Instituto Juan March de Estudios e Investigaciones

116

CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Comparison of the Mechanisms of Cellular Vesicle and Viral Membrane Fusion

Organized by

J. J. Skehel and J. A. Melero

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L. V. Chernomordik
F. S. Cohen
J. M. Edwardson
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F. X. Heinz
R. A. Lamb
B. R. Lentz
A. Mayer
J. A. Melero
J. L. Nieva
H. R. B. Pelham
J. E. Rothman
R. H. Scheller
J. J. Skehel
D. C. Wiley
J. Zimmerberg

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Introduction

J. J. Skehel and J. A. Melero

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The fusion of membranes is an event shared by many biological processes, such as oocyte fertilisation, compartmentalisation, endocytosis, secretion synaptic transmission and entry of enveloped virus into cells. Whereas fusion of two lipid bilayers is the common ground in all these processes, the promotion of membrane fusion and the regulation of membrane mixing has specific characteristics in each biological system. In some processes, such as vesicle fusion, there is reversibility of the fusion event and recycling of the membranes. In others, such as virus and cell membrane fusion, disassembly of the membranes does not occur. Despite these differences, understanding the molecular mechanism of membrane fusion in a given process may provide important clues for other biological systems. For this reason, the Workshop sponsored by the Fundación Juan March brought together leading world experts with different backgrounds to discuss the mechanisms of membrane fusion.

Several presentations faced the mechanism of viral and cell membrane fusion at the initial stages of the infectious cycle in different enveloped viruses. In every case, a particular glycoprotein of the viral membrane appears to promote membrane fusion by inserting, through hydrophobic sequences, into the target membranes. This event requires structural reorganisations of the viral glycoproteins that, in some cases, are triggered by a drop in the pH of the vesicles through which the virus particle is endocytosed. In other cases, fusion of the virus and cell membranes occurs at the cell surface and the triggering event for fusion seems to be mediated by the interaction of the attachment proteins of the respective viruses with specific cell receptors.

Concurrently with the rearrangements of viral glycoproteins and their insertion into the cell membranes, there is an apposition of the two membranes that is proposed to favour, first, the interchange of lipid molecules between the outer leaflets (hemifusion) and, later on, mixing of the two lipid bilayers. Completion of membrane fusion may require the formation of multiprotein complexes of the viral glycoproteins, once inserted into the target membranes.

Similar to the process of viral membrane fusion, apposition of vesicle and target membranes is an indispensable intermediate step for membrane fusion within cells. In this process, a complex is assembled between proteins inserted in the vesicle and target membranes. Generally, the formation of such complexes involves refolding of the proteins and formation of intermolecular helical bundles that bring the two membranes into close

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proximity. The interaction of proteins present in the surface of vesicle and target membranes is highly specific, orchestrating the fusion of membranes within the cell. Other proteins inserted into the vesicle membranes or interacting with them *in vivo* contribute to generate the chemical energy needed for membrane fusion and to regulate the reversibility and specificity of the process.

The lipid composition and the disposition of lipid molecules in the membranes are also important factors that contribute to the fusion process. This was highlighted by the results obtained with simplified model systems such as phospholipid vesicles treated with polyethyleneglycol or phospholipases. It is possible that fusion of natural membranes also requires alteration of the lipid composition at the sites where fusion pores are formed.

The unquestionable success of the meeting was greatly due to the kind hospitality of the Fundación Juan March and the efficient work of its personnel. The informal atmosphere of the meeting, promoted by our hosts, favoured lengthy discussions that crystallised in a few general ideas. Hopefully these ideas will keep all of us busy in our respective places for the next few years.

J. J. Skehel and J. A. Melero

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Session 1: Viral proteins that mediate membrane fusion
Chair: Joshua Zimmerberg

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Viral entry and the mechanism of membrane fusion

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The membrane fusion potential of influenza HA, like many viral membrane-fusion glycoproteins, is generated by proteolytic cleavage of a biosynthetic precursor. The cleavage site of the HA was revealed by X-ray crystallography to be a prominent surface loop adjacent to a novel cavity; cleavage results in structural rearrangements in which the non-polar amino acids near the new amino-terminus bury ionizable residues in the cavity that are implicated in the low-pH-induced conformational change.

The structures a group of virus membrane fusion proteins, like that of Ebola virus GP2, HIV-1 gp41, and influenza virus HA are similar to the recently determined structure of a protein complex involved in neurotransmitter release and intracellular vesicle trafficking. In each case hydrophobic sequences, embedded in the membranes to be fused, are located at the same end of a rod-shaped molecular complex composed of a bundle of long α-helices. This molecular arrangement is proposed to cause close membrane apposition as the complexes are assembled for membrane fusion.

By studying a recombinant form of the influenza virus HA2 subunit (HA2(23-185)), we discovered a domain composed of N- and C-terminal residues that form an N-Cap terminating both the N-term a-helix and the central coiled coil. The structure implies that continuous helices are not required for membrane fusion at either the N- or C-termini. The difference in stability between recombinant molecules with and without the N-Cap sequences, suggests that additional free energy for membrane fusion may become available after the formation of the central triple-stranded coiled coil and insertion of the fusion peptide into the target membrane.

The structure of the envelope glycoprotein of influenza C virus (HEF) was determined by X-ray crystallography and separate receptor binding and receptor-destroying enzyme (9-*O*-acetylesterase) sites identified with receptor analogues. The segregation of the glycoprotein's three functions into structurally distinct domains suggests that the entire stem region, including sequences at the N- and C-termini of HEF1 preceding the posttranslational cleavage site between HEF1 and HEF2, forms an independent fusion domain probably derived from an ancestral membrane fusion protein.

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Structural organization of the flavivirus fusion machinery

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There are at least two different structural classes of viral fusion proteins. Class 1 fusion proteins are characteristic of virus families such as myxo-, paramyxo-, retro-, and filoviridae and were shown to share a number of structural and functional properties. These include the formation of a trimeric spike, the requirement for proteolytic cleavage to prime fusion activity, the presence of amino-terminal or amino-proximal fusion peptides, and the triggered transition into a lower energy state involving trimeric alpha-helical coiled coils. The functional homologs of flaviviruses do not share the salient features of class 1 viral fusion proteins but differ in several significant aspects. They have a different oligomeric organization in the virion envelope, priming of fusion activity requires the proteolytic cleavage of an accessory protein but not of the fusion protein itself, they are not predicted to form coiled coils, and the available evidence indicates the presence of an internal fusion peptide. The envelope protein E of tick-borne encephalitis (TBE) virus (together with yellow fever, Japanese encephalitis, Dengue, and West Nile virus one of the major human pathogenic flaviviruses) represents the prototype of this second class of viral fusion proteins, because so far it is the only one for which a high-resolution structure is available.

In contrast to class 1 viral fusion proteins the flavivirus protein E is a head to tail dimer that is oriented parallel rather than perpendicular to the membrane. Recent cryo EM studies of a recombinant subviral particle (RSP) (I. Ferlenghi, S. Fuller et al., submitted) show that the protein E dimers form an icosahedral network, and preliminary cryo EM data (Fuller et al.; unpublished) as well as structural and biochemical evicence suggest that whole virions also have icosahedral structure. As shown by in vitro liposome fusion assays, TBE virus fusion is characterized by an extremely fast fusion rate (40% per second) and - at least at 37°C - the absence of a measurable lag phase. A comparison of the TBE virus fusion properties with those of other viruses such as influenza and Semliki Forest virus revealed that TBE virus has the fastest and most efficient fusion machinery of all enveloped viruses analyzed to date.

Flavivirus fusion is triggered by acidic pH which also leads to a complete oligomeric reorganization of the virion envelope and an irreversible conversion of E-dimers into E-trimers. As revealed by sedimentation and cross-linking experiments, exposure to acidic pH first leads to a dissociation of the E-dimer, thus probably exposing those structural elements that make the first contact with the target membrane. A highly conserved sequence of amino acids that is buried and protected in the neutral pH structure by interactions with the second monomeric subunit in the E-dimer has been hypothesized to function as an internal flavivirus fusion peptide. Using the X-ray structure as a guide we have introduced specific mutations at this site in RSPs, taking advantage of the fact that they exhibit similar fusion properties as whole virions. The analyses of these mutants in liposome-binding and fusion assays provide more direct evidence that the mutated sequence indeed functions as an internal fusion peptide.

The organization of the flavivirus fusion machinery thus has a number of features that are distinct from those of enveloped viruses with class 1 viral fusion proteins. At the same time they share several of these distinguishing features with alphaviruses such as Sindbis, Semliki Forest, and Ross River virus. Both groups of viruses have an icosahedral envelope structure, their fusion proteins are synthesized as a complex with a second protein that has to be cleaved for priming fusion activity, and they both have internal fusion peptides. It will be interesting to see whether these fusion proteins also exhibit structural homologies such as those found in class 1 viral fusion proteins.

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Paramyxovirus fusion (F) protein-mediated membrane fusion

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The paramyxovirus fusion (F) protein mediates membrane fusion. The biologically active F protein consists of a membrane distal subunit F_2 and a membrane anchored subunit F_1. We have identified a highly stable structure comprised of peptides derived from the F_1 heptad repeat A, which abuts the hydrophobic fusion peptide (peptide N-1), and the F_1 heptad repeat B, located 270 residues downstream and adjacent to the transmembrane domain (peptide C-1). Biochemical and biophysical and electron microscopic evidence indicates that these peptides formed a six-helix bundle that is extremely stable. We suggest that this α-helical trimer of heterodimer complex represents the core most stable form of the F protein that is either fusion competent or forms after fusion has occurred. The crystal structure of the core trimer at 1.4 Å resolution revealed a 96 Å long coiled coil surrounded by three antiparallel helices. This structure places the fusion and transmembrane anchor of SV5-F in close proximity with a large intervening domain at the opposite end of the coiled coil. Six amino acids, potentially part of the fusion peptide, form a segment of the central coiled coil, suggesting that this structure extends into the membrane. Deletion mutants of SV5-F indicate that putative flexible tethers between the coiled coil and the viral membrane are dispensable for fusion. The lack of flexible tethers may couple a final conformational change in the F protein directly to the fusion of two bilayers.

We have found that whereas the F protein of the W3A strain of SV5 causes syncytia formation without coexpression of HN, the F protein of the WR strain of SV5 requires coexpression of HN for fusion activity. The two strains differ by two amino acid residues at residues 22 and 443. There is a complex interrelationship between the differing residues. A mutational analysis indicates that some mutants have enhanced fusion kinetics with a faster rate and greater extent and a lower temperature of activation. In contrast other mutants do not cause fusion under physiological conditions but fusion can be activated at elevated temperatures. Although strain WR requires coexpression of HN to cause fusion at 37°C and does not cause fusion when coexpressed with influenza virus HA, at elevated temperatures coexpression of WR F protein with HA resulted in fusion activation. The SV5 F protein is presumed initially to exist in a metastable native fusion-inactive state; thus, the temperature dependence of F-mediated fusion suggests that the energy barrier for the conformational change from the metastable state to the fusion active conformation is lower for some mutants than for wt W3A. Conversely, the energy barrier for other mutants is much higher than for W3A F protein. Thus, these data suggest that prolines at residues 22 and 443 (the latter which maps on the core trimer) may destabilize the F protein and thereby decrease the energy required to trigger the presumptive conformational change to the fusion active state. Thus, the simplest explanation for the interaction of HN and F is that it causes a change in free energy to activate the F protein

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To obtain reagents that should discriminate between internal and external domains of the SV5 F protein and hence be capable of showing a rearrangement of domains upon a conformational change, we have generated a panel of F-specific anti-peptide sera derived from across the linear protein sequence. By using a modified F protein (F3R) whose cleavage can be regulated by addition of exogenous trypsin and the site-specific sera our data suggests there is a major rearrangement of the F protein on cleavage: 13 different antipeptide sera recognize F_0 but only three recognize cleaved F. Furthermore, the site-specific sera do not recognize the core trimer. Thus, taken together, these data indicate that the core complex is not present in the precursor molecule F_0, and that significant conformational changes occur subsequent to cleavage of the F protein.

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Rabies virus-induced membrane fusion pathway

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Rabies virus-induced membrane fusion is mediated by the viral transmembrane glycoprotein G which is organized in trimers (three monomers of 65 kDa each). Fusion is triggered at low pH, optimal around pH 5.8-6 and is not detected above pH 6.3. Fusion of rabies virus with liposomes is preceded by a lag time, the duration of which increases with lower temperature and higher pH (up to the pH threshold for fusion). Preincubation of the virus below pH 6.75 in the absence of a target membrane leads to inhibition of viral fusion properties. However, this inhibition is reversible and readjusting the pH to above 7 leads to the complete recovery of the initial fusion activity (1).

Low pH-induced conformational changes of the glycoprotein and their relationships with fusion activity have been studied. It has been demonstrated that G can assume at least three different states (1): the native (N) state detected at the viral surface above pH 7; the activated (A) hydrophobic state and the fusion inactive conformation (I). There is a pH-dependent equilibrium between these states which is shifted toward the I state at low pH. The A state is detected immediately after acidification, induces the formation of viral aggregates stabilized at low pH and low temperature and interacts with the target membrane as a first step of the fusion process. The domain of G involved in this interaction, the so-called fusion peptide, has been located between aminoacids 102 and 179 using hydrophobic photolabeling (2). Finally, the I state is detected after prolonged incubation at low pH. In the I conformation, G is longer than in the N conformation but also antigenically distinct and more sensitive to proteases.

Monoclonal antibodies were used to characterize the equilibrium between the different conformational states of G. These experiments have shown that the structural transition toward the I-state is associated with the cooperative binding of 3 protons, most probably on each of the three histidines 397 of the trimer. Furthermore, the comparison of the kinetics of the structural transition toward the I state with those of viral fusion inactivation indicated that 6 trimers constitute the minimal fusion complex.

Rabies virus-induced fusion pathway was also studied by investigating the effects of exogeneous lipids having various dynamic molecular shapes on the fusion process (3). Inverted cone-shaped lysophosphatidylcholines (LPCs) blocked fusion at a stage subsequent to fusion peptide insertion into the target membrane. Consistent with the stalk-hypothesis, LPC with shorter alkyl chains inhibited fusion at lower membrane concentrations and this inhibition was compensated by the presence of oleic acid. However, under suboptimal fusion conditions, short chain LPCs, which were translocated in the inner leaflet of the membranes, considerably reduced the lag time preceding membrane merging resulting in faster kinetics of fusion. This indicated that the rate limiting step for fusion is the formation of a fusion pore in a diaphragm of restricted hemifusion.

The cold-stabilized prefusion complex (1, 2) was also characterized. This intermediate is at a well-advanced stage of the fusion process when hemifusion diaphragm is destabilized

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but lipid mixing is still restricted, probably by a ring-like complex of 6 glycoproteins. This state has a dynamic character and its lipid organization can reverse back to two lipid bilayers (3).

These results are very similar to those observed with influenza virus (4, 5). Thus, despite important differences between their fusion machineries, membrane fusion probably follows a similar pathway for both viruses suggesting that the mechanism of membrane rearrangements leading to fusion is probably universal.

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Entry of vesicular stomatitis virus: Multiple regions of VSV G protein involved in cell fusion and virus entry.

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The transmembrane envelope glycoprotein G of the negative-stranded RNA containing vesicular stomatitis virus (VSV) is essential for the infectivity of the virus. Glycoprotein G contains a single transmembrane domain of 20 amino acids near the carboxy-terminus, a carboxy-terminal cytoplasmic tail of 20 residues and an ectoplasmic domain of 461 amino acids containing two N-linked oligosaccharide. Glycoprotein G expressed on the cell surface can induce membrane fusion at low pH and is essential for the entry of the virus into the host-cell via an endocytotic pathway. G protein unlike other viral fusion proteins does not require a post-translational cleavage event before becoming fusion active and the conformational change involved in fusion is reversible.

Studies from our laboratory have identified a highly conserved amino-terminal region (H2) spanning residues 123 to 137 of VSV G glycoprotein as an internal fusion peptide which, unlike other viral fusion peptides is not hydrophobic. A carboxy-terminal H10 region (residues 395 to 418), conserved in vesiculoviruses was identified as a domain controlling low-pH induced conformational change. The fusogenic activity of the G protein required membrane anchoring by a hydrophobic peptide sequence but specific amino acid sequence was not important. Recent experiments demonstrated that a membrane proximal domain of 11 amino acids spanning residues 451 to 461, which is highly conserved in vesiculoviruses and is unusually rich in tryptophan, is also required for fusion. Deletion or substitution of the 11 amino acids or mutagenesis of the conserved residues blocked the fusogenic activity without affecting transport or membrane anchoring ability. Recently coiled coil structures have been shown to be involved in both intracellular vesicle and viral membrane fusion. Analyses of VSV G protein sequence by a number of secondary structure programs, such as Coils, Multi-Coil, Pair-Coil or Learn-Coil, however, failed to predict the presence of any coiled-coil structure. The mechanism of membrane fusion induced by VSV G protein may, therefore, involve new principle(s).

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Structure-function of pneumovirus fusion protein

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Full-length fusion (F) protein of human respiratory syncytial virus (HRSV) and a truncated anchorless mutant (F_{TM}^-) lacking the C-terminal 50 amino acids were expressed from vaccinia recombinants and purified by immunoaffinity chromatography. Western blot of F and F_{TM}^- revealed three bands that contained the F_1 chain and that corresponded to the uncleaved (F_{1+2}) precursor, a partially processed product (F_{1+2t}) with the last 27 amino acids of the F_2 chain covalently bound to F_1 and the fully processed F_1 chain. The proportions of the three forms were $F_1 > F_{1+2t} > F_{1+2}$. Site-directed mutagenesis confirmed that Arg 108 and 109 were needed for generation of the F_{1+2t} product. These results suggest that proteolytic processing of the F protein precursor may involve cleavage at two different sites, eliminating a C-terminal segment of the F_2 chain from the mature protein.

Electron microscopy of the full-length F protein in the absence of detergents revealed micelles (i.e., rosettes) containing two distinct type of rods, one cone-shaped and the other lollipop-shaped. Analysis of anchorless F molecules showed individual cone-shaped rods with a low proportion of rosetted lollipop-shaped rods. Limited trypsin digestion of anchorless F, under conditions that cleaved F_{1+2} and F_{1+2t}, showed a transition from individual cone-shaped rods to rosetted lollipop-shaped rods. A similar transition from cones to lollipops was observed in the micelles of full-length F after limited trypsin digestion. These results indicate that the rods and lollipops may represent different conformations of the F molecule and that proteolytic processing may trigger transition from one conformation to the other. Complexes of F protein with antibodies of known specificity provided information on the three-dimensional organisation of both full-length F and membrane anchorless F.

Transfection of BSR T7-5 cells, which express constitutively the T7 polymerase, with plasmids carrying the F gene under a T7 promoter, was sufficient for induction of syncytia. Site-directed mutagenesis of the F gene is providing evidence of the structural requirements for membrane fusion. Data will be presented which link mutations at certain sites of the F molecule, particularly the proteolytic processing sites, with inhibition of the membrane fusion activity.

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Structure-function analysis of the Sendai virus glycoprotein cytoplasmic domain: a different role for the two proteins in virus particle production

Nathalie Fouillot-Coriou and Laurent Roux

The role of the cytoplasmic domain (cytd) of the Sendai virus HN and F glycoproteins in the process of virus assembly and budding are evaluated. Recombinant Sendai virus (rSeV) mutants are generated carrying modifications in the cytd of each of the glycoprotein separately. The modifications include increasing truncations and/or amino acid sequence substitutions. The virions in the cell supernatants are measure relative to the extent of the infection, assessed by the intracellular N protein signal. For both the F and HN ctd truncation mutants, the largest cytd deletions lead to a 20 to 50-fold reduction in virion production. This reduction cannot be explained by a reduction of the cell surface expression of the glycoproteins. For the F protein mutants, the virions produced in reduced amount always exhibit a normal F protein composition. It is then concluded that a threshold level of F is required for SeV assembly and budding. The rate or the efficiency with which this threshold is reached up appears to depend on the nature of the F cytd. A minimal cytd length is required, as well as a specific sequence. The analysis of HN protein mutants brings to light an apparent paradox. The larger cytd truncations result in significant reduction of virion production. On the other hand, a normal virion production can take place with an under representation of or, even, an undetectable HN in the particles. The HN uptake in virion is confirmed to depend on the previously proposed cytd SYWST signal (Takimoto et al., 1998).

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The fusion protein of Semliki forest virus

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Alphaviruses are enveloped viruses containing an external icosahedral T=4 glycoprotein shell which covers almost entirely the lipid bilayer. Glycoproteins E1 and E2 associate as trimers of a non-covalent E1/E2 heterodimer to form 80 spikes. The lateral interactions between these spikes at the viral surface result in a protein scaffold, called the "skirt", outside the viral membrane. While E2 has a receptor recognition function, E1 has an internal fusion peptide and is responsible for the low pH triggered membrane fusion step during entry. Our preliminary crystallographic results on the ectodomain of E1 (residues 1 to 390) from Semliki Forest Virus (SFV) show that its overall fold is remarkably similar to the the fold of the fusion glycoprotein of flaviviruses. Inspection of the recently published 9A resolution cryo-EM reconstruction of SFV (Mancini et al, 2000) reveals that the long rod shaped E1 monomer fits in density corresponding to the skirt, lying horizontally about the quasi-twofold (Q2) axes that relate adjacent spikes in the viral surface. The association of E1 monomers about Q2 axes results in head-to-tail dimers strikingly reminiscent of the flavivirus fusion protein dimer found by X-ray crystallography (Rey et al, 1995). The overall distribution of these 120 E1 dimers in the alphavirus surface is also similar to the T=3 model (containing 90 dimers) propósed for the flavivirus surface (Ferlenghi et al, 2000). Our data thus suggest that the fusion proteins of flaviviruses and alphaviruses belong to a different class of fusion proteins which "lie down" as dimers on the viral membrane surface at neutral pH and re-associate into a trimeric fusogenic form at low pH.

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Session 2: Triggering of viral mediated membrane fusion
Chair: Hugh R.B. Pelham

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The thermostability of influenza haemagglutinin and its biosynthetic precursor in relation to membrane fusion

Skehel JJ,Stevens D,Calder L,Wharton S,Steinhauer D, Wiley DC.

The subunits of the Influenza haemagglutinin trimer are biosynthesized as precursors that are proteolytically cleaved following trimerization, into two disulphide-linked polypeptides. Cleavage, which is required for membrane fusion, results in haemagglutinin molecules that are primed for subsequent activation of their fusion potential at low pH in endosomes. Activation can also result *in vitro* from incubation at elevated temperature at neutral pH; 62 °C for X31 HA that is activated at pH 5.6 at 37C.These observations of the temperature dependence of fusion led to analyses of the relative thermostabilities of primed and activated HAs which have now been extended to comparisons with the thermostability of uncleaved precursor HA0. Heat induced changes in structure monitored by fluorescence spectroscopy, CD, EM, and proteolytic susceptibility,will be described and compared with those that occurr at fusion pH at physiological temperature.

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Mutagenesis studies on the influenza HA fusion peptide

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Membrane fusion mediated by the influenza virus hemagglutinin (HA) occurs following structural rearrangements of the molecule that are triggered by the acidification of endosomes. These conformational changes function to relocate the highly conserved N-terminus of the HA2 subunit toward the endosomal membrane. Subsequent interactions of this "fusion peptide" domain with the target membrane are generally regarded as being critical for fusion. In common with functionally homologous domains of many other viral and cellular fusion proteins, the HA fusion peptide contains a number of large hydrophobic residues and several interspersed glycine residues. We have generated a large panel of HA mutants with changes to the first 10 residues of HA2 in attempts to determine the requirements for fusion of specific amino acids at particular positions, and to analyze the relevance of the spacing among glycines and large hydrophobic residues in this region. These mutants were analyzed functionally and biochemically using expressed proteins, and the infectivity and growth characteristics of mutant influenza viruses generated by reverse genetics was also assessed. The fusion activity and infectivity of several mutants was found to be severely restricted, despite the observation that the HAs were capable of undergoing acid-induced conformational changes and associate with target membranes. We also observed that, without exception, every mutation to residues within the 10 N-terminal HA2 positions resulted in an elevated pH at which conformational changes were induced. This suggests that selective pressure to maintain the stability of the native HA structure as well as the capacity for fusion may be operating on this highly conserved domain.

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Modular organization of the Friend murine leukemia virus envelope protein: implications for the mechanism of infection

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The modular organization of the envelope protein of C type retroviruses has been exploited to investigate how binding of the surface subunit (SU) to receptors on susceptible cells triggers fusion mediated by the trans-membrane subunit (TM). We have shown that infection by Friend murine leukemia virus (Fr-MLV) is abolished by deletion of RBD, the domain in SU that binds to receptor. Infection is restored, however, by supplying RBD as a soluble protein. This demonstrates that a fusion-competent envelope protein can be assembled in the absence of RBD, indicating that this domain is not a suppressor of the fusion mechanism that is inactivated by receptor binding. Fr-MLV that expresses an envelope protein in which the hormone, erythropoietin (Epo), has been inserted in place of RBD is able to attach to cells that express the Epo receptor, but infection occurs only when soluble RBD is added and only on cells that express the receptor for RBD. These experiments indicate that upon binding to receptor, RBD establishes a functional interaction with the remainder of SU and/or TM that activates fusion. Based on similarities in the organization and function of the envelope proteins of other viruses, including influenza, Ebola, HIV and Sendai, we speculate this mechanism of receptor-dependent infection has been widely adopted.

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A hypothesis on coiled coil protein mediated membrane fusion

Dr. Joe Bentz

The hypothesis that formation of the extended coiled coil by HA creates a hydrophobic defect in the viral envelope provides a simple tranduction of the energy released by the formation of the coiled coil to the energy needed to create and stabilize the high energy intermediates of fusion (Bentz, 2000b). The same model can apply to the SNARE proteins and predicts a natural asymmetry between the requirements of the v- and t-SNARE anchors. The data of Melikyan et al. (1995), for the kinetics of first fusion pore formation between HA expressing cells and planar bilayers containing gangliosides, has been analyzed using a comprehensive kinetic model for fusion (Bentz, 2000a). The committed step in influenza HA mediated fusion begins with an aggregate of at least 8 HAs, called the fusogenic aggregate. The slow conformational change of 2 or 3 of these HAs yields the formation of the first fusion pore.

It was proposed for the HAs in the fusogenic aggregate that some of their HA2 N-termini, aka fusion peptides, are embedded into the viral bilayer. The HAs in the fusogenic aggregate are packed so closely that lipids from the viral envelope are restricted from diffusing into the center of the aggregate. The conformational change to the extended coiled coil extracts the fusion peptides from the viral bilayer. When this extraction occurs from the center of the site of restricted lipid flow, it exposes acyl chains and parts of the HA transmembrane domains, i.e. a hydrophobic defect is created. This would be the "transition state" of the committed step of fusion. The defect is stabilized by a "dam" of HAs, which are inhibited from diffusing away by the rest of the HAs in the fusogenic aggregate. Recruitment of lipids from the apposed target membrane can heal this hydrophobic defect, initiating lipid mixing and fusion.

We have probed the structure of the fusion site further using the data of Danieli et al. (1996) for lipid mixing between HA expressing cells and R18 labeled RBCs using the kinetic model described in Bentz (2000a). The results are compared with those of the first fusion pore kinetics. The minimal fusion unit, i.e. the number of HA trimers at the fusion site which must undergo the essential conformational change slowly, is 2-3 for both sets data. However, the ratio of fusogenic aggregates for the same pair of cell lines was =10-fold less for the lipid mixing data. This can be explained by accumulation of HAs bound to sialates on glycophorin within the area of apposition between a RBC and HA expressing cell. The number of fusogenic aggregates, each composed of both bound and free HA, increases with this accumulation. Using the known HA1-sialate binding constant to estimate the glycophorin-HA binding constant yields that the ratio of fusogenic aggregates would be reduced by the amount predicted from these data. The binding of HA to gangliosides in the planar bilayer is predicted to be much weaker, likely due to the closeness of the sialate to the bilayer surface. Remarkably, the number of unbound HA per fusogenic aggregate is equal to the minimal fusion unit. Evidently, the unbound HAs within the fusogenic aggregate create the initial defect via the conformational change to the extended coiled coil, while the other HAs, whether bound or free, stabilize the defect.

It is clear that both viral and intracellular membrane fusion proteins contain a minimal set of domains which must be deployed at the appropriate time during the fusion process. The

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model proposed in Bentz (2000b) has been extended in Bentz & Mittal (2000) to identify these domains and their functions for the four best described fusion systems: influenza HA, sendai virus F1, HIV gp120/41 and the neuronal SNARE core composed of synaptobrevin (syn), syntaxin (stx) and the N- and C- termini of SNAP25 (sn25), together with the Ca2+ binding protein synaptotagmin (syt). By comparing these domains, we have proposed a minimal set which appears to be adequate to explain how the conformational changes can produce a successful fusion event, i.e. communication of aqueous compartments.

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Membrane rearrangements and protein refolding in influenza hemagglutinin mediated fusion

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To elucidate the pathway of hemagglutinin (HA)-mediated fusion we dissect it into a sequence of distinct membrane structures and protein conformations (Chernomordik et al., 1998). In (Leikina and Chernomordik, 2000) to study early fusion intermediates at physiological temperature we slowed down the formation of advanced fusion intermediates by decreasing the number of low pH-activated HA molecules. We identified a new fusion intermediate, with the set of properties expected from restricted hemifusion. While lipid flow between the membranes was restricted, the contacting membrane monolayers were apparently transiently connected as detected by the transformation of this fusion intermediate into complete fusion with treatments known to destabilize the hemifusion diaphragm. These reversible connections disappeared within 10-20 min after low pH application indicating that after the energy released by HA refolding dissipated, the final low pH conformation of HA did not support membrane merger. Since formation of the transient restricted hemifusion sites at physiological temperature was as fast as a fusion pore opening and required less HA, we hypothesize that fusion starts with formation of multiple RH sites, only few of which then evolve to an expanding fusion pore.

Lipid flow between membranes in restricted hemifusion intermediates and in initial fusion pores is apparently hindered by multiple low pH-activated hemagglutinins. To address the possible role of HA interactions in fusion we studied the effects of the surface density of HA molecules on the percentage of HA activated at given pH. We found that the rate of low pH activation of HA considerably increases with the surface density of HA, suggesting that HA activation is facilitated by either direct or indirect trimer-trimer interactions. Cooperativity of HA activation can facilitate formation of functional multiprotein complexes at a stage of actual fusion. Our results are consistent with the hypothesis that HA-mediated fusion proceeds through the same membrane structures as fusion of protein-free bilayers (Chernomordik et al., 1995). Formation of these intermediates, stalks and pores, apparently involves bending of membrane lipid monolayers within a ring-like complex of activated fusion proteins.

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The formation of the six-helix bundle of HIV-1 gp41 requires membrane merger. Fredric S. Cohen[1], Ruben M. Markosyan[1], Mary K. Delmedico[2], Dennis M. Lambert[2], Grigory B. Melikyan[1]. [1]Rush Medical College, Chicago, IL 60612; [2]Trimeris Inc., Durham, NC 27707.

Many viral fusion proteins exhibit six-helix bundles. There is little question that these bundles are critical to the fusion process, but the precise role they play is not known. The creation of the bundles could bring membranes into close contact, but not participate further in the process. In this case, either the protein structure directly mediates fusion or additional protein conformational changes induce fusion. Alternatively, bundle formation and fusion could occur virtually simultaneously. If this is the case, the movement of the protein into the bundle would cause hemifusion and/or fusion. We are using Env of HIV-1 to address the role of the bundle in the fusion process.

Effector cells expressing Env were fused to target cells expressing CD4 and CXCR4 chemokine receptors. Fusion was monitored by spread of aqueous and lipid fluorescent dyes between effector and target cells or by electrical capacitance measurements. A temperature-arrested stage (TAS) of fusion was established by binding the effector and target cells together for several hours at 23°C. Fusion was significantly faster when temperature was raised to 37°C from TAS than when raised immediately upon binding the cells. At this intermediate of fusion, Env had undergone its CD4-dependent conformational changes but had not yet completed the CXCR4-dependent ones. It is well known that binding of the peptides T20 and T21 to Env prevent gp41 from forming a six-helix bundle and that, for fusion systems in general, incorporation of lyso-phosphatidylcholine (LPC) in outer leaflets prevents hemifusion. The six-helix bundle had not yet formed by TAS, but the peptide binding sites had become exposed. As expected, by incorporating LPC in membranes after creating TAS, fusion did not result upon raising temperature to 37°C. The effect of LPC was reversible: lowering temperature and washing out the LPC led to fusion when temperature was again increased. But the six-helix bundle had not formed at the LPC arrested stage: adding T20 or T21 after washing out LPC prevented fusion when temperature was raised. Because LPC prevents hemifusion, these results indicate that the bundle cannot form without membrane merger. Independent experiments showed that if, after creating TAS, 37°C was maintained for times too short to induce fusion, the addition of T20 or T21 still prevented subsequent fusion. This indicates that the bundle does not form until the fusion pore is created. If bundle formation is essential for pore formation, it follows that membrane merger and formation of the six-helix bundle are tightly coupled processes, occurring simultaneously.

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Session 3: Vesicle fusion
Chair: John J. Skehel

Instituto Juan March (Madrid)

A general principle for intracellular membrane fusion

James E. Rothman

Membrane-enveloped vesicles travel among the compartments of the cytoplasm of eukaryotic cells, delivering their specific cargo to programmed locations by membrane fusion. The pairing of vesicle v-SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) with target membrane t-SNAREs has a central role in intracellular membrane fusion. We have tested all of the potential v-SNAREs encoded in the yeast genome for their capacity to trigger fusion by partnering with t-SNAREs that mark the Golgi, the vacuole and the plasma membrane. Here we find that, to a marked degree, the pattern of membrane flow in the cell is encoded and recapitulated by its isolated SNARE proteins, as predicted by the SNARE hypothesis".

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SNAREs and exocytosis in the pancreatic acinar cell

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In the pancreatic acinar cell, digestive enzymes are packaged in a condensed form into zymogen granules (1). Secretagogue stimulation of the cell causes an elevation in intracellular Ca^{2+} concentration (2), which in turn triggers fusion of the granules with the apical domain of the plasma membrane and secretion of the granule contents. Despite extensive study, the mechanism underlying zymogen granule exocytosis is still far from clear; however, some information is beginning to emerge about the proteins that mediate the exocytotic membrane fusion event.

We have developed an *in vitro* assay in which isolated zymogen granules fuse with pancreatic plasma membranes and with each other. Both fusion events are Ca^{2+}-dependent, although they have different Ca^{2+}-sensitivities. By exploiting the ability of botulinum toxin C1 to cause specific cleavage of the Q-SNARE syntaxin, we have shown that syntaxin 2, present on the apical plasma membrane, is essential for fusion between the zymogen granules and the plasma membrane, while syntaxin 3, on the granule membrane, mediates homotypic fusion between granules (3). Surprisingly, complete cleavage of the zymogen granule R-SNARE synaptobrevin 2 by tetanus toxin had only a minor effect on fusion.

An attempt to identify novel syntaxin binding partners on the zymogen granule membrane led to the isolation of a 16-kDa protein, named syncollin. Syncollin binds to syntaxin in a Ca^{2+}-sensitive manner, and recombinant syncollin inhibits zymogen granule-plasma membrane fusion *in vitro* (4). Further, syncollin binds most efficiently to syntaxin 2, the apical membrane Q-SNARE. In light of these observations we proposed that syncollin was involved in the control of exocytotic membrane fusion in the pancreatic acinar cell. More recently, we have shown that syncollin has an N-terminal signal sequence that directs its translocation across the membrane of the endoplasmic reticulum (5). The zymogen granule contains two pools of syncollin: one free in the lumen and the other bound to the luminal surface of the granule membrane. Membrane-bound syncollin is resistant to salt-washing of the granule membranes, but is removed by sodium carbonate. These results call into question the likely function of syncollin, and in particular the significance of its interaction with syntaxin.

Removal of cholesterol from the granule membrane by treatment with methyl-β-cyclodextrin causes the detachment of syncollin, and this effect is enhanced at a high salt concentration. Purified syncollin is able to bind to brain liposomes at pH 7.6, but not at pH 11.0, a condition that also causes its extraction from granule membranes. Syncollin binds only poorly to dioleoylphosphatidylcholine liposomes, but binding is dramatically enhanced by the inclusion of cholesterol.

In taurodeoxycholate extracts of zymogen granule membranes, syncollin behaves as a 120-kDa oligomer. Imaging of purified syncollin by negative-stain electron microscopy reveals doughnut-shaped structures of outer diameter approximately 10 nm. The doughnuts are

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reminiscent of structures found previously in the zymogen granule membrane by freeze-fracture, which were proposed to represent pores. We tested the idea that syncollin has pore-forming properties by adding purified syncollin to liposomes that had been loaded with a self-quenching concentration of carboxyfluorescein. We found that syncollin caused a concentration-dependent de-quenching, as a consequence of leakage of the probe from the liposomes. The ability of syncollin to permeabilize the liposomes was abolished by boiling the protein, and by raising the incubation pH to 11.0, conditions under which syncollin cannot bind to liposomes.

We conclude that syncollin is able to interact directly with membrane lipids, and to insert into the granule membrane in a cholesterol-dependent manner. Further, we suggest that syncollin is able to penetrate the membrane completely, to form trans-membrane pores. If this is the case, then it may after all interact with syntaxin within the acinar cell.

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Membrane traffic between endosomes and the Golgi apparatus

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The small genome and relatively simple endomembrane system of yeast means that a complete description of the membrane trafficking pathways in this organism is achievable. The complete set of SNAREs is known, as are the Ypt (rab) GTPases and many other components that contribute to vesicle docking and fusion events.

Seven distinct sites of specific budding and/or fusion can be defined, namely the ER, early Golgi, late Golgi, plasma membrane, early endosomes, late endosomes and vacuoles. Typically, each contains a different member of the syntaxin family of SNAREs. However, these SNAREs, like other integral membrane proteins, cycle between different compartments and thus do not have an absolutely defined location.

The cycling of proteins makes it particularly difficult to distinguish early endosomes from the late Golgi, since it appears that all membrane proteins that are considered late Golgi "residents" in fact cycle through early endosomes. Furthermore, these two compartments share a single syntaxin, Tlg2p. Perhaps because of this, early endosomes have only recently been recognised as distinct organelles (1). They are defined functionally as the site from which certain endocytosed proteins such as the SNARE Snc1p are retrieved from the endocytic pathway.

Given the cycling of SNAREs, how do vesicles choose their targets correctly *in vivo*? It is likely that this is achieved not by integral membrane proteins but by peripheral proteins that recognise particular features of the organelles, such as their content of phosphorylated lipids.

Ypt6p is a GTPase that appears to be present on both early endosome and late Golgi membranes, and is required for endosome-Golgi traffic. By genetic and biochemical studies we have identified a complex of two peripheral membrane proteins, Ric1p and Rgp1p, which form a specific nucleotide exchange factor for Ypt6p (2). This complex is restricted to Golgi membranes, and thus ensures that Ypt6p is activated only there. We have also identified two proteins that bind specifically to the GTP form of Ypt6p. These too are peripheral membrane proteins and indirect evidence suggests that at least one of them is present on endosomes. Thus, we hypothesise that docking of endosome-derived vesicles with the Golgi is mediated by the interaction of activated Ypt6p on the Golgi with an effector on the vesicles, and that this is followed by SNARE engagement and membrane fusion.

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Yeast exocytic SNAREs

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Exocytosis in yeast requires assembly of the secretory vesicle v-SNAREs Snc1p or Snc2p and the plasma membrane t-SNAREs Sec9p and Sso1p or Sso2p into a SNARE complex. Mutations that block either secretory vesicle delivery or tethering prevent SNARE complex assembly. By contrast, wild-type levels of SNARE complexes persist in the sec1-1 mutant after a secretory block is imposed, suggesting a role for Sec1p after SNARE complex assembly. In the sec18-1 mutant, cis-SNARE complexes containing HA-Snc2p accessible to surface iodination accumulate in the plasma membrane. Thus, one function of Sec18p is to disassemble SNARE complexes on the post-fusion membrane. High-level expression of mutant Snc1 or Sso2 proteins that have a C-terminal geranylgeranylation signal instead of a transmembrane domain inhibits exocytosis. The mutant SNARE proteins are membrane associated, correctly targeted, assemble into SNARE complexes, and do not interfere with the incorporation of wild-type SNARE proteins into complexes. Mutant SNARE complexes recruit GFP-Sec1p to sites of exocytosis and can be disassembled by the Sec18p ATPase. Heterotrimeric SNARE complexes assembled from both wild-type and mutant SNAREs are present in heterogeneous higher-order complexes also containing Sec1p which sediment at greater than 20S. Based on a structural analogy between geranylgeranylated SNAREs and the GPI-HA mutant influenza virus fusion protein, we propose that the mutant SNAREs are fusion proteins unable to catalyze fusion of the distal leaflets of the secretory vesicle and plasma membrane. Mutation of a methionine-based sorting signal in the cytoplasmic domain of either Sncp inhibits Sncp endocytosis and prevents recycling of Sncp to the Golgi after exocytosis. snc1-M43A mutant yeast have reduced growth and secretion rates and accumulate post-Golgi secretory vesicles and fragmented vacuoles. However, cells continue to grow and secrete for several hours after de novo Snc2p or Snc2-M42Ap synthesis is repressed. Thus, v-SNARE recycling facilitates membrane fusion, but may not be essential.

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Pathway of SNARE complex assembly

Dirk Fasshauer

Assembly of SNARE proteins between two opposing membranes into ternary complexes is thought to be a key event in intracellular membrane fusion. *In vitro*, complex forming domains of the individual SNARE proteins are largely unstructured. Complex formation is associated with major conformational rearrangements and an increased thermal stability. The central domain of the ternary synaptic SNARE complex consists of an extended, parallel four-helix bundle. To this bundle synaptobrevin and syntaxin each contribute a single and SNAP-25 two helices. Syntaxin and SNAP-25 can form a "binary" complex consisting of two syntaxins and one SNAP-25. This binary complex consists of a four-helix bundle similar to the ternary complex, where the second syntaxin occupies the position of synaptobrevin. To investigate the molecular events and energetics of the assembly reaction in more detail, thermodynamic and kinetic studies on these complexes were carried out. The ternary SNARE complex unfolds at temperatures above 80°C. In contrast, refolding occurs only below 65°C. A similar hysteresis between ternary complex formation and unfolding was observed in the presence of denaturant. Hence, it is likely that the pathways for ternary SNARE complex formation and unfolding are different. Interestingly, the binary complex formed by SNAP-25 and syntaxin is less stable and reversibly unfolds at temperatures around 45 °C. In addition, kinetic data imply that successful interaction of syntaxin and SNAP-25 must precede to allow for ternary complex formation. Together, these data suggest that the binary complex is an intermediate for ternary complex formation.

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Control of the terminal phase of vacuole fusion

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Fusion of intracellular membranes requires inital recognition and docking of the membranes and subsequent bilayer mixing. Most components identified so far kinetically map to the early binding and recognition events (1). Using *in vitro* fusion of yeast vacuoles as a model reaction we have identified a Ser/Thr protein phosphatase as a novel fusion factor associated with the vacuolar membrane. The phosphatase is essential for life. In contrast to SNARE complexes, whose function in a physiological membrane is restricted to earlier phases of the reaction, the phosphatase acts after recognition and binding of the membranes to each other, very close to or during bilayer/contents mixing (2). It is essential not only for membrane fusion at the vacuole but also for ER to Golgi transport and for endocytic trafficking. The protein is part of a high molecular weight complex which does not cofractionate with SNAREs but contains calmodulin. Calmodulin serves as Ca2+ receptor for an efflux of luminal calcium which is triggered by the docking event (3). We hence postulate that there is a universal apparatus controlling the final step of fusion - bilayer mixing - which is distinct from SNARE complexes. We propose that calmodulin and the Ser/Thr phosphatase are constituents of this apparatus. Further components of this complex are being identified and will be presented.

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Do presynaptic pla2 neurotoxins promote the fusion of synaptic vesicles at nerve terminals?

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Snake presynaptic neurotoxins with phospholipase A2 activity block nerve terminals in an unknown way. They induce the release of acetylcholine followed by blockade of the terminal, which degenerates. carateristically, the entire plasma membrane of the terminal poisoned by these neurotoxins is decorated with clathrin-coated omega-shaped invaginations. We propose a novel hypothesis to explain the mechanism of action of these snake toxins. They are suggested to enter the lumen of synaptic vesicles, following endocytosis, and hydrolyse phospholipids of the inner leaflet of the membrane. The trans-membrane pH gradient promotes the translocation of fatty acids to the cytosolic monolayer, leaving lysophospholipids on the lumenal layer. Such vesicles are highly fusogenic and release neurotransmitter upon fusion with the presynaptic membrane, but cannot be retrieved because of the high local concentration of fatty acids and lysophospholipids, which prevents vesicle neck closure, giving rise to the omega-shaped membrane structures.

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Session 4: Protein-lipid and lipid-lipid interactions
Chair: Don C. Wiley

Instituto Juan March (Madrid)

Poly(ethylene glycol)-mediated vesicle fusion: A mechanism in common with exocytotic and viral fusion

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Protein machines and lipid bilayers both play central roles in cell membrane fusion, a process crucial to life. My lab models the role of lipids in fusion by examining the behavior of model membrane vesicles brought into close inter-vesicle contact by poly(ethylene glycol) [PEG] (*3*). I summarize key observations and propose a common mechanism by which very different fusion machines (from lipid-enveloped viruses and synaptic vesicles) may function to produce compartment-joining pores. This mechanism presumes that protein fusion machines use stored conformational energy to assemble closely juxtaposed lipid bilayers, bend these to fusion-competent structures, stabilize unfavorable lipid structures, and destabilize a committed intermediate to drive fusion pore formation. Our studies aim to define changes in the lipid structures that occur during the fusion process and how these structural changes might be facilitated by a protein fusion machine.

Our most recent progress has been in three areas:

1] We have identified a mix of lipids that optimizes the fusion capacity of membrane vesicles. The particular mix of phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and cholesterol (CH) that optimizes PEG-mediated fusion of small, unilamellar vesicles turns out to be roughly the same mix seen in synaptic vesicles. The role of each lipid component of this mix will be discussed.

2] The sequence of lipid structural changes that accompany fusion in small, unilamellar vesicles has been defined. Rapid mixing of lipids between the outer leaflets of contacting lipid bilayers is the fastest process observed. Minor leakage of aqueous contents between fusing compartments accompanies this event. Directed lipid movement from hemi-fused outer leaflets to unfused inner leaflets is the next process we detected. The final stage of the process was defined by extensive mixing of vesicle trapped aqueous contents and mixing of the inner leaflets of fusing bilayers. This series of events agrees with that previously reported for less highly curved vesicles (*1, 2*), although the intermediates were less stable and thus less well defined in the most highly curved membranes.

3] We have examined the influence of various forms of membrane stress (curvature, osmotic, and hydrophobic perturbant) on PEG-mediated fusion. A negative osmotic gradient (high osmotic pressure outside) favored fusion, while a positive gradient inhibited fusion. The effect of a negative gradient was not due to induction of curvature in osmotically distorted vesicles. Hydrophobic perturbants largely overcame the

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inhibition due to a positive osmotic gradient. We have interpreted these results by calculating the free energies of the structures our results suggest are present during the fusion process. We propose that both hydrophobic perturbants and a negative osmotic gradient lower the free energy of hydrophobic interstices that dominate the free energy of fusion intermediates. Finally, we have examined the ability of different fluorescent probes to detect interstices and have used one of these to follow interstice build-up and disappearance during the fusion process.

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Membrane fusion induced by phospholipases C

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In the past years we have described the fusion of lipid vesicles induced by: bacterial PC-preferring phospholipase C (1), sphingomyelinase (2), a combination of phospholipase C and sphingomyelinase (3), and, more recently, by PI-specific phospholipase C (4).

The purpose of this presentation is threefold: (a) to review the common aspects of the above fusion processes, (b) to explore possible underlying similarities between phospholipase-induced and virus-induced fusion, and (c) to put these findings in the context of the physiological processes of membrane fusion.

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Target-membrane partitioning regions within ectodomains of viral fusogenic envelope glycoproteins

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Fusion mediated by envelope viral proteins is an example of a process driven by highly regulated protein-lipid interactions. We suggest here that the initial interaction of the viral fusion protein with the target cell membrane can be analyzed in terms of a partitioning process of the protein ectodomain between the aqueous phase and the outer lipid monolayer of the target membrane. In order to analyze in a quantitative way the energetics of partitioning of prototypical ectodomains into membranes we have adopted a novel approach, namely the evaluation of the peptide interfacial hydrophobicity. This approach is based on experimental measurements of the free energy of partitioning into membrane interfaces of the individual amino acids as determined by Wimley and White (reviewed in: 1).

Interfacial hydrophobicity analysis of the primary structure of several envelope products reveals the presence within protruding ectodomains of two membrane-partitioning regions, namely, the "fusion peptide" (2) and the "pretransmembrane" stretch (3), separated by a collapsible intervening sequence (4).

The partitioning energetics of known secondary structure elements can also be analyzed taking into account the per-residue free energy change upon peptide folding (5). According to our free energy computations, protein-membrane complex formation would be favored against formation of coiled-coil structures by N-terminal heptad domains.

Finally, several proposed mechanisms for ectodomain-target membrane interactions will be discussed in view of the partitioning free energy distribution within three dimensional structures determined by X-ray crystallography. We conclude that the local organization and spatial distribution of membrane partitioning regions within viral fusion protein ectodomains might represent common structural motifs related to the fusogenic function.

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Molecular mechanisms of membrane trafficking and exocytosis

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The organization of membrane compartments:

If SNARE complex formation is indeed the driving force behind membrane fusion, perhaps the specificity of vesicle trafficking is, at least in part, determined by the selective pairing of SNAREs localized to particular membranes (Söllner et al., 1993; Bennett and Scheller, 1993). If this hypothesis is true, several criteria should be met. First, there should be a large number of SNARE proteins expressed in cells and second, they should be specifically localized to the various membrane compartments within cells. Third, the various SNARE proteins should form specific sets of complexes consistent with the known membrane trafficking pathways within cells. We have initiated a series of studies to rigorously test this hypothesis.

The first step is to determine the number and organization of SNAREs in mammalian cells. Since the number of SNARE proteins has turned out to be quite large, this has been a labor-intensive, although interesting, project. We have used several approaches to characterize SNARE proteins. The first approach makes use of an observation from the nervous system that reveals immunoprecipitation of a particular SNARE results in the coprecipitation of interacting proteins. This approach was used to characterize many of the SNAREs important in vesicle trafficking between the ER and the Golgi (Hay et al., 1997). Another approach has been to define potentially new SNARE proteins through the expressed sequence tag database. In this approach, we use EST sequence information to define predicted proteins and then, through epitope tagging and generation of specific antibodies, characterize the protein (Advani et al., 1998; Steegmaier et al., 1998). We have now defined the localization of numerous SNARE proteins within the secretory pathway of mammalian cells at the light and electron microscopic levels. We certainly conclude at this stage that many, if not all, membrane compartments have a distinct composition of SNARE proteins consistent with their having important roles in determining the specificity of membrane compartment organization. We are also actively engaged in studying the function of these proteins using permeabilized cell systems. Recently we have been successful in devising systems to study the recycling of transferrin receptors and the degradation of EGF as probes of the endocytic membrane trafficking pathways within cells (Prekeris et al., 1998).

Now that we have characterized many SNARE proteins, we are in a position to ask if their binding to each other is consistent with the known trafficking pathways within cells. We demonstrated that 21 pairs of complexes formed *in vitro* and that they were all of high, although somewhat variable, thermal stability. From these studies, we conclude that the information for the specificity of membrane fusion is not defined by the ability of the proteins to form specific complexes (Yang et al., 1999). This does not rule out a role for the SNAREs in determining the specificity of membrane fusion events, nor do we conclude that SNARE pairing is not specific *in vivo*. We simply conclude that the information for the specificity is likely not encoded by the ability of the proteins to form complexes.

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Membrane fusion complexes in exocytosis and viral entry

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The wave of intracellular calcium at fertilization causes ~15,000 cortical secretory vesicles to exocytose. An *in vitro* preparation of these vesicles and the plasma membrane fuse in response to calcium. The vesicles are all docked and fully primed. By combining physiological and molecular approaches, we have characterized the final steps of calcium-triggered exocytosis in sea urchin eggs. The non-linear relationship between the free calcium concentration and the rate of exocytosis can be explained solely by the calcium-dependence of the distribution of active fusion complexes at vesicle docking sites. The properties of this active complex are compared with the properties of the heterotrimeric SNARE protein complex that is present in the cortical vesicle system. SNARE proteins have multiple trypsin cleavage sites, we can test whether trypsin-specfic loss of one or more of the SNAREs correlates with the loss of Ca^{2+} sensitivity or fusion. Increasing concentrations of trypsin produced a progressive rightward shift in the curve of the Ca^{2+} sensitivity of fusion and a gradual decline in the extent of fusion, eventually resulting in a complete block of triggered fusion. The loss of the SNARE proteins did not correlate with the loss of fusion, rather with calcium sensitivity. Thus, although the SNAREs may be necessary for optimal Ca^{2+} sensitivity, they are not sufficient to catalyze membrane fusion. Another trypsin-sensitive protein must be essential for calcium-triggered membrane fusion other than the SNAREs. The SNARE complex appears to promote the calcium sensitivity of fusion, possibly by defining or delimiting a localised, focal membrane fusion site that ensures rapid and efficient exocytosis *in vivo*.

In viral fusion, we can visualize a contact site mediated by the ectodomain of influenza HA. Membrane fusion intermediates induced by HA and lipid-anchored HA (GPI-HA) were investigated by rapidly frozen, freeze-substitution, thin section electron microscopy, and with simultaneous recordings of whole-cell admittance and fluorescence. Upon triggering, the previously separated membranes developed, when viewed by electron microscopy, numerous hourglass shaped contact sites comprised of both membranes (~10-130 nm waist). Stereo pairs showed close membrane contact at peaks of complementary protrusions, arising from each membrane. With HA, there were fewer contacts, with wide fusion pores. Physiological measurements showed fast lipid dye mixing between cells after acidification, and either fusion pore formation or the lack thereof (true hemifusion, more often for GPI-HA). Our findings are consistent with a pathway wherein conformational changes in the ectodomain of HA pulls membranes towards each other to form a contact site, then hemifusion and pore formation initiate in a small percentage of these contact sites. Finally, the transmembrane domain of HA is needed to complete membrane fusion for macromolecular content mixing.

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Interactions of the SFV internal fusion peptide with membranes. Role of membrane composition and protein environment

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The alphavirus Semliki forest (SFV), penetrates into cells trough receptor-mediated endocytosis. Exposure to the low pH present in the lumen of late endosomes promotes fusion of virions with the membrane of these organelles following a yet unresolved mechanism. The putative fusogenic subdomain of the SFV El spike protein is an internal peptide including residues 75DYQCKVYTGVYPFMWGGAYCFCD97. This sequence is predicted to partition into membranes and adopt a characteristic topology on the basis of the Wimley-White scales of free energies of transfer from water to membrane-interfaces and to octanol [Wimley, W. C., and White, S. H. (1996) Nature Struct. Biol. 3, 842-848]. A synthetic peptide representing this region penetrates into membranes and destabilizes them at acidic pH 5.5 but not at neutral pH 7.4. A recombinant fragment comprising El residues 1-151 also interacts with membranes depending on the pH. Synthetic fusion peptides and recombinant fragments containing the G91D substitution, known to abrogate E1-induced fusion activity, lack the ability to interact with and perturb membranes. We conclude that the predicted SFV fusion peptide may spontaneously interact with membranes and that this ability may be indeed expressed within the context of the neighbouring E1 sequences.

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The role of different signalling pathways in acetylcholine-induced zymogen granule exocytosis

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Secretion in rat pancreatic acinar cells induced by 6 min applications of acetylcholine (ACh) can be sub-divided into an initial burst, probably representing the fusion of docked vesicles, and a slower phase, that can result from the translocation of vesicles from an intracellular pool (Campos-Toimil et al., J. Physiol., in press). Here we investigate the effects of 2,3-butanedione monoxime (BDM, a myosin ATPase inhibitor), SB 203580 (SB, a p38 MAP kinase inhibitor) and bisindolylmaleimide I hydrochloride (BIS, a PKC inhibitor) on ACh-evoked secretion. Quantification of zymogen granule exocytosis was done by time-differential analysis of digital images. Changes in [Ca2+]i were measured by imaging fura-2 fluorescence. We also investigated the effects of these drugs on the agonist-induced redistribution of actin filaments using FITC-phalloidin staining. BDM and SB inhibited the second phase of the exocytotic response to ACh, while BIS was without effect. Both SB and BIS reduced the plateau of ACh-induced changes in [Ca2+]i, but BDM had no effect. Interestingly, BDM both inhibited the redistribution of actin filaments after stimulation with ACh and slightly delayed the exocytotic burst. In conclusion, the results with BDM imply a direct role for myosin ATPase in the final stages of exocytosis in rat pancreatic acinar cells, while the effects of SB may support a role for p38 MAP kinase in the mobilization of a reserve pool of zymogen granules. In contrast, the lack of effect of BIS suggests that PKC plays no part in the final steps of the secretory pathway in these cells.

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Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection

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HIV-1 infection triggers lateral membrane diffusion following interaction of the viral envelope with cell surface receptors. We show that these membrane changes are necessary for infection, as initial gp120-CD4 engagement leads to redistribution and clustering of membrane microdomains, enabling subsequent interaction of this complex with HIV-1 coreceptors. Disruption of cell membrane rafts by cholesterol depletion before viral exposure inhibits entry by both X4- and R5 HIV-1 strains, although viral replication in infected cells is unaffected by this treatment. This inhibitory effect is fully reversed by cholesterol replenishment of the cell membrane. These results indicate a general mechanism for HIV-1 envelope glycoprotein-mediated fusion by reorganization of membrane microdomains in the target cell, and offer new strategies for preventing HIV-1 infection.

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Modification of the infectivity and membrane fusion properties of the VHS rhabdovirus using synthetic combinatorial peptide libraries

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Synthetic peptides were previously shown to interfere infectivity and membrane fusion processes for different enveloped virus (Kliger et al, 2000; Ferrer M et al , 1999; Lambert et al, 1996) but not yet for rhabdovirus. In this work, we tested the ability of a mixture-based combinatorial peptide libraries (6 and 16-mer) in a positional scanning format to inhibit the entry/fusion of the VHS rhabdovirus. By the contrast, we found using a microneutralization assay (Lorenzo et al 1996), that two hexadecapeptides (p005 and p006) strongly enhanced the viral infectivity on susceptible cells (3-4 fold). A similar viral infectivity enhancement caused by RANTES has also reported for HIV-1 (Gordon et al, 1999; Trkola et al , 1999). Since the infectivity enhanced capacity could be the result of direct interaction with specific viral or cellular components, we investigated the possible mode of interaction of these peptides with lipid bilayers and with the G glicoprotein of the VHSV by biophysical and biochemical techniques. Preliminary results have shown that the peptide p006 destabilizes membranes as it was previously reported for an N-terminal region of G protein of VHSV (Fr#11, aa 56 to 110) (Estepa et al, submitted) and increases the viral-cell fusion, while the p005 seems to interact specifically with the surface glicoprotein of the virus. The present findings could be important theoretical and practical implications. First, understanding the underlying mechanisms might throw light on fundamental processes of early steps of viral infections, in particular for rhabdovirus. Second, opening perspectives for to obtain new recombinant rhabdovirus vectors with high rate of infection for displaying foreign antigens, delivering therapeutic genes, vaccines, etc. Furthermore, this enhancing effect induced by the peptides could be used to improve sensitivity of rhabdovirus detection tests based on viral infectivity.

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Role of the NH2-terminal β-hairpin of ribotoxins on its ability to degrade RNA and to interact with phospholipid membranes

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Alpha-sarcin and restrictocin are two ribotoxins which sequences differ in only 19 residues. The most significant changes are located in the loops and the amino-terminal beta-hairpin. This amino-terminal structure is absent in the other microbial non-toxic RNases. The three-dimensional structure of alpha-sarcin is well known (1) but, unfortunately, the restrictocin structure lacks electron density for this amino-terminal hairpin and therefore its conformation it is not known (2). Two of these residues of alpha-sarcin have been substituted by the equivalent amino acids in restrictocin. Two single mutants (K11L and T20D) and the corresponding K11L/T20D variant have been produced in *E. coli* and purified to electrophoretical homogeneity. The spectroscopical characterization of the purified proteins reveals that the original native alpha-sarcin structure is preserved. Indeed their ability to specifically inactivate ribosomes it is also retained. However, the activity of K11L against non-specific substrate analogs such as poly(A) is significantly reduced. Much smaller changes are observed when Thr 20 is the residue substituted. On the other hand, although the three mutants studied interact with DMPG vesicles, K11L showed a different behaviour when compared with the wild-type protein in terms of aggregation, lipid-mixing, and leakage of the model vesicles employed. The results obtained allow to propose that the amino-terminal beta-hairpin is needed to maintain a ribonucleolytic competent conformation against non-specific substrates, such as poly(A) or ApA. More interestingly, this protruding structural element does participate in the protein-lipid interactions, including fusion of the lipid model systems, as minor conformational changes can alter the behaviour of ribotoxins when interacting with membranes.

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Maturation of human respiratory syncytial virus fusion protein involves a complex proteolytic processing

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Proteolytic processing of a truncated anchorless mutant form (F_{TM}-) of the fusion protein (F) of Human Respiratory Syncytial Virus (HRSV) has been studied. F_{TM}- lacks the C-terminal 50 amino acids of the F protein. F_{TM}- was expressed from vaccinia recombinants and purified by inmunoaffinity chromatography. SDS-PAGE of F_{TM}- revealed three bands that contain the F_1 chain. Characterization of the three bands was done by western blotting with a rabbit serum raised against a peptide of the F_2 chain (amino acids 104-117); N-terminal sequencing of the bands and mass spectrometry of their tryptic peptides. The results obtained demonstrated that the three bands corresponded to different products of proteolytic cleavage: i) the uncleaved precursor (F_{1+2}), ii) a partially processed product with the last 27 amino acids of the F_2 chain (starting at the amino acid 110) covalently bound to F_1 (F_{1+2t}), and iii) the fully processed F_1 chain. In addition, mass spectrometry of the tryptic peptides of the F_2 chain suggests that the mature form of this chain ends at amino acid 109. Site-directed mutagenesis at residues Arg 108 and Arg 109 indicated that both residues were needed for generation of F_{1+2t} product. These results suggest that proteolytic processing of the F_{TM}- protein precursor may involve the cleavage at two different sites, one after amino acid 109 and the other after the polybasic sequence (KKRKRR) after amino acid 137.

F protein expressed from vaccinia recombinants and HRSV were also purified by inmunoaffinity chromatography. Both preparations of full-length F protein also contain the F_{1+2t} product, as seen in western blot. In addition, the size of the F_2 chain of the F protein is the same as that of F_{TM}-. These results suggest that proteolytic cleavage of the full-length F protein is similar to that of F_{TM}-.

Electron microscopy of the full-length F protein in the absence of detergents revealed micelles (i.e., rosettes) containing two distinct types of rods, one cone-shaped and the other lollipop-shaped. Analysis of F_{TM}- molecules showed individual cone-shaped rods and a low proportion of rosetted lollipop-shaped rods. Limited trypsin digestion of F_{TM}-, under conditions that cleaved F_{1+2} and F_{1+2t}, showed a transition from individual cone-shaped rods to rosetted lollipop-shaped rods. A similar transition from cones to lollipops was observed in the micelles of full-length F after limited trypsin digestion. These results indicate that the rods and lollipops may represent different conformations of the F molecule and that proteolytic processing may trigger transition from one conformation to the other.

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Cloning and expression of sprint, a *Drosophila* homologue of RIN1

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The small GTPase Ras is critical for regulation of growth and differentiation during development. The mammalian protein RIN1 is a potential Ras effector protein, which can also interact with the Abelson tyrosine kinase. However, its biological function is unknown. We have identified the *Drosophila* homologue of RIN1, called *sprint*, for SH2, poly-proline containing Ras interactor. The *sprint* locus is very large and contains at least two differentially expressed isoforms (*sprint -a* and *sprint -b*). Both isoforms are expressed in the ovary and maternal mRNA is deposited into embryos. In addition, *sprint* is zygotically expressed in the developing midgut, amnioserosa and in a specific subset of CNS neurons. In the embryo, the expression patterns of the two *sprint* isoforms are temporally distinct suggesting that the isoforms may have unique functions.

The *sprint* locus encodes a large, multidomain molecule with no obvious catalytic activity. It consists of an SH2 domain, a proline-rich region, a domain similar to the Rab5 GDP/GTP exchange factors RABEX and VPS9, and a Ras-binding domain. In *Drosophila* Schneider cells overexpressed Sprint protein is localised to structures which may be vesicles. The structure and subcellular localisation of Sprint suggest that it might provide a link between tyrosine kinase/Ras signaling and Rab-mediated vesicle dynamics.

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Complementation of defective transmissible gastroenteritis virus by packaging cell lines

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Coronavirus are enveloped positive-strand RNA viruses. Co-expression of the E and M proteins from transmissible gastroenteritis virus (TGEV) leads to the formation of the virus membrane, releasing virus-like particles (VLPs) from the cells, indicating that these two proteins constitute the minimal assembly machinery of virus envelope. Packaging cell lines stably espressing individual TGEV structural proteins (E, M, N, and 7) were established by using the non-cytopathic Sindbis virus replicon expression vector pSINrep21 (Frolov, I., et al., 1996). The cell lines were generated by transfection of these constructs in BHK-21 cells that constitutively expressed porcine aminopeptidase-N, the major receptor for TGEV (Delmas, B. et al., 1992). The expression of TGEV proteins was detected by Western blot and immunocitochemistry and could be mantained at least during ten passages in cell culture. The presence of the Sindbis virus replicon did not interfere the production of infective TGEV viral particles in these cell lines, as assessed by virus titration (108 p.f.u./ml). Knocked-out TGEV genomes are being generated from the cDNA generated in our laboratory (Almazan, F. et al., 2000) in order to produce defective viral particles that can be complemented in trans by the packaging cell lines. This system will be very helpful to understand coronavirus assembly and budding, and also the role of the viral proteins in TGEV morphogenesis.

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Role of the vaccinia virus H3L protein in viral infectivity and virus-induced cell fusion

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The vaccinia virus H3L gene encodes for a 35 kDa protein that is expressed late during infection and is inserted in the outer membrane of intracellular mature virus (IMV). This protein has been implicated in the increased viral infectivity after phosphatidyl-serine treatment and may be importat in the process of viral penetration. We have obtained monoclonal antibodies with a low capacity for virus neutralization and inhibition of virus-induced cell fusion. Although, repeated attemps to generate a deletion mutant by replacing most of the coding sequence by the xanthine guanine phosphoribosyl transferase gene were negative, we have obtained recombinant viruses with the H3L protein expression highly repressed by the LacI repressor/operator system of *E. coli*. Analysis of these virus recombinants showed that under conditions of maximal repression of H3L protein expression the plaque size was only slightly reduced. This result suggested that this gene is not essential for viral multiplication *in vitro*. However, the production of infectious IMV was reduced to less than a 10%, but the EEV yield was almost similar to that obtained with wild type virus. The similar capacity of this mutant to induce cell fusion "from within" might support a correct virus release. The reduced infectivity of IMV was related with a reduced adsorption ability. The absence of the H3L protein on the surface of IMV made this virus more resistant to heparan sulfate-induced inhibition of vaccinia virus binding to cells. There is a transcript expressed early that initiates inside the H3L gene and encodes for the carboxi terminus of the 35 kDa protein. In light of the results obtained during the generation of deletion and inducible mutants, we suggest that the expression of this early transcript would be sufficient for correct viral dissemination *in vitro*. On the other hand, the amino terminus of the 35 kDa protein would contain a region essential for IMV adsorption to cells, most likely via a heparan sulfate-containing receptor.

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Steps involved in Paramyxovirus fusion mediated by the SV5 fusion (F) protein

Charles J. Russell

While only differing by three amino acids, the Fusion (F) proteins of the SV5 WR and W3A strains either do or do not require, respectively, their homotypic binding proteins (HN) to promote fusion. Mutations at the three differing residues show native prolines contribute to faster fusion kinetics, lower activation temperatures, and HN-independent fusion. WR F did not require coexpression of HN for fusion at elevated temperature. For paramyxoviruses, the requirement for a homotypic binding protein for fusion may be circumvented by either thermal activation or destabilizing mutations which overcome energetic barriers of presumptive metastable native states of F. Peptides from heptad repeat regions of the W3A F ectodomain were used for inhibition studies. The N-1 peptide inhibited fusion after target cell binding at a temperature-arrested stage only when F was coexpressed with HN, suggesting specific interactions between HN and F lead to a conformational change in F in the absence of thermal activation. The C-1 peptide only inhibited fusion transiently after thermal activation. Reversible lipidic fusion inhibitors were used to show that C-1, but not N-1, inhibits fusion until the actual membrane merger. The results suggest the peptide-binding regions of F undergo changes in accessibility and conformation during target cell binding, fusion activation, and membrane merger. Apparently, 6-helix bundle formation is directly coupled to fusion. The coupling of helix-bundle formation and membrane merger may be similar for fusion by HIV gp41 and SNARE complexes but different for fusion by influenza HA.

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Membrane-partitioning domains of ebola fusion protein: detection and characterization of the sequence proximal to the transmembrane anchor

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We report here the detection and characterization of a membrane-interacting domain located preceding the transmembrane anchor of GP2, the fusogenic subunit of Ebola glycoprotein. Detection has been carried out using a new method consisting in the analysis of the interfacial affinity displayed by the polypeptide sequence (Wimley-White). Experimental characterization, including intrinsic fluorescence changes, leakage of aqueous contents from vesicles and penetration into lipid monolayers, showed that the Ebola GP pretransmembrane sequence partitioned into membranes of different lipid compositions, but required the presence of phosphatidylinositol to perturb them and induce efficient permeabilization. Structural analysis by infrared spectroscopy confirmed that: i) the sequence in solution was partially folded; ii) partitioning into membranes stabilized an a-helical conformation. Evaluation of the exclusion surface pressure for insertion demonstrated that penetration was more effective into lipid monolayers containing phosphatidylinositol. We conclude that the Ebola GP2 ectodomain contains two main membrane-partitioning domains, the fusion peptide and the pretransmembrane sequence.

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A small cysteine-flanked hydrophobic region and a conserved region within the 40 residue ectodomain of P10 are essential for fusion activity

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We have identified two novel proteins (p10) encoded by non-enveloped reoviruses capable of inducing cell-to-cell fusion from within. P10 is an extremely small (10Kda) non-structural accessory protein, not essential for viral entry and replication. These unique characteristics suggest that unlike the fusion proteins of enveloped viruses, p10 may not require mechanisms that regulate the fusion activity and may contain the minimal determinants required to direct membrane localization, destabilization and fusion. P10 is a surface localized integral membrane protein with an N-terminal out topology. The direct involvement of the ectodomain in fusion is supported by the inhibitory action of N-terminal specific antibodies on the fusion activity of p10. The 40 residue ectodomain lacks an extended heptad repeat and therefore it is unlikely that in p10-mediated fusion, extensive conformational changes (such as those that accompany membrane fusion induced by certain enveloped viruses) provide the energy to overcome the thermodynamic barriers to membrane fusion. Sequence comparison between the p10 proteins of avian and Nelson Bay reoviruses revealed two conserved regions within the p10 ectodomain. Nine residues downstream of the N-terminus is a moderately hydrophobic 11-amino acid sequence flanked by conserved cysteines. Mutational analysis shows that both cysteines are absolutely essential for p10-mediated fusion despite continued p10 surface expression. The cysteines are not involved in intermolecular disulphide bonds, but may form a disulfide-bonded loop. An amphipathic loop structure has been proposed for several internal fusion peptides of enveloped viruses in addition to some antibacterial peptides and lipid exchange and lipase proteins, perhaps indicating a new common motif for lipid interactions. Several methods are presently underway to demonstrate the presence of a loop. The overall hydrophobicity of this motif is considerably less than that of N-terminal fusion peptides of enveloped viral fusion proteins according to the normalized consensus scale of Eisenberg. Mutations that alter the hydrophobic distribution within this region destroy fusogenic activity of p10 and will be discussed. The second motif within the ectodomain of p10, a linear sequence of 10 residues with absolute conservation, is also essential for p10-mediated fusion as demonstrated by several site-specific mutations. P10 ectodomain-lipid interactions are presently under investigation in attempt to designate roles to the hydrophobic and conserved domains. The ectodomain of p10 may reflect the minimal requirements for destabilization of, and fusion with, acceptor membranes, devoid of complex conformational changes necessary for regulation and specificity of fusion.

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Membrane interface-interacting sequences within the ectodomain of the HIV-1 envelope glycoprotein: putative role during viral fusion

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We have identified a region within the ectodomain of the fusogenic HIV-1 gp41, different from the fusion peptide, that interacts strongly with membranes (1,2) . This conserved sequence, that immediately precedes the transmembrane anchor, is not highly hydrophobic according to the Kyte-Doolittle hydropathy prediction algorithm, yet it shows a high tendency to partition into the membrane interface as revealed by the Wimley-White interfacial hydrophobicity scale (3). We have investigated here the membrane effects induced by NH2-DKWASLWNWFNITNWLWYIK-CONH2 (HIVc) the membrane interface-partitioning region at the C-terminus of gp41 ectodomain, in comparison to those caused by NH2-AVGIGALFLGFLGAAGSTMGARS-CONH2 (HIVn) the fusion peptide at the N-terminus of the subunit. Both HIVc and HIVn were seen to induce membrane fusion and permeabilization, although lower doses of HIVc were required for a comparable effect to be detected. Experiments in which equimolar mixtures of HIVc and HIVn were used, indicated that both peptides may act in a cooperative way. Peptide-membrane and peptide-peptide interactions underlying those effects were further confirmed by analyzing the changes in fluorescence of peptide Trp residues. Substitution of the first three Trp residues by Ala, known to render a defective gp41 phenotype unable to mediate both cell-cell fusion and virus entry, also abrogated the HIVc ability to induce membrane fusion or form complexes with HIVn but not its ability to associate with vesicles. Hydropathy analysis indicated that the presence of two membrane-partitioning stretches separated by a collapsible intervening sequence is a common structural motif among other viral envelope proteins. According to our experimental results, such a feature might be related to their fusogenic function.

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Functional analysis of cell surface-expressed hepatitis C Virus glycoproteins E1 and E2 and their involvement in the virus-cell fusion

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The hepatitis C virus (HCV) glycoproteins (gps), E1 and E2, exist as a heterodimer and are believed to be responsible for initiating viral attachment to the cell surface via specific receptors and mediating fusion of the virus and cell membranes (2). A truncated form of E2 interacts with CD81 which has been postulated to be a putative receptor for HCV attachment (4, 6, 7). A stretch of hydrophobic amino acids within E1 displays similarity to flavi- and paramyxo-virus fusion peptides and may therefore mediate fusion post E2-CD81 mediated attachment (3). However, both E1 and E2 gps are retained in the endoplasmic reticulum (ER), via independent signals within their transmembrane and cytoplasmic regions (1, 5). In order to study cellular interactions a number of chimeric proteins were constructed where the predicited E1 and E2 ectodomains were fused to the transmembrane and cytoplasmic domains of either influenza HA or vesicular stomatitis G protein. Chimeric gps were analysed for: oligomerisation; antigenic conformation; affinity for CD81; sensitivity to low pH and for their ability to mediate cell-cell fusion. We will discuss the implication of E2-CD81 interaction and heterodimerization of E1 and E2 for HCV attachment and entry.

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36 **Workshop on Flower Development.**
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*37 **Workshop on Cellular and Molecular Mechanism in Behaviour.**
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*40 **Workshop on Nuclear Oncogenes and Transcription Factors in Hematopoietic Cells.**
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*41 **Workshop on Three-Dimensional Structure of Biological Macromolecules.**
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42 **Workshop on Structure, Function and Controls in Microbial Division.**
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*43 **Workshop on Molecular Biology and Pathophysiology of Nitric Oxide.**
Organizers: S. Lamas and T. Michel.

*44 **Workshop on Selective Gene Activation by Cell Type Specific Transcription Factors.**
Organizers: M. Karin, R. Di Lauro, P. Santisteban and J. L. Castrillo.

45 **Workshop on NK Cell Receptors and Recognition of the Major Histocompatibility Complex Antigens.**
Organizers: J. Strominger, L. Moretta and M. López-Botet.

46 **Workshop on Molecular Mechanisms Involved in Epithelial Cell Differentiation.**
Organizers: H. Beug, A. Zweibaum and F. X. Real.

47 **Workshop on Switching Transcription in Development.**
Organizers: B. Lewin, M. Beato and J. Modolell.

48 **Workshop on G-Proteins: Structural Features and Their Involvement in the Regulation of Cell Growth.**
Organizers: B. F. C. Clark and J. C. Lacal.

*49 **Workshop on Transcriptional Regulation at a Distance.**
Organizers: W. Schaffner, V. de Lorenzo and J. Pérez-Martín.

50 **Workshop on From Transcript to Protein: mRNA Processing, Transport and Translation.**
Organizers: I. W. Mattaj, J. Ortín and J. Valcárcel.

51 **Workshop on Mechanisms of Expression and Function of MHC Class II Molecules.**
Organizers: B. Mach and A. Celada.

52 **Workshop on Enzymology of DNA-Strand Transfer Mechanisms.**
Organizers: E. Lanka and F. de la Cruz.

53 **Workshop on Vascular Endothelium and Regulation of Leukocyte Traffic.**
Organizers: T. A. Springer and M. O. de Landázuri.

54 **Workshop on Cytokines in Infectious Diseases.**
Organizers: A. Sher, M. Fresno and L. Rivas.

55 **Workshop on Molecular Biology of Skin and Skin Diseases.**
Organizers: D. R. Roop and J. L. Jorcano.

56 **Workshop on Programmed Cell Death in the Developing Nervous System.**
Organizers: R. W. Oppenheim, E. M. Johnson and J. X. Comella.

57 **Workshop on NF-κB/IκB Proteins. Their Role in Cell Growth, Differentiation and Development.**
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58 **Workshop on Chromosome Behaviour: The Structure and Function of Telomeres and Centromeres.**
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59 **Workshop on RNA Viral Quasispecies.**
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61 **Workshop on Oxygen Regulation of Ion Channels and Gene Expression.**
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63 **Workshop on TGF-β Signalling in Development and Cell Cycle Control.**
Organizers: J. Massagué and C. Bernabéu.

64 **Workshop on Novel Biocatalysts.**
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65 **Workshop on Signal Transduction in Neuronal Development and Recognition.**
Organizers: M. Barbacid and D. Pulido.

66 **Workshop on 100th Meeting: Biology at the Edge of the Next Century.**
Organizer: Centre for International Meetings on Biology, Madrid.

67 **Workshop on Membrane Fusion.**
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68 **Workshop on DNA Repair and Genome Instability.**
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69 **Advanced course on Biochemistry and Molecular Biology of Non-Conventional Yeasts.**
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73 **Workshop on Development and Evolution.**
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*74 **Workshop on Plant Viroids and Viroid-Like Satellite RNAs from Plants, Animals and Fungi.**
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76 **Workshop on Initiation of Replication in Prokaryotic Extrachromosomal Elements.**
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83 **Workshop on Bacterial Transcription Factors Involved in Global Regulation.**
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84 **Workshop on Nitric Oxide: From Discovery to the Clinic.**
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85 **Workshop on Chromatin and DNA Modification: Plant Gene Expression and Silencing.**
Organizers: T. C. Hall, A. P. Wolffe, R. J. Ferl and M. A. Vega-Palas.

86 **Workshop on Transcription Factors in Lymphocyte Development and Function.**
Organizers: J. M. Redondo, P. Matthias and S. Pettersson.

87 **Workshop on Novel Approaches to Study Plant Growth Factors.**
Organizers: J. Schell and A. F. Tiburcio.

88 **Workshop on Structure and Mechanisms of Ion Channels.**
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89 **Workshop on Protein Folding.**
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91 **Workshop on Eukaryotic Antibiotic Peptides.**
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93 **Workshop on Cell Cycle Regulation and Cytoskeleton in Plants.**
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99 **Workshop on Specificity in Ras and Rho-Mediated Signalling Events.**
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100 **Workshop on the Interface Between Transcription and DNA Repair, Recombination and Chromatin Remodelling.**
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Organizers: K. Roberts and P. Vera.

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103 **Workshop on the Neural Mechanisms of Addiction.**
Organizers: R. C. Malenka, E. J. Nestler and F. Rodríguez de Fonseca.

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105 **Workshop on the Molecules of Pain: Molecular Approaches to Pain Research.**
Organizers: F. Cervero and S. P. Hunt.

106 **Workshop on Control of Signalling by Protein Phosphorylation.**
Organizers: J. Schlessinger, G. Thomas, F. de Pablo and J. Moscat.

107 **Workshop on Biochemistry and Molecular Biology of Gibberellins.**
Organizers: P. Hedden and J. L. García-Martínez.

108 **Workshop on Integration of Transcriptional Regulation and Chromatin Structure.**
Organizers: J. T. Kadonaga, J. Ausió and E. Palacián.

109 **Workshop on Tumor Suppressor Networks.**
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111 **Workshop on Dendrites.**
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112 **Workshop on the Myc Network: Regulation of Cell Proliferation, Differentiation and Death.**
Organizers: R. N. Eisenman and J. León.

113 **Workshop on Regulation of Messenger RNA Processing.**
Organizers: W. Keller, J. Ortín and J. Valcárcel.

114 **Workshop on Genetic Factors that Control Cell Birth, Cell Allocation and Migration in the Developing Forebrain.**
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115 **Workshop on the Chaperonins: Structure and Function.**
Organizers: W. Baumeister, J. L. Carrascosa and J. M. Valpuesta.

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