Edited by
JOE BENTZ

Viral Fusion MECHANISMS

Edited by

JOE BENTZ, Ph. D.
Associate Professor
Bioscience and Biotechnology
Drexel University
Philadelphia, Pennsylvania

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INTRODUCTION

Today there are two important questions in membrane fusion: How do the membrane proteins of the enveloped animal viruses accomplish it and to what extent are the mechanisms utilized by viral fusion proteins relevant to the membrane fusion mechanisms utilized by cells? Since enveloped animal viruses express only a few different membrane proteins, it has been possible in many cases to identify which of these proteins are responsible for mediating the fusion event itself. This book is our first attempt to put together what we know about viral fusion sites. Hopefully, this will help us to think about how eukaryotic fusion sites might be assembled, even if it is true that eukaryotic fusion proteins are more sophisticated and are assembled more artfully. Perhaps, it will even help us to think about how nonenveloped viruses pass through the cell membrane.

In principle, the fusion site between the viral envelope and the target membrane can involve multiple copies of several different proteins. To know the molecular mechanism of fusion implies knowing which viral protein is required for fusion, whether other viral or target membrane proteins play a role in that fusion, which parts of the proteins at the fusion site are responsible for bilayer destabilization, and finally, which parts of the proteins at the fusion site are responsible for completing the fusion event. This is more than we know about any viral fusion protein, although the discussion of influenza hemagglutinin is getting close to the mark.

The chapters in this book fall roughly into two categories. The first category is the general experimental and theoretical methodologies for elucidating fusion mechanisms at the molecular level, and the second category is the application of these methodologies to particular viruses. The arrangement of the chapters is (roughly) general experimental methodologies, followed by the chapters on particular viruses, and ending with the general theoretical methodologies.

EXPERIMENTAL METHODOLOGIES

Chapter 1 is a description of growing virus in cell culture by Len Benade. This is the first step in most studies and a good place to begin. Of particular interest is the care that must be exercised to obtain a virus that is uncontaminated and unbroken, see also Chapters 9 and 14. While plaque assays appear to be largely undisturbed by "membranous" debris, including " pen-strep" inebriated bacteria, quantitative kinetic studies are difficult (to say the least) without clean preparations. The remainder of the book is adequate testimony that quantitative kinetic studies are required to elucidate a mechanism at the molecular level.

Since membrane fusion involves only a small fraction of the membrane proteins and lipids for a short period of time, it would appear that the most direct method for determining the architecture of the fusion site is cryoelectron

microscopy, which is described in Chapter 2 by Frank Booy. The virtue of cryomicroscopy is that the sample is neither fixed nor stained, and frozen ultrafast. The problem is the lack of contrast and resolution. Contrast image processing is discussed as one way to overcome this problem.

Many viral fusion proteins have ''fusion peptides'', i.e., amphipathic sequences that appear to be required for fusion. Just how these peptide sequences execute this role while attached to the protein has yet to be resolved. Understanding why and how amphipathic peptides insert into lipid domains is described in the next two chapters. In Chapter 3, Jim Lear and Maria Rafalski describe the interactions between peptides and lipid monolayers. Nearly all amphipathic peptides bind to liposomes, including those which correspond to the ''fusogenic'' N-terminus of influenza hemagglutinin, aka HA2-homologs. However, the homologs typically create a greater surface pressure at air-lipid monolayer interfaces than ''mutant'' sequences, i.e., those which would not be fusogenic within the protein. Whether this is why the homologs are fusogenic is not known, but the correlation is very intriguing. (See also Chapter 7.)

In general, we would like to know how the fusion peptide, as well as the rest of the fusion protein, interacts with the target membrane during fusion. One powerful means of addressing this question is photolabeling using phospholipid-linked probes, which is described in Chapter 4 by Josef Brunner and Masato Tsurudome (see also Chapter 5). This chapter provides a bridge between the methodology section and the chapters on influenza virus. A very clear discussion of the advantages and disadvantages of this technique is followed by data on influenza HA (PR8 strain) at low pH and 0°C. The N-terminus of HA2 inserts into ganglioside containing target membranes, probably as an α-helix and at an oblique angle, but there is no lipid mixing. Evidently, insertion alone is not sufficient to induce fusion.

THE VIRUSES

The fusion mechanism of only a few viruses has been studied. For each virus in this book, there is a brief description of its life cycle and epidemiology. The viruses are discussed in several consecutive chapters, and information on them is also in the first chapter. The chapters are arranged in alphabetical order, except that influenza virus comes first. The reason for this is not ''fluphilia'', rather it is because the influenza virus fusion mechanism is the current paradigm for viral fusion mechanisms. Like all paradigms, this one is instructive only as long as we constantly evaluate how well our experimental and theoretical techniques are sensitive to the differences between influenza virus and the other viruses.

Influenza Virus

The four primary articles on influenza virus (Chapters 5 to 8) clearly show the depth of our understanding of this fusion mechanism. There is a

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set of basic facts including (1) only sialic acid (either on a protein or ganglioside) is required for binding; (2) fusion is initiated at low pH after exposure of the N-terminus of HA2 (the "fusion peptide"); (3) there is more than one HA trimer at the fusion site; and (4) the trimer remains together during fusion, i.e., the complete dissociation of the HA1 tops is a postfusion event. However, there are disagreements on how these facts are best fitted together into a molecular mechanism. For example, how do the "fusion peptides" induce bilayer destabilization? Are they inserted into the membranes in order to draw the two bilayers together or are they acting between the bilayers to induce a "wetting" of the fusion proteins?

Many approaches have been taken to dissect the fusion process of influenza hemagglutinin. In Chapter 5, Toon Stegmann and Ari Helenius describe the fusion of influenza virus (X31 strain) with ganglioside liposomes at low temperature where the fusion process occurs via several slow steps. They found a long delay time before lipid mixing starts, during which HA2 can be photolabeled. In Chapter 6, Michael Clague, Christian Schoch and Robert Blumenthal describe how influenza HA-expressing fibroblast cells fusing with erythocyte ghosts show a similar delay time. After this delay time, the pH can be raised without stopping the subsequent fusion. While it is generally agreed that the fusion site is being assembled during this delay time, it has not yet established what this assembly looks like or how it works. This is the heart (or brains?) of the fusion mechanism.

Simplifying the membranes will simplify our work, assuming that we keep sight of the physiologically relevant issues. Since influenza virus only needs sialic acid for binding, the ganglioside containing liposomes serve as very simple model target membranes, and whether influenza virus is bound to glycoproteins or gangliosides in the endosome is unknown. In Chapter 7, Jan Wilschut and Romke Bron describe simplifying the viral envelope by reconstituting the influenza hemagglutinin by detergent dialysis, i.e., making virosomes. A major advantage of virosomes is that more rigorous fusion assays can be used. Of course, it remains to be proven that influenza hemagglutinin works the same in virosomes as it does in virions.

Determining how many fusion proteins are required at the fusion site (the minimal fusion unit) is as important as establishing the conformational changes of the fusion protein. Varying the surface density of the fusion protein is the most rigorous way of determining the minimal fusion unit (see Chapter 19). Unfortunately, this cannot be reliably done with the native virus or (as yet) with virosomes. In Chapter 8, Harma Ellens, Dennis Alford and I describe the usage of cell lines with different surface densities of influenza HA to determine that more than one HA was required at the fusion site and that the HAs bound to glycophorin in the target membranes were probably not involved in fusion.

Detailed cartoons on the steps of HA-mediated fusion are proposed in Chapters 5, 7, 8, and 20. All begin with the presumption that the N-terminus

of HA2 cannot extend very far from the HA trimer body due to tertiary structure constraints (see Chapter 8). In Chapter 5, Stegmann and Helenius propose that the N-terminus must insert into the target membrane to initiate the fusion process and the HAs must bend over to permit this insertion. In Chapter 20, Siegel continues the model of Stegmann and Helenius with a proposal that the inserted N-termini lead the lipids to form a stalk between the apposed bilayers. In Chapter 7, Wilschut and Bron have the HAs standing up, but propose that the propensity of the HA2 N-termini to insert into both target and host membranes leads to their ''tilting'', which induces a large bilayer curvature and fusion. In Chapter 8, we also leave the HAs standing up and propose that the N-termini initiate fusion by inducing the lipids to wet the inner surface of the collar composed of aggregated HAs. We discuss how tilting or bending of HA would require consideration of its hydrophobic transmembrane domain as a potential element of the fusion sight architecture. While multiple chapters on the same virus lead to some duplication, it is my hope that juxtaposing detailed models on HA-mediated fusion will facilitate the design of conclusive experiments.

Other Viruses

In Chapter 9, Pat Spear describes herpes simplex virus (HSV). This virus is the current leader with respect to known complexity by having up to fifteen different envelope glycoproteins, of which at least three appear essential for infection. HSV may require only proteoglycan-linked heparin sulfate as an initial receptor. This is consistent with its tissue tropism in human and other vertebrates, but it seems paradoxically unsophisticated as a receptor for such a complex virus. Whether or not additional host receptors are required for fusion is unknown.

There are two articles on human immunodeficiency virus (HIV), which has become a sort of lingua franca of virology. In Chapter 10, John Moore, Brad Jameson, Robin Weiss and Quentin Sattentau present a comprehensive review of the HIV fusion literature. They particularly focus on the possibility of auxiliary molecules that may be required to complete the fusion initiated by CD4 and gp120/41 (the sole HIV envelope glycoprotein). In Chapter 11, Harma Ellens and Charles Larsen describe the sequence of conformational changes initiated by CD4 binding to gp120/41, which leads in the end to the dissociation of gp120 from gp41. They propose that fusion proceeds from an intermediate of this dissociation process. In their view, auxiliary molecules enhance the efficiency of fusion without being absolutely required.

There are two chapters on Sendai virus. In Chapter 12, Philip Yeagle proposes a structure for the F(for fusion)-protein, based upon biochemical and immunoprecipitation data. He also describes how small hydrophobic peptides inhibit Sendai virus fusion and alter lipid phase behavior, which he proposes have a common link in the lipid intermediates of both processes. In Chapter 13, Yoav Henis proposes that both the F-protein and HN (for hem-

agglutinin and neuraminidase activity)-protein on the Sendai envelope must be mobile for fusion to occur. This is consistent with more than one envelope glycoprotein being required at the fusion site, not to mention more than one type of glycoprotein.

In Chapter 14, Anne Walter and Lou Ann Downing discuss respiratory syncitial virus (RSV). While a member of the Paramyxoviradae, RSV is unusual in having no neuraminidase or hemagglutinating activities. This provides a good comparison system for assessing the advantages of HN activities with respect to fusion. In Chapter 15, Margaret Kielian describes the fusion of alphaviruses, e.g., Sindbis and Semliki Forest virus, which are so far unique in requiring cholesterol in their target membrane. Whether this is involved in species or tissue tropism is an open question. In Chapter 16, Rafael Blasco and Bob Doms describe the fusion of vaccinia virus, which has become a primary vector for transfection of eukaryotic cells with exogenous genes. This virus may be even more complex than herpes simplex virus, in that it appears to have two independent sets of fusion proteins, one of which acts intracellularly and the other acts extracellularly. In Chapter 17, John Lenard describes vesicular stomatitis virus (VSV) and the usage of γ-ray inactivation to estimate how many G-proteins, its fusion glycoprotein, are required at the fusion site. While the answer is not fixed, it seems that more than one G-protein is required.

THEORETICAL METHODOLOGIES

Any microscopic or biochemical evidence of fusion steps or "intermediates" must be strongly correlated with the fusion kinetics, both in terms of the times of formation and the overall number found. Otherwise, one could be tracking secondary reactions. In Chapter 18, Shlomo Nir, Maria Pedroso de Lima, Charles Larsen, Jan Wilschut, Dick Hoekstra, and Nejat Düzgünes describe the kinetic analysis of virus-cell fusion from which the virus-cell (macroscopic) fusion rate constant can be obtained. They particularly focus on the kinetics of viral inactivation. This is a very important issue, because it gives us a time scale to use when judging whether certain protein conformations are likely to be relevant to fusion.

In Chapter 19 the "generic" kinetic properties of multistep membrane fusion mechanisms are illustrated, such as have been proposed in many of the chapters. It is possible to measure the number of proteins at the fusion site, although probably not via the kinetics of the delay time before fusion (see, however, Chapter 7). The delay time need not be due to the aggregation of HA, even when the delay time depends upon HA surface density. It is shown how the transition kinetics between "microscopic" fusion intermediates generate the "macroscopic" fusion rate constants, as well as the inactivation kinetics, discussed by Nir et al. in the preceding chapter. While the fusion rate constant is our best measure for the activation energy of fusion, as discussed by David Siegel in the next chapter, Arrhenius plots of protein-

mediated fusion data are likely to be either misleading or inconclusive. Multistep processes can yield remarkably straight Arrhenius plots, regardless of whether any intermediate step is rate limiting, with slopes bearing little predictive value about the activation energies of the underlying fusion intermediates.

Finally, in Chapter 20, David Siegel discusses what lipid physical chemistry can (and should) contribute to our cartoons of membrane fusion intermediates. This topic is quite speculative, but some important ground rules are emerging. In particular, it is clearly demonstrated that keeping track of the lipids, with respect to their packing and exposure to water, is almost certainly as important thermodynamically as what we ask the protein to do. He describes recent theoretical work on pure lipid systems that suggest the preferred initial defect between apposed bilayers is a lipid stalk, and proposes a model for HA-mediated fusion based upon this stalk mechanism.

What's the conclusion? We are getting close to agreeing on how influenza HA actually induces bilayer destabilization and fusion. This means that the experiments required to iron out the differences will be more difficult. The reward for these hard experiments will come in their application to other viral systems, since we must discover the extent to which the influenza HA mechanism is relevant to them. Certain similarities seem likely, such as more than one fusion protein at the fusion site. Certain dissimilarities seem likely, such as target membrane protein involvement at the fusion site, as (probably) in the case of CD4 with gp120/41 from HIV. Other dissimilarities seem certain, such as different viral proteins at the fusion site, as (probably) in the case of HSV, or specific lipid requirements in the target membrane, as in the case of Semliki Forest virus. The involvement of amphipathic peptide chains or surfaces is certain, but much individuality could be discovered. It will be very important to focus on the generic properties of all these fusion sites if what we discover about viral fusion mechanisms is to help us with cellular fusion mechanisms.

Penultimately, I would like to thank all of the authors for putting real work into these chapters and for being as prompt as humanly possible. Since protein-mediated membrane fusion is a new field, mechanistically speaking, the success of this book will be measured by the work it inspires, not in the correctness of the speculations contained herein. Seeing the chapters all finished and in order, I know it was worth the effort. A similar feeling arises upon seeing my children, Michael and Emily, quietly sleeping at night. Never mind the chaos of the day. Ultimately, I thank Harma Ellens who contributed both at work and at home.

Joe Bentz
Jeffersonville, Pennsylvania

THE EDITOR

Joe Bentz, Ph.D., is Associate Professor of Bioscience and Biotechnology at Drexel University in Philadelphia.

Dr. Bentz received his B.A. degree from Reed College in Portland, Oregon in 1974 and his Ph.D. degree from the Department of Biophysical Sciences, State University of New York at Buffalo in 1979. After a year of postdoctoral work, he was appointed as a Cancer Research Scientist at Roswell Park Memorial Institute in Buffalo. In 1982, he was appointed Assistant Professor of Pharmacy and Pharmaceutical Chemistry at the University of California at San Francisco (UCSF). In 1988, he was promoted to Associate Professor at UCSF. In 1989, he moved to Philadelphia and was appointed as Associate Professor in the Department of Bioscience and Biotechnology at Drexel University.

Dr. Bentz is a member of the American Chemical Society, the American Society of Cell Biology and the Biophysical Society. He is currently an advisor to the American Red Cross on prevention of viral contamination in blood products. He has had continuous support of his research by the National Institutes of Health and has published more than forty papers. His research interest is membrane fusion.

CONTRIBUTORS

Dennis Alford, B.S.
Sr. Research Specialist
Department of Bioscience and Biotechnology
Drexel University
Philadelphia, Pennsylvania

Leonard E. Benade, Ph.D.
Transmissible Diseases Laboratory
American Red Cross
Rockville, Maryland

Joe Bentz, Ph.D.
Department of Bioscience and Biotechnology
Drexel University
Philadelphia, Pennsylvania

Rafael Blasco, Ph.D.
Laboratory of Viral Diseases
National Institutes of Health
Bethesda, Maryland

Robert Blumenthal, Ph.D.
Centro de Investigacion y Techologia
INIA – Sanidad animal
Madrid, Spain

Frank Peter Booy, Ph.D.
Laboratory of Structural Biology
National Institute of Arthritis, Musculoskeletal, and Skin Diseases
National Institutes of Health
Bethesda, Maryland

Romke Bron, Ph.D.
Department of Physiological Chemistry
University of Groningen
Groningen, The Netherlands

Josef Brunner, Ph.D.
Department of Biochemistry
Swiss Federal Institute of Technology Zürich
Zürich, Switzerland

Michael J. Clague, Ph.D.
European Molecular Biology Laboratory
Heidelberg, Germany

Robert W. Doms, M.D., Ph.D.
Department of Pathology and Laboratory Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Lou Ann Downing, M.Sc.
Department of Physiology and Biophysics
Wright State University
Dayton, Ohio

Nejat Düzgüneş, Ph.D.
Department of Microbiology
University of the Pacific School of Dentistry
San Francisco, California

Harma Ellens, Ph.D.
Department of Drug Delivery
SmithKline Beecham Pharmaceuticals
King of Prussia, Pennsylvania

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Ari Helenius, Ph.D.
Department of Cell Biology
Yale University School of Medicine
New Haven, Connecticut

Yoav I. Henis, Ph.D.
Department of Biochemistry
George S. Wise Faculty of Life Sciences
Tel Aviv University
Tel Aviv, Israel

Dick Hoekstra, Ph.D.
Department of Physiological Chemistry
University of Groningen
Groningen, The Netherlands

Bradford A. Jameson, Ph.D.
Jefferson Cancer Institute
Philadelphia, Pennsylvania

Margaret Kielian, Ph.D.
Department of Cell Biology
Albert Einstein College of Medicine
Bronx, New York

Charles Larsen, Ph.D.
Department of Bioscience and Biotechnology
Drexel University
Philadelphia, Pennsylvania

James D. Lear, Ph.D.
DuPont-Merck Pharmaceuticals
Experimental Station
Wilmington, Delaware

John Lenard, Ph.D.
Department of Physiology and Biophysics
University of Medicine and Dentistry, NJ
Robert Wood Johnson Medical School
Piscataway, New Jersey

John Moore, Ph.D.
Department of Cell and Molecular Biology
Chester Beatty Laboratories
The Institute of Cancer Research
London, England

Shlomo Nir, Ph.D.
Seagram Centre of Soil and Water Sciences
Faculty of Agriculture
Hebrew University of Jerusalem
Rehovot, Israel
and
The Aaron Diamond AIDS Research Center
New York, New York

Maria C. Pedroso de Lima, Ph.D.
Center for Cell Biology
Department of Chemistry
University of Coimbra
Coimbra, Portugal

Maria Rafalski, Ph.D.
DuPont-Merck Pharmaceuticals
Experimental Station
Wilmington, Delaware

Quentin Sattentau, Ph.D.
HIV Group
CIML, Luminy Science Park
Marseille, France

Christian Schoch, Ph.D.
Institute of Medical Microbiology
University of Berne
Berne, Switzerland

David Paul Siegel, Ph.D.
Procter & Gamble Company
Miami Valley Laboratories
Cincinnati, Ohio

Patricia G. Spear, Ph.D.
Department of Microbiology and Immunology
Northwestern University Medical School
Chicago, Illinois

Toon Stegmann, Ph.D.
Department of Biophysical Chemistry
Biocenter of the University of Basel
Basel, Switzerland

Masato Tsurudome, D.V.M., Ph.D.
Department of Biochemistry
Swiss Federal Institute of Technology
Zürich, Switzerland

Anne Walter, Ph.D.
Department of Physiology and Biophysics
Wright State University
Dayton, Ohio

Robin A. Weiss, Ph.D., HonMRCP, FRCPath
Department of Cell and Molecular Biology
Chester Beatty Laboratories
The Institute of Cancer Research
London, England

Jan Wilschut, Ph.D.
Department of Physiological Chemistry
University of Groningen
Groningen, The Netherlands

Philip L. Yeagle, Ph.D.
Department of Biochemistry
University at Buffalo
School of Medicine and Biomedical Sciences
Buffalo, New York

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Chapter 1

GROWTH AND ASSAY OF VIRUSES

Leonard E. Benade

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I. CELL CULTURE

A. GENERAL REQUIREMENTS

Cell culture and embryonated chicken eggs are the primary means by which viruses are isolated, grown, and studied. However, of the two, cell culture is by far the more sensitive and adaptable, and will be emphasized in this chapter. The following rather basic discussion of virus culture technique is slanted toward those with limited or no virology/cell culture experience. It is hoped that the reader will get a useful overview of the requirements for establishing the technology in his laboratory, as well as an understanding of the basics of several viral models and their assay systems.

Establishing the capability for cell and virus culture is not an insignificant undertaking. The most important equipment items are the biological safety cabinet ("the hood") in which most cell and virus culture operations are performed and the incubator (preferably with CO_2 capability) in which the culture vessels are maintained.

There are three classes of biological safety cabinet: I, II, and II. The class III cabinet is totally enclosed and gas tight; access is gained through arm-length rubber gloves attached to the front panel. While this cabinet affords the highest level of protection for laboratory personnel and cell culture activities, it is not the most practical choice, nor is its high level of containment warranted unless very hazardous materials are to be used. Both class I and II cabinets are suitable for working with moderate- to high-risk viruses, provided, of course, that one adhers to recommended microbiological technique. Both of these cabinets have open fronts, with negative pressure drawing air flow into the cabinet; outward air flow is exhausted through high-efficiency particulate (HEPA) filters. For most purposes, the best choice is the class II. The design of this cabinet is such that air is recirculated within the chamber according to a vertical "laminar flow" scheme, which offers the significant advantage of increased protection of cell culture activities from outside contamination. Another consideration is that this cabinet can be set up to exhaust filtered air into the workspace, requiring no additional ductwork and attendant construction costs. It is important to be aware that no open-front cabinet offers absolute protection of workers and materials. Care must be taken to avoid activities or placement of materials that disrupt or otherwise alter the airflow in and around the cabinet. When work is being carried out in the hood, undue clutter on the working surface must be avoided, as must unnecessary traffic of personnel nearby, opening and closing of doors in the vicinity, etc.

The advantage of using a CO_2 incubator (as opposed to an incubator which simply maintains the desired temperature) is to create a 95% air/5% CO_2 atmosphere for pH control. One must otherwise rely on the addition of bicarbonate to the culture medium. It is then also necessary to blow a stream of CO_2 into the tissue culture flask (dishes cannot be used in this case) and

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