen using Emp47p to find new mutants affected in retrograde transport. This screen is based on a colony-blot and we are looking for *decreased* levels of Emp47p in mutant cells. In the informative subset of such mutants the decreased level of Emp47p results from the mislocalisation of the protein to the vacuole.

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Genetics for the Brain Dead: A Mutant Screen for Defects in Synaptic Function and Synaptogenesis in *Drosophila melanogaster*.

Sean T. Sweeney*, Richard P. Auburn*, Peter Deak# and Cahir J. O'Kane*

*Department of Genetics, University of Cambridge, Downing St, Cambridge, England, CB2 3EH (e.mail STS: ss2@mole.bio.cam.ac.uk)
#Department of Anatomy and Physiology, Old Medical School, University of Dundee, Dundee, Scotland, DD1 4HN

We have initiated a screen in *Drosophila melanogaster* with the aim of isolating mutations that affect synaptogenesis and synaptic function. In previous studies, late embryos carrying identified mutations in genes involved in synaptic function have been found to be paralysed or uncoordinated. We have used this phenotype (late embryonic paralysis/loss of coordination of the body wall musculature) to screen through a collection of 1145 third chromosome P-element embryonic lethal insertions (the third chromosome represents ~40% of the *Drosophila* genome). We have so far screened through 40% of the collection and have found 91 paralysed/uncoordinated lines. We are currently screening through these lines to assess synaptic, neuronal and muscle development. If the morphology of any of these mutants is found to be normal, we will then initiate molecular and electrophysiological investigations. Initial molecular work on 22 of the paralysed lines has identified mutations in some known genes, two of which are involved in neuronal development (*castor* and *prospero*), one mutation affecting muscle contraction, (tropomyosin II), one mutant affecting muscle attachment (*wings held out*) and two mutants in a previously identified neuronal RNA binding protein, mutations in which result in hypoactivity (*couch potato*). We have also isolated mutations in two unexpected genes, one in the protein phosphatase 2A regulatory subunit and another in the small heat shock protein 23.

Christine Wandelt, Institute of Molecular Biology, University of Jena.

Membrane fusion is induced by a peptide from a sea urchin sperm protein:
- a molecular model to study fertilization ?!

Marlies Otter, Charles Glabe, Dick Hoekstra, Christine Wandelt, Anne Ulrich

Fertilization between sperm and egg proceeds via several stages of cell-cell adhesion and membrane fusion. In sea urchin, the acrosomal protein "bindin" is known to play a key role in the recognition and binding of the sperm membrane to the egg cell receptor. The same protein also appears to be resonsible for the actual fusion event between the two membranes, since isolated bindin was shown to induce rapid fusion between lipid vesicles.

We are using a minimal peptide model (18 amino acids) derived from the fertilization protein bindin (24 kDa), in order to study the mechanism of membrane fusion on a molecular level and to derive structural information. The highly conserved hydrophobic sequence in the central domain of bindin is responsible for attaching the protein peripherally to the membrane. Peptides from this region are known to be biologically active as potent inhibitors of fertilization *in vitro*. We find that the peptide LGLLLRHLRHHSNLLANI behaves very similar to the whole protein in terms of its interaction with lipid vesicles and by being capable of triggering membrane fusion.

Using fluorescence and light scattering assays, the peptide-induced fusion, aggregation and leakage kinetics have been characterized for large unilamellar vesicles composed of sphingomyelin/cholesterol. Zinc ions are found to be necessary for fusion, presumably by leading to an initial aggregation of the membrane-bound peptides. The prominent histidine residues in the middle of the peptide are responsible for coordinating the metal ion, and they are also likely to form a zinc binding site in the intact protein. The binding of the peptide to the lipid bilayer appears to be driven by hydrophobic interactions of the seven leucine sidechains, and is accompanied by some considerable leakage of vesicle contents. We suggest that fusion proceeds via the formation of a zinc bridge between two membrane-bound peptides on apposing vesicles. That way, the vesicles are drawn closer together, while the bilayer surfaces are being destabilized by the peripheral interaction of the hydrophobic peptides. A detailed structural investigation of the peptide in the membrane bound state is being carried out by CD and NMR spectroscopy, to contribute to a better understanding of lipid-protein interactions and membrane fusion.

Complex Regulation of the N-type Calcium Channel Synprint Site

C.T. Yokoyama, Z.-H. Sheng, S. Mochida, J. Rettig, and W.A. Catterall
Department of Pharmacology, University of Washington, Seattle, WA USA

Voltage-gated calcium channels are an essential element of regulated exocytosis in presynaptic termini of central and peripheral neurons. In response to membrane depolarization, they produce a rapid and localized calcium signal which interacts with calcium sensors on the exocytotic apparatus to initiate vesicle fusion and neurotransmitter release. Biophysical and biochemical studies suggest a tight association of calcium channels to sites of vesicle docking, priming, and fusion. Recent work from our group has identified the synaptic protein interaction (synprint) site in the intracellular loop II-III of N-type calcium channels. This site binds with high specificity, affinity, and stoichiometry to the plasmalemma SNARE proteins, syntaxin and SNAP-25, and also to the putative calcium sensor and vesicle membrane protein, synaptotagmin. Furthermore, synprint peptides introduced into cultured sympathetic neurons inhibit the fast, synchronous phase of neurotransmitter release, consistent with a requirement for direct interaction of calcium channels with docked vesicles. Protein interactions at the synprint site from N-type calcium channels are subject to complex regulation. Calcium stimulates binding of syntaxin and SNAP-25 at concentrations between 10 μM and 30 μM and inhibits binding at higher concentrations. In contrast, there is no apparent calcium-dependent modulation of the interaction of synaptotagmin with the synprint. Syntaxin and synaptotagmin compete for binding to the synprint, and the competition favors syntaxin at the optimal free calcium concentration for syntaxin binding. Phosphorylation of the synprint by the protein kinase C and Ca^{2+}/calmodulin kinase II inhibits interactions with syntaxin, SNAP-25, and synaptotagmin. Calcium at concentrations which are optimal for syntaxin and SNAP-25 association does not restore binding to the phosphorylated synprint. Together, these studies suggest that in addition to providing the critical calcium signal to trigger membrane fusion, voltage-gated calcium channels bind, via the synprint site, to essential elements of the fusion machinery, from both the plasmalemma and vesicle membranes, and this interaction is essential for rapid, synchronous exocytosis. Furthermore, they reveal a complex regulatory code for SNARE protein binding at the synprint site, with affinities that are a graded, dynamic function of the calcium level, protein kinase activation, and protein binding competition. In addition to the well-characterized regulation of neurotransmission via the modulation of calcium channel gating, these studies predict that the synprint site on voltage-gated calcium channels may actively participate in the exocytotic mechanism by ensuring vesicle docking near the source of calcium and that the gain on the calcium sensitivity of membrane fusion may be regulated through this mechanism.

List of Invited Speakers

Workshop on

MEMBRANE FUSION

List of Invited Speakers

Wolfhard Almers	Department Molekulare Zellforschung, Max-Planck-Institut für Medizinische Forschung, Jahnstrasse 29, Heidelberg (Germany). Tel.: 49 6221 48 63 50 Fax : 49 6221 48 63 25
Guillermo Alvarez de Toledo	Dept. Physiology and Biophysics, University of Seville, 4 Sánchez Pizjuan Ave., 41009 Sevilla (Spain). Tel.: 34 5 455 17 70 Fax : 34 5 455 17 69 E-mail: Gatoledo@cica.es
Robert D. Burgoyne	The Physiological Laboratory, University of Liverpool, Crown Street, Liverpool L69 3BX (U.K.). Tel.: 44 151 794 53 05 Fax : 44 151 794 53 37 E-mail: burgoyne@liverpool.ac.uk
Dieter Gallwitz	Max-Planck-Institute for Biophysical Chemistry, Department of Molecular Genetics, D-37070 Göttingen (Germnay). Tel.: 49 551 201 14 96 Fax : 49 551 201 17 18 E-mail: dgallwil@gwdg.de
Yukiko Goda	The Salk Institute, Molecular Neurobiology Lab., 10010 North Torrey Pines Road, La Jolla, CA. 92186-5800(USA). Tel.: 1 619 453 41 00 Fax : 1 619 450 21 72 E-mail: goda@axp1.salk.edu
Reinhard Jahn	HHMI, Departments of Pharmacology and Cell Biology, Yale University School of Medicine, BCMM, 295 Congress Ave., New Haven, CT. 06510 (USA). Tel.: 1 203 737 44 58 Fax : 1 203 737 17 63 E-mail: Reinhard.Jahn@Yale.edu
Martin Latterich	The Salk Institute,Molecular Biology and Virology Lab., 10010 North Torrey Pines Road, La Jolla, CA. 92037 (USA). Tel.: 1 619 453 41 00 Fax : 1 619 457 47 65 E-mail: mlatterich@aim.salk.edu

Vivek Malhotra	Department of Biology 0347, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA. 92093-0347(USA). Tel.: 1 619 534 89 10 Fax : 1 619 534 05 55 E-mail: malhotra@jeeves.ucsd.edu
Thomas F.J. Martin	Department of Biochemistry, University of Wisconsin, 420 Henry Mall, Madison, WI. 53706-1569(USA). Tel.: 1 608 263 24 27 Fax : 1 608 262 34 53 E-mail: tfmartin@facstaff.wisc.edu
Ira Mellman	Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, PO Box 208002, New Haven, CT. 06520 (USA). Tel.: 1 203 785 50 58 Fax : 1 203 785 72 26 E-mail: ira.mellman@yale.edu
Peter Novick	Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT. 06510 (USA). Tel.: 1 203 785 58 71 Fax : 1 203 785 72 26
Hugh Pelham	MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (U.K.). Tel.: 44 1223 40 22 90 Fax : 44 1223 41 21 42 E-mail: hp@mrc-lmb.cam.ac.uk
Richard H. Scheller	Department of Molecular and Cellular Physiology, Stanford University, Beckman Center B-155, Stanford, CA. 94305-5426 (USA). Tel.: 1 415 723 90 75 Fax : 1 415 725 44 36 E-mail: scheller@cmgm.stanford.edu
Kai Simons	EMBL. European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg (Germany). Tel.: 49 6221 387 334 Fax : 49 6221 387 512
Thomas C. Südhof	Howard Hughes Medical Institute and Department of Molecular Genetics, The University of Texas Southwestern Medical Ctr., 5323 Harry Hines Blvd., Dallas, TX. 75235-9050 (USA). Tel.: 1 214 648 50 22 Fax : 1 214 648 64 26
Angel Velasco	Department of Cell Biology, Faculty of Biology, University of Seville, Reina Mercedes Ave., 41012 Sevilla (Spain). Tel.: 34 5 455 70 38 Fax : 34 5 461 02 61 E-mail: E-mail: avelasco@cica.es

Graham Warren

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX (U.K.).
Tel.: 44 171 269 35 61
Fax : 44 171 269 30 78
E-mail: warren@icrf.icnet.uk

Marino Zerial

EMBL. European Molecular Biology Laboratory, Meyerhofstrasse 1, 69017 Heidelberg (Germany).
Tel.: 49 6221 38 73 34
Fax : 49 6221 38 75 12

Joshua Zimmerberg

Laboratory of Cellular and Molecular Biophysics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD. 20892-1855 (USA).
Tel.: 1 301 496 65 71
Fax : 1 301 594 08 13
E-mail: joshz@helix.nih.gov

List of Participants

Workshop on

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List of Participants

Alicia Alonso	Grupo Biomembranas (Unidad asociada al CSIC) Dept. de Bioquím. y Biol. Molec., Univ. del País Vasco. Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00 Fax : 34 4 464 85 00
Miguel A. Alonso	Centro de Biología Molecular "Severo Ochoa", CSIC, Fac. de Ciencias, Univ. Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 80 37 Fax : 34 1 397 80 87
Rebecca Ellis Dutch	Dept. of Biochemistry, Molec. Biology and Cell Biology, Howard Hughes Medical Inst. , Northwestern University, 2153 North Campus Drive, Evanston, IL. 60208-3500 (USA). Tel.: 1 847 491 54 33 Fax : 1 847 491 24 67 E-mail: ralamb@nwu.edu
Gustavo Egea	Dept. Biologia Cel.lular i Anatomia Patològica. Fac. de Medicina, Universitat de Barcelona, 08036 Barcelona (Spain). Tel.: 34 3 402 19 09 Fax : 34 3 402 19 07 E-mail: egea@medicina.ub.es
Zvulun Elazar	Dept. Membrane Research & Biophysics, The Weizmann Institute of Science, Rehovot 76100 (Israel). Tel.: 972 8 934 36 82 Fax : 972 8 934 41 12 E-mail. bmzevi@weizmann.ac.il
Rafael Fernández-Chacón	Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, Dallas, TX., 75235-9050 (USA). Tel.: 1 214 648 50 99 Fax : 1 214 648 50 66
Jeffrey E. Gerst	Dept. of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100 (Israel). Tel.: 972 8 934 39 70 Fax : 972 8 934 41 08 E-amil: lvjeff@weizmann.weizmann.ac.il

Benjamin S. Glick	Dept. of Molecular Genetics and Cell Biology, The University of Chicago, 920 East 58th Street. Chicago, IL. 60637 (USA). Tel.: 1 773 702 53 15 Fax : 1 773 702 31 72 E-mail: bsglick@midway.uchicago.edu
Félix M. Goñi	Grupo Biomembranas (Unidad asociada al CSIC) Dept. de Bioquím. y Biol. Molec., Univ. del País Vasco. Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00 Fax : 34 4 464 85 00
Luis M. Gutiérrez	Dept. de Neuroquímica, Fac. de Medicina, Universidad de Alicante, Campus de San Juan 03013 Alicante (Spain). E-mail: luisguti@ua.es
Manfred Heckmann	Physiologisches Institut der Technischen Universität München, Biedersteiner Straße 29, D-80802 München (Germany). Tel.: 49 89 41 40 31 44 Fax : 49 89 41 40 33 77 E-mail: heckmann@physiol.med.tu-muenchen.de
Josefina Hidalgo	Department of Cell Biology, Faculty of Biology, University of Seville, 41012 Sevilla (Spain). Tel.: 34 5 455 70 44 Fax : 34 5 461 02 61
Benjamin Lewin	Cell, Editorial Offices, 1050 Massachusetts Avenue, Cambridge, MA. 02138 (USA). Tel.: 1 617 661 7057 Ext. 129 Fax : 1 617 661 70 61 E-mail: Editor@cell.com
Alberto G. Luini	Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Chieti (Italy). Tel.: 39 872 57 03 55 Fax : 39 872 57 82 40 E-mail: luini@cmns.mnegri.it
Franz Martín	Department of Physiology and Institute of Neurosciences, University of Alicante, Aptdo. 374, 03080 Alicante (Spain). Tel.: 34 6 590 34 00 Fax : 34 6 590 39 62 E-amil: franz.martin@ua.es

Sonia Martínez Arca

Centro de Biología Molecular "Severo Ochoa", Lab. 302, Fac. de Ciencias, Univ. Autónoma, Cantoblanco, 28049 Madrid (Spain).
Tel.: 34 1 397 50 70
Fax : 34 1 397 47 99
E-mail: smartinez@trasto.cbm.uam.es

Imanol Martínez Padrón

Instituto Cajal, CSIC, Ave. Doctor Arce 37, 28002 Madrid (Spain).
Tel.: 34 1 585 47 38
Fax : 34 1 585 47 54
E-mail: ISRMP54@pinar1.csic.es

Andreas Mayer

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37-39, D-72076 Tübingen (Germany).
Tel.: 49 7071 60 18 50
Fax : 49 7071 60 14 55
E-mail: anma@mailer1.mpib-tuebingen.mpg.de

Amaia Menéndez

Unidad de Bioquímica, Facultad de Ciencias Químicas, Apartado 1072, 20080 San Sebastián (Spain).
Tel.: 34 43 21 66 00 - Ext. 108
Fax : 34 43 21 22 36
E-mail. qpbmemaa@sc.ehu.es

Faustino Mollinedo

Instituto de Biología y Genética Molecular, CSIC, Fac. de Medicina, Univ. de Valladolid, c/Ramón y Cajal 7, 47005 Valladolid (Spain).
Tel.: 34 83 42 30 62
Fax : 34 83 42 35 88

Miguel Morales

Instituto Cajal, CSIC, Dr. Arce 37, 28002 Madrid (Spain).
Tel.: 34 1 585 47 38
Fax : 34 1 585 41 54
E-mail: isrfm76@fresno.csic.es

Fernando Moya

Dept. de Genética y Microbiología e Instituto de Neurociencias, Universidad de Alicante, Campus de San Juan, Apdo. 374, 03080 Alicante (Spain).
Tel.: 34 6 590 38 64
Fax : 34 6 590 38 67
E-mail: Fmoya@ua.es

Josep Rizo

Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX. 75235-9041 (USA).
Tel.: 1 214 648 90 26
Fax : 1 214 648 86 73
E-mail: jose@arnie.swmed..edu

Mª Begoña Ruiz-Argüello	Grupo Biomembranas (Unidad asociada al CSIC) Dept. de Bioquím. y Biol. Molec., Univ. del País Vasco. Apdo. 644, 48080 Bilbao (Spain). Tel.: 34 4 464 77 00 Fax . 34 4 464 85 00 E-amil: gbbruarm@lg.ehu.es
Roger Schneiter	Dept. of Biochemistry, Technical University Graz, Petersgasse 12, A-8010 Graz (Austria). Tel.: 43 316 873 64 54 Fax : 43 316 873 69 52 E-mail: Schneiter@fochsg02.tu-graz.ac.at
Stephan Schröder-Köhne	Department of Molecular Genetics,Max-Planck-Institute for Biophysical Chemistry, P.O. Box 2841 D-37018 Göttingen (Germany). Tel.: 49 551 201 17 15 Fax : 49 551 201 17 18 E-mail. sschroe1@gwdg.de
Bernat Soria	Department of Physiology and Institute of Neuroscience, School of Medicine, University of Alicante, Aptdo. 374, 03080 Alicante (Spain). Tel.: 34 6 590 39 61 Fax : 34 6 590 39 62 E-mail: bernat.soria@ua.es
Christine Sütterlin	Dept. of Biochemistry, Biozentrum of the University, Klingelbergstr. 70, CH-4054 Basel (Switzerland). Tel.: 41 61 267 21 53 Fax : 41 61 267 21 48 E-mail: suetterlinc@ubaclu.unibas.ch
Sean Sweeney	University of Cambridge, Dpt. of Genetics, Darwin College, Downing Str., Cambridge CB2 3EH (U.K.). Tel.: 44 1223 333 976 Fax : 44 1223 333 992 E-mail: ss2@mole.bio.cam.ac.uk
Christine Wandelt	Friedrich-Schiller-University Jena, Institute of Molecular Biology, Winzerlaer Str. 10, D-07708 Jena (Germany) Tel.: 49 3641 65 75 73 Fax : 49 3641 65 75 20 E-mail: wandelt@imb-jena.de
Lois S. Weisman	Department of Biochemistry, University of Iowa, 4403 Bowen Science Bldg., Iowa City, IA. 52242-1109 (USA). Tel.: 1 319 335 85 81 Fax : 1 319 335 95 70 E-amil: lweisman@blue.weeg.uiowa.edu

Charles T. Yokoyama

Dept. of Pharmacology and Program in Neurobiology, Univ. of Washington, Box 357280, Seattle, WA. 98195-7280 (USA).
Tel.: 1 206 685 22 33
Fax : 1 206 685 38 22
E-mail: charlest@u.washington.edu

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