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Cell Entry of Enveloped Viruses

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Enveloped viruses penetrate their cell targets following the merging of their membrane with that of the cell. This fusion process is catalyzed by one or several viral glycoproteins incorporated on the membrane of the virus. These envelope glycoproteins (EnvGP) evolved in order to combine two features. First, they acquired a domain to bind to a specific cellular protein, named “receptor.” Second, they developed, with the help of cellular proteins, a function of finely controlled fusion to optimize the replication and preserve the integrity of the cell, specific to the genus of the virus. Following the activation of the EnvGP either by binding to their receptors and/or sometimes the acid pH of the endosomes, many changes of conformation permit ultimately the action of a specific hydrophobic domain, the fusion peptide, which destabilizes the cell membrane and leads to the opening of the lipidic membrane. The comprehension of these mechanisms is essential to develop medicines of the therapeutic class of entry inhibitor like enfuvirtide (Fuzeon) against human immunodeficiency virus (HIV). In this chapter, we will summarize the different envelope glycoprotein structures that viruses develop to achieve membrane fusion and the entry of the virus. We will describe the different entry pathways and cellular proteins that viruses have subverted to allow infection of the cell and the receptors that are used. Finally, we will illustrate more precisely the recent discoveries that have been made within the field of the entry process, with a focus on the use of pseudoparticles. These pseudoparticles are suitable for high-throughput screenings that help in the development of natural or artificial inhibitors as new therapeutics of the class of entry inhibitors.

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membrane fusion, which preserves the integrity of the cell membrane. The fusion of two separate lipid bilayers in a nonaqueous environment first requires that they come into close contact. This is followed by an intermediate stage characterized by the merger of only the closest contacting monolayer, a process called hemifusion. Third, the fully completed fusion results in whole bilayer merging, followed by the opening of the pore. To achieve this process, the envelope glycoproteins (EnvGP) have evolved in order to combine two features. On the one hand, they acquired a domain to bind to a specific cellular protein, named “receptor.” On the other hand, they developed in a different manner, according to the genus of the virus, a function of fusion that allows the destabilization of the membrane and the opening of a pore through which the genetic material will enter the cell.

Despite three different classes of fusion protein having been described so far, three common main steps are described for achieving the pre- to post-conformational changes. The first one, after EnvGP activation upon receptor binding or acidification of the endosomal compartment, exposes the fusion peptide that is projected toward the top of the glycoprotein, allowing the initial interaction with the target membrane. The second one is the folding back of the C-terminal region onto a trimeric N-terminal region that leads to the formation of a postfusion protein structure. The final and third step also requires further refolding of the membrane proximal and transmembrane regions in order to obtain a full-length postfusion structure where both membrane anchors (fusion peptide and tm domains) are present in the same membrane.

The viral glycoprotein-induced fusion must be controlled to allow the virus to leave the cell, to prevent the aggregation of the viruses, and to release genetic material next to a compartment that will permit the continuity of the infectious cycle. This regulation is executed principally by the inactivation or the masking of the fusion machinery that directly disorganizes the lipid bilayer. On one hand, most of these EnvGP are synthesized as a precursor that requires a cleavage by a cellular protease (like furin) and prepares the molecules for the subsequent necessary changes of conformation for the fusion process. On the other hand, the activation of the conformational changes is induced by interaction with the receptor and/or the action of the pH (that protonates some amino acids), modifying the interactions of the EnvGP and their structure. In addition, some proteases or other enzymes are necessary to achieve some complementary priming of the EnvGP to make it competent for fusion (such as cathepsin B and L).

Two types of fusion mechanisms can occur, namely, pH independent and pH dependent. In the first case, the recognition between virus and receptor directly triggers conformational changes in the EnvGP that leads to the direct fusion between the two membranes (viral and plasma) and to the liberation of the viral genetic material. This activation of EnvGP at neutral pH allows the fusion in vitro and in vivo of EnvGP-expressing cells with receptor-expressing cells. This fusion leads to the merging of cell cytoplasms and to the generation of
multinucleated cells called syncytia. In the second case, for pH-dependent fusion, the interaction between the EnvGP and the receptor leads to the obligatory endocytosis of the virus–receptor complex, and the acidification of endosomes triggers conformational changes in the EnvGP. For the pH-dependent virus, such a fusion can be reproduced in cell culture in vitro or in a liposome–virus fusion assay in the tube after decreasing the pH, but cannot occur in vivo.

Research during the last few years has greatly advanced our understanding of the cell surface receptors for viruses and has provided many surprising insights. These advances were achieved largely by identification and molecular cloning of the cell surface or cytoplasmic proteins that have been subverted for use as viral receptors or cofactor, and by parallel advances in studies of the viral EnvGP that bind to the receptors.

Another key area of new insights concerns the physical–chemical process of viral adsorption and of pulling the virus closely onto the cellular membrane. Indeed, adsorption is a severely limiting step in infections of cultured cells, and the initial attachment often does not involve the receptors that ultimately mediate infections (Andreadis et al., 2000; Guibinga et al., 2002; Pizzato et al., 1999, 2001; Ugolini et al., 1999). Thus, we need to distinguish cell surface molecules such as heparan sulfate proteoglycans, DC-SIGN, or integrins that can enhance infections by concentrating retroviruses onto cells (Bounou et al., 2002; Geijtenbeek et al., 2000; Jinno-Oue et al., 2001; Mondor et al., 1998; Pohlmann et al., 2001; Saphire et al., 2001) from authentic receptors that induce conformational changes in EnvGP that are a prerequisite for fusion of the viral and cellular membranes. In contrast to other cell surface components such as lectins or proteoglycans that influence infections indirectly by enhancing virus adsorption onto specific cells, the true receptors induce conformational changes in the viral EnvGP that are essential for infection. However, it appears that more and more intracellular proteins have roles in controlling viral host ranges, and the proteins involved in traffic of intracellular vesicles like endosomes, play a critical role in entry. Therefore, proteins from pathways specifically characterized might be considered as a cofactor as their role is not to mediate direct contact with EnvGP but is crucial for entry. In addition, some viruses are able to use more than one endocytic pathway and the cellular proteins involved thus direct the virus to the entry door beneficial for the virus under a particular condition.

Pseudoparticles are retroviridae cores which incorporate heterogeneous envelope protein from a different virus, possibly of a different family. Their entry process mimics precisely that of the wild-type viruses from which the envelope glycoprotein was derived. They represent a useful tool to study molecular processes of envelope viruses as they are very flexible, allowing the analysis of numerous mutants. Moreover, they allow a precise measurement of the infectivity that depends on the envelope glycoprotein for the entry step and they allow the establishment of virus–liposome fusion assays.
We will first introduce envelope architecture and structure of the viral fusion machineries that have been developed to achieve the fusion and entry steps. Despite many differences of structure, they share a common refolding process that activates different fusion domains found in most fusion proteins. We will then illustrate the different fusion regulation processes that have been developed by viruses to lead to functional virions, both in terms of cleavage activation of fusion proteins during exit and in terms of activation during binding and endocytosis. Despite some differences between distinct players, some common principles can be proposed for all fusion processes. We will illustrate the molecular details characterizing the maturation of the different fusion proteins, defined by the following three characteristics: the cleavage of an envelope protein precursor, the presence and triggering of the exposition of a fusion peptide, and an association as a trimeric complex association in its active fusion conformation. The progression of these structural rearrangements slows down the kinetic barrier between hemifusion and fusion-pore formation. In a second part, we will present the different entry pathways and cell proteins that are used by viruses to infect cells. Viruses have emerged as valuable tools for the study of endocytic mechanisms. These properties have been crucial for the development of pseudoparticles for their use in terms of vector for gene therapy and for their use in terms of tool for receptor cloning, transgenesis, or transduction. Finally, we will give examples of strategies that have been developed in vivo and in vitro to inhibit the entry step of the enveloped viruses which have led, in the case of HIV, to the development of inhibitors that are used in the clinic.

II. ARCHITECTURE AND STRUCTURE OF THE VIRAL FUSION MACHINERY

A. Membrane fusion according to the stalk-pore model

EnvGP are responsible for bringing the membranes closer, triggering the link and the destabilization of the outer leaflet, the merging of the whole membrane, and the opening of a pore through the cell and virus membrane to allow the core to enter the cytoplasm of the cell. The hypothesis of the pore model in viral membrane fusion mechanism is supported by experimental results. The first evidence for a hemifusion intermediate was achieved by studying influenza virus entry that occurs after the hemagglutinin glycoprotein binding to the host cell. The substitution of the hemagglutinin transmembrane domain by a glycosylphosphatidylinositol (GPI) revealed the importance of the transmembrane region for the fusion pore opening and expansion. Hemifusion structures are connections between outer leaflets of apposed membranes, whereas the inner leaflets remain distinct. This is a transient structure that either dissociates or
gives rise to the fusion pore (Chernomordik and Kozlov, 2008). Interestingly, the helix breaker residues within the tm domain are critical for the fusogenicity of different retroviral Env, such as HIV (Owens et al., 1994) and Mo-MLV (Taylor and Sanders, 1999), being important for both the hemifusion and pore opening step of the fusion process. In addition, hemifusion intermediate has been detected in the case of HIV Env-mediated fusion (Munoz-Barroso et al., 1998) by using peptide inhibitors that target a prefusion or prehairpin structure such as HIV-1 gp41 T-20. Once the pore is formed, it allows a connection between two compartments initially separated by the apposed membranes.

The ability of the membrane to hemifuse and develop a fusion pore has been found to depend on the lipid microdomain composition, for example, cholesterol (Chernomordik and Kozlov, 2003). Indeed, a potential lipid dependence of virus entry processes was first deduced from experiments on influenza virus, implying a role for detergent-resistant lipidic microdomain (Takeda et al., 2003). For retroviruses, the tm palmitoylations which contribute to the Env localization in detergent-resistant lipidic microdomain domains (Li et al., 2002) indirectly influence the fusion process (Ochsenbauer-Jambor et al., 2001). As an alternative to the lipidic pore hypothesis, a direct fusion mechanism has also been proposed. The fusion pore is a full proteic channel-like structure dependent only on the transmembrane domains of the glycoproteins. In this model, the pore is opened by the joining of two hemipores located on each membrane (Chernomordik and Kozlov, 2005, 2008).

After fusion pore opening and enlargement (Melikyan et al., 2005), the genetic material enters the cytoplasm of the cell, instigating the virus cell cycle.

**B. pH-dependent and -independent molecular switches**

The fusion of the viruses that enter directly at membrane plasmic level (as with the paramyxoviruses and most retroviruses) is triggered by the activation of the viral envelope protein at neutral pH. In this case, only the binding of the virus to its receptor activates the fusogenic potential of the complexes of EnvGP. This mechanism is pH-independent. In the case of retroviruses, the binding of the surface subunit (SU) to its receptor induces conformational changes not only in itself but also in the TM with which it interacts, thus inducing fusion. This activation at neutral pH permits the envelope glycoprotein of pH-independent viruses, in certain experimental conditions or in vivo, to induce the fusion between the cells expressing the envelope glycoprotein and the cells expressing the receptor (Harrison, 2008; Kielland and Rey, 2006; Weissenhorn et al., 2007). This intercellular fusion, by merging their plasma membranes, places the cell cytoplasms in continuity, and one or more cells become giant multinucleated cells named syncytia.
In contrast, the fusion of Fig. 4.1 most other viruses depends strictly on their internalization into one of the numerous endocytic pathways such as the clathrin-dependent, clathrin-independent, and caveolae-independent, as well as the macropinocytosis (Vaccinia virus) (Mercer and Helenius, 2008) or the phagocytose (as recently described for Equine herpesvirus 1 virus (Frampton et al., 2007) and the HIV virus (Trujillo et al., 2007), although in this case not resulting in a productive infection; Marsh and Helenius, 2006; Sieczkarski and Whittaker, 2002; Figure 4.1). As described so far, the enveloped viruses that use these itineraries have fusion reactions that require exposure to a moderately acid pH in the different endocytosis vesicles (pH-dependent viruses). Classical examples of viruses that fuse at low pH with the membrane of endosomes or artificial membranes in the test tube include the influenza orthomyxovirus, the dengue or the tick-borne encephalitis (TBEV) flaviviruses, and the Semliki forest alphavirus (SFV) (Harrison, 2008; Kielian and Rey, 2006; Roche et al., 2008; Weissenhorn et al., 2007).

Interestingly, an intermediate mechanism has been described for two retroviruses, ASLS and JSRV. The proposed mechanism of ASLV virion entry occurs in two steps involving a receptor-priming step that induces Env conformational changes, thus allowing the Env to become sensitive to the action of acid pH (Mothes et al., 2000). This hybrid mechanism does not lead to cell–cell fusion in vivo. JSRV also uses receptor priming for fusion activation of JSRV Env at a low pH, but the mechanism differs slightly to ASLV, requiring dynamin-associated endocytosis (Bertrand et al., 2008).

So far, many different endocytosis pathways have been described (Marsh and Helenius, 2006; Mercer and Helenius, 2009; Mercer et al., 2010b) as being used by both pH-dependent and pH-independent viruses. However, reinvestigations of these entry pathways are clearly needed for many pH-independent viruses that were originally thought not to rely on endocytosis. For example, Nipah paramyxoviruses induce fusion between cells at neutral pH and were considered as a pH-independent virus not reliant on endocytosis for entry. However, recently, it was proposed that Nipah viruses (NiVs) use macropinocytosis for entry (Pernet et al., 2009). It should be noted that viruses that use a pH-independent mechanism of activation of EnvGP may still enter the cell by endocytosis without any imperative requirement for acidification activation of EnvGP in the endosomes.

Clathrin-dependent endocytosis is the best-characterized pathway of entry and is the major itinerary of entry for pH-dependent viruses. This process is initiated by the formation of the characteristic invaginations of membrane, known as clathrin-coated pits (CCP) (Fig. 4.1). The CCP assembly takes place on the internal face of the plasma membrane following a signal induced by the activation of a receptor. This clathrin-dependent method of internalization is used by the Semliki forest and Sindbis alphaviruses, the rubella rubivirus, and the
Figure 4.1. Viruses exploit different endocytosis pathways to enter host cells. Multiple mechanisms have been defined as pinocytic, that is, they are involved in the uptake of fluids, solutes, and small particles. These include clathrin-mediated, macropinocytosis, caveolar/raft-mediated mechanisms, as well as several novel mechanisms. Large particles are taken up by phagocytosis, a process restricted to a few cell types. Though poorly documented, the entry of some viruses can be mediated by numerous cargoes which can be endocytosed by mechanisms that are independent of the clathrin coat protein and the fission GTPase, dynamin. These pathways include RhoA- (IL-2 pathway), ARF6-, and CDC42-regulated pathway (GEEC pathway). After binding of the particles to the cell surface entry receptor, genetic material is delivered into the cytoplasm of the cell via a specific endocytosis pathway. Recently, macropinocytosis has emerged as an important entry pathway for viruses (1). The entry of most pH-dependent viruses is mediated by the use of clathrine-coated endocytic vesicles (2). Other viruses penetrate host cells via the formation of vesicles covered by caveola molecule (3). Using this pathway, the viral particles are targeted to neutral pH caveosomes or to early endosomes with moderately acid pH. Alternately, the virus can use non-clathrin, non-caveolin-dependent pathways, both dynamin-dependent or independent (4, 5, 6). These pathways are differentiated by the implication of different GTPases (RhoA, CDC42, or ARF6) and their different compositions (cholesterol, flotillin, TEM, etc).
Hantaan hantavirus, for example (DeTulleo and Kirchhausen, 1998; Helenius et al., 1980; Jin et al., 2002; Kee et al., 2004). Macropinocytosis is another endocytosis pathway utilized by viruses belonging to vaccinia, adeno, picorna, and other virus families. Macropinocytosis is an endocytic mechanism normally involved in fluid uptake induced by growth factors, phorbol ester. However, the binding of virus particles can also activate signaling pathways that trigger actin-mediated membrane ruffling and blebbing. This is followed by the formation of large vacuoles (macropinosomes) at the plasma membrane, internalization of virus particles, and penetration by the viruses or their capsids into the cytosol through the limiting membrane of the macropinosomes (Mayor and Pagano, 2007; Mercer and Helenius, 2009). A variety of evidences suggest that macropinocytosis can be a mechanism for HIV-1 and Epstein-Barr herpes virus entry in some primary cells or cell lines (Marechal et al., 2001; Miller and Hutt-Fletcher, 1992). Based on the more defined criteria of macropinocytosis (Mercer and Helenius, 2009), these conclusions are probably premature and may depend on the experimental conditions that use very concentrated viruses which may influence the utilization of less specific pathways. It should be noted that different strains of the same virus can elicit dramatically different responses in host cells during entry, and different macropinocytic mechanisms are possible in the same cell line through subtle differences in the activating ligand (Mercer et al., 2010a).

An additional endocytosis pathway, whilst badly defined, is known as a clathrin- and caveola-independent pathway. The dependence on different GTPase and cargo proteins (dynamin, Rho, CDC42, ARF6, etc.) defines this endocytosis pathway. Several viruses are using this clathrin- and caveola-independent pathway, including the influenza orthomyxovirus (as a second possible pathway), the SARS-CoV coronavirus, the lymphocytic choriomeningitis arenavirus (LCMV), and picornaviruses (Madshus et al., 1987; Matlin et al., 1981; Wang et al., 2008). Finally, other vesicles (characterized by caveolin or caveosome, with a high concentration in cholesterol and sphingolipids) are clathrin-independent but dynamin-dependent and do not have acid compartments before their merging with early endosomes (Mayor and Pagano, 2007). The nonenveloped SV40 virus and some human enteroviruses are the prototypes of the pH-independent viruses that use this endocytosis pathway. So far, only two enveloped viruses have been described to use this pathway, the Newcastle disease paramyxovirus (NDV) (Cantin et al., 2007) and Ebola virus (Empig and Goldsmith, 2002; Sanchez, 2007). Most viruses have been shown to use only one entry pathway; however, there are more and more recent examples that indicate that some viruses use multiple endocytosis pathways to enter their target cells (influenza, HIV). For example, Ebola viruses are known to enter cells by clathrin-mediated endocytosis (Sanchez, 2007), but lipid raft-associated, caveolin-mediated endocytosis has also been proposed as a mechanism of Ebola virus uptake (Empig and Goldsmith, 2002).
In terms of the activation process of the membrane fusion and the need for particular microdomains, it is not always clear why the viruses are using so many different pathways. It is possible that the viruses that evolved to fuse intracellularly have a selective advantage to release their genome to specific intracellular sites that will allow the rapid and efficient establishment of the infectious cycle. One selective advantage could also be the requirement for particular lipid microdomains, like those enriched in cholesterol. Indeed, it was shown that contrary to the class II flavivirus dengue virus and the yellow fever virus, the E1 fusion protein from Semliki forest virus (SFV) binds cholesterol which explains its dependence for cholesterol and compartment containing cholesterol (Umashankar et al., 2008). For other viruses, the dependence on cholesterol is linked to bulk effects on membrane fluidity and the maintenance of particular microdomains where receptors are located and where they have some particular diffusion.

C. The entry process can be achieved by different number of EnvGP

In addition to these dichotomies of pH-independent or pH-dependent fusion process, of plasma or endosomes membrane fusion, the mechanisms of activation of the fusion proteins of the different enveloped viruses are very diverse. Some reactions of fusion are triggered by the interaction of one envelope glycoprotein with a unique receptor. One example is the interaction of the envelope glycoprotein SU–TM of the γ-retrovirus with the multitransmembrane receptor (Table 4.1). In another case, one unique envelope glycoprotein can interact with several receptors. For example, the fusion of HIV-1 is triggered by the sequential interaction of the SU gp120 with the CD4 receptor, followed by interaction with a coreceptor such as CCR5 or CXCR4, chemokine receptors of seven transmembrane domains (Hunter, 1997). Previously, it was believed that binding to receptors directly triggered a series of conformational changes in the viral EnvGP culminating in fusion of the viral and cellular membranes. However, new evidence suggests that γ-retroviral association with receptors triggers an obligatory cooperative interaction or cross-talk between EnvGP on the viral surface for HIV and MLV (Tailor et al., 2003). If this intermediate step is prevented, infection fails. Conversely, in several circumstances, this cross-talk can be induced in the absence of a cell surface receptor for the virus, in which case, infection can proceed efficiently. This new evidence strongly implies that the role of cell surface receptors in infections of γ-retroviruses (and perhaps of other enveloped animal viruses) is more complex and interesting than was previously imagined.

In all these cases, the EnvGP have a double function attachment to the receptor and an exclusive role in fusion in the same protein. However, for other viruses, these two functions are filled by different proteins. The E2 protein of the
| Virus Family | Virus                                           | Envelope Glycoprotein | Receptor               | Function                                      |
|--------------|------------------------------------------------|-----------------------|------------------------|-----------------------------------------------|
| pH-dependant |                                                |                       |                        |                                              |
| Alpha        | Semliki forest virus (SFV)                     | E3-E2-6K-E1           | MHC-I                  | Immune recognition (1 tmd)                    |
|              | Chikungunya                                    | E3-E2-6K-E1           |                        |                                               |
| Flavi        | Tick-borne encephalitis virus (TBEV)           | PrM-E                 | HS                     | Glycoaminoglycan (1 tmd)                      |
|              | Dengue                                          | PrM-E                 | MR (CD206)             | Mannose receptor                              |
|              |                                                |                       | HS, DC-SIGN?           | Glycoaminoglycan, lectin                      |
|              |                                                |                       |                        | Tetraspanin (adhesion, activation…) (4 tmd)   |
|              |                                                |                       |                        | HDL Receptor (2 tmd)                          |
|              |                                                |                       |                        | Tight junction (4md)                           |
|              |                                                |                       |                        | Tight junction (4md)                           |
| Hepaci       | Hepatitis C virus (HCV)                        | E1–E2                 | CD81, SRB1, Claudin,   |                                              |
|              |                                                |                       |                        | Occludin                                      |
|              |                                                |                       |                        |                                               |
| Orthomyxo    | Influenza A                                    | HA1–HA2               | Sialic acid            | Carbohydrate                                  |
| Rhabdo       | Vesicular stomatitis virus (VSV)               | G                     | PS?                    | Phosphatidylerine                             |
| Bunya        | Haanta virus                                   | G1–G2                 | beta 3 integrin        | Integrin (1tmd)                               |
|              |                                                |                       | DAF(CD55)              | Complement system                             |
| Filo         | Ebolavirus                                     | GP1–GP2               | L-DC-SIGN, hMGL        | C-type lectin (1tmd)                          |
|              |                                                |                       |                        | Endosomal protease                            |
|              |                                                |                       |                        | Folate receptor α (1tmd)                       |
|              |                                                |                       |                        | Phospholipids                                 |
|              |                                                |                       |                        | Glycolipids                                   |
|              |                                                |                       |                        |                                               |
| Rubella      | Rubellavirus                                   | E2-E1                 | PS, PI, PE, PC ganglioside | LDL-R homology (1tmd)                        |
| Retro        | Avian leukemia and sarcoma virus (ALSV)        | Gp85–gp37             | Tva                    | Laminin receptor extracellular matrix         |
| Arena        | Lymphocytic choriomeningitis virus (L-CMV)     | GP1–GP2               | α-Dystroglycan         | Laminin receptor extracellular matrix         |
|              | Lassa virus                                    | GP1–GP2               | α-Dystroglycan         | Laminin receptor extracellular matrix         |

(Continues)
| Virus Family | Virus | Envelope Glycoprotein | Receptor | Function |
|--------------|-------|----------------------|----------|----------|
| pH-Independent Retro | Human immunodeficiency virus 1 (HIV-1) | Gp120–gp41 | CD4, CCR5, CXCR4 | Immune recognition (1 tmd) |
| | Amphotropic Murine leukemia virus (A-MLV) | Gp70–p15 | Pit-2 | Chimiokine receptor (7 tmd) |
| | Feline leukemia virus (FeLV) | Gp70–p15 | Pit-1 | Na-Pi cotransporter (10 tmd) |
| | Gibbon ape leukemia virus (GaLV) | Gp70–p15 | Pit-1 | Na-Pi cotransporter (10 tmd) |
| | Pig endogenous retrovirus A (PERV-A) | Gp70–p15 | HuPAR2, GHBh1 | G-protein coupled receptor (10 tmd) |
| | Feline endogenous retrovirus (RD114) | Gp70–p15 | ASCT-2, SLC1A5 | Cotransport Na-a.a. neutres (7 tmd) |
| | Human T-cell lymphotropic virus type 1 (HTLV-1) | Gp46–gp21 | GluT-1, Neuropilin 1, HS | Transport glucose (12tmd) |
| Paramyxoviruses | Measle virus | H, F | CD46, Sialic acid | Glycoaminoglycan (1 tmd) |
| | Newcastle disease virus | HN, F | SLAM (CD150) | Complement regulator; Ig-like, CD4 regulation |
| | Human parainfluenza virus | H, F2-F1 | Sialic acid | On gangliosides and glycoproteins |
| Alpha-herpes | Herpes simplex virus 1 (HSV-1) | gB, gD, gL, gH | HEM, nectin 1α et β, HS | Ig-like; adherens-junction, synapse (1 tmd) |
| Beta-herpes | Epstein-Barr virus (EBV) | gB, gL, gH | CD21 | Glycoaminoglycan (1 tmd) |
| | Vaccinia virus | L1, A27L, D8L, A33, H3L, B5, etc. | HS, EGF receptor | Complement cascade component |
| | Murine hepatitis virus 4 (MHV-4) | S | Bgp (biliary gp) | Ig-like (1 tmd) |
| | SARS-CoV | S | ACE-2 | Metallocarboxypeptidase |
| | | | | Endosomal protease |
SFV alphavirus binds the receptor that permits the endocytosis of the virus in a compartment where the acid pH will activate the E1 protein that will induce the fusion (Table 4.1). For most paramyxoviruses (including Newcastle parainfluenza virus, human parainfluenza virus type 3 (HPIV-3), mumps virus, bovine RSV (BRSV), ovine RSV (ORSV), and human RSV (HRSV)), the hypothesis is that the binding of HN or G/SH to their receptor induced not only conformational changes in the receptor but also in the F protein with which it interacts. These changes make F competent for the membrane fusion, with the exception of the F protein of the simian parainfluenza virus 5 (SV5), the BRSV, ORSV, and HRSV that do not strictly require HN or G/SH to induce the fusion (though the fusion is greatly increased by HN or G/SH, respectively). This complexity in the distribution of the functions is still more evident in, for example, the alphaherpesvirus (HSV-1, HSV-2) (Rey, 2006). In this case, the binding of the gD glycoprotein to one of its three receptors (HVEM for herpesvirus entry molecule mediator, or nectin 1, or a specific heparan sulfate) activates a complex of gB trimers associated to gL and gH proteins, all the three being essential to the fusion (Gianni et al., 2006; Table 4.1). In the same way as for the hepatitis C virus, the E1E2 heterodimer induces entry following the recognition of at least four receptors, CD81, SRB1, claudin, and ocludine (Zeisel et al., 2009).

Another interesting variation of activation of fusion is the artificial reverse process. In some cases, it was described that some exosomes or pseudoparticle-incorporating receptors were able to enter cells expressing envelope. For example, the multitransmembrane receptor mCAT-1 from ectropic murine leukemia virus (MLV) or the Tva receptor for avian sarcoma-leukosis virus (ASLV-A) can be used to infect cells that express their respective retroviral EnvGP (Balliet and Bates, 1998). Similarly, the incorporation of both CD4 and one other coreceptor of human immunodeficiency virus (HIV), CCR5 (Endres et al., 1997) or CXCR4 (Endres et al., 1997; Mebatsion et al., 1997; Schnell et al., 1997), allows the production of viral particles infecting cells that express simian immunodeficiency virus (SIV) or HIV envelope glycoprotein. Interestingly, it was shown that Nef, an accessory protein of human immunodeficiency virus type 1 (HIV-1) that enhances the infectivity of progeny virions when expressed in virus-producing cells, significantly enhanced the infectivity of CD4-chemokine receptor pseudotypes for cells expressing HIV-1 Env. Surprisingly, Nef also increased the infectivity of HIV-1 particles pseudotyped with Tva, even though Nef had no effect if the pH-dependent Env protein of ASLV-A was used for pseudotyping (Pizzato et al., 2008). This process indicates that the difference of superficial tension between the cell (weak because the radius of the cell is big) and the virus (strong as the radius of the virus is small) is not crucial for the development of an oriented fusion process with EnvGP on the one side and receptor on the other. Having said that, the cell is not an empty bubble, and this efficacy of the fusion process regardless of which membrane harbors the receptor
or the EnvGP may reflect the adaptation of the virus to optimize the membrane merging independently of the superficial tension. Cells are not completely round, and superficial tension depends on the localization where the virus binds. Moreover, the plasma membrane has different lipid compositions and interacts with the cytoskeleton which will attenuate or increase the superficial tension at certain localizations.

D. Structural classification: Class I, class II, and class III fusion proteins

Glycoproteins from enveloped viruses have evolved to combine two main features. First, they have the capacity to bind with a specific cellular receptor and second, they include a fusion domain (peptide fusion and transmembrane domain) that can be activated to mediate the merging (fusion) of viral and cellular membranes.

Three different classes of viral fusion proteins have been identified to date based on key structural features at pre- and postfusion stages. Many studies have demonstrated that the structural transition from a pre- to a postfusion conformation leads to a stable hairpin conformation. This includes class I fusion proteins, characterized by trimers of hairpins containing a central alpha-helical coiled-coil structure, and class II fusion proteins, characterized by trimers of hairpins composed of beta structures. A third class of fusion proteins has been described recently, that also forms trimers of hairpins by combining the two structural elements alpha-helix and beta-sheet structures (Roche et al., 2006, 2007).

The synthesis and the conformation of these classes I, II, or III fusion proteins are different. Class I viral fusion proteins of diverse virus families, including Retroviridae, Filoviridae, Orthomyxoviridae, Paramyxoviridae, and Coronaviridae, differ greatly in size and amino acid sequence, but their membrane-anchored domains share common structural features that are essential for membrane fusion, including two heptad repeats (called HR-1 and HR-2), preceded by a hydrophobic fusion peptide. Class I membrane-fusion reaction is mediated by the refolding of the fusion protein to a highly stable rod-like structure with a central trimeric α-helical coiled coil. Such coiled-coil structures are emblematic of class I proteins, and physical demonstration or computer prediction of such a structure is frequently used to help define a fusion protein as belonging to class I. The envelope glycoprotein of a retrovirus is generated by the cleavage of its precursor in one SU and one transmembrane subunit containing an anchoring sequence to the membrane (Hunter, 1997). This maturation, essential to the process of fusion, frees a hydrophobic sequence to the N terminal extremity of the TM, named fusion peptide, as it is supposed to insert into the target membrane and initiates the fusion. Initially, the fusion peptide is masked inside the trimer of EnvGP, a form competent for the fusion (Fig. 4.2A).
In general, the cleavage of the fusion proteins is mandatory for most class I proteins to render them competent for fusion (Earp et al., 2005; Harrison, 2005). Exceptions are the envelope glycoprotein from Ebola or SARS-CoV viruses, classified as class I on the grounds of the three-dimensional structure of one of their fragments, which are not cleaved but yet are functional. Though an N-terminal fusion peptide is predicted in the potential transmembrane subunit (localized by analogy to homologous viruses), their functionality can be explained also by the presence of an internal fusion peptide (Ebola). Another explanation of their functionality is their requirement for the L or B cathepsins that cleave the EnvGP during endocytosis (see below). The number of complexes on the surface of viruses harboring class I fusion proteins is very variable. It seems that lentiviruses have only 10–20 SU–TM trimers, whereas the coronaviruses have hundreds of trimers coating the surface, giving them their characteristic morphology of “crown” which is the origin of the name of this family (Tables 4.1 and 4.2).

The process of assemblage and biosynthesis of class virus II is very different to the class I proteins (Kielian and Rey, 2006) (Fig. 4.2B). During biosynthesis, the alphavirus (E1) and flavivirus (E) fusion proteins fold cotranslationally with a companion or regulatory protein, termed p62 (or PE2) for alphaviruses and prM for flaviviruses (Garoff et al., 2004). This heterodimeric interaction is important for the correct folding and transport of the fusion protein. Both p62 and prM are cleaved by the cellular protease furin late in the secretory pathway, in a maturation reaction that is a crucial regulatory step for subsequent virus fusion (Salminen et al., 1992; Stadler et al., 1997; Wengler, 1989; Zhang et al., 2003a). Though this cleavage in the envelope glycoprotein complex is important, contrary to class I fusion protein, it is not crucial, since mutated alphavirus and flavivirus for which the regulating proteins are not cleaved have only a decreased infectivity (Salminen et al., 1992; Zhang et al., 2003b). In the case of the SFV alphavirus, the fusion process induced by the uncleaved fusion proteins can be a trigger after treatment of the virus at pH5 or less, rather than the normal fusion threshold pH 6 of the wild-type virus (Salminen et al., 1992; Zhang et al., 2003a). This variation in the threshold of pH of the fusion is necessary for the dissociation of the heterodimer. The class II fusion proteins are either homodimers or heterodimers (E homodimer for the flaviviruses or E2–E1 heterodimer for alphaviruses) that form an envelope netting the viral membrane (Lescar et al., 2001; Rey et al., 1995). The internal fusion peptide is masked at the interface of the dimers at the extremity of a long beta sheet. One important difference between these two groups of viruses is the budding site (Garoff et al., 2004). In alphaviruses, the p62–E1 complex is transported to the plasma membrane, and the heterodimer interaction is maintained after p62 processing. New virions bud at the plasma membrane, in a process that is driven by lateral contacts between E2 and E1 heterodimers (E2 being the mature companion protein) to induce the required curvature of the lipid bilayer, in combination
Figure 4.2. Possible fusion models have evolved for class I (A), class II (B), and class III (C) fusion proteins from their pre- to postfusion conformations. Ambiguities remain regarding certain events in membrane fusion promoted by a viral fusion protein. Despite the diversity in the structure of the fusion proteins, the major steps of the fusion process are similar. The first step, after EnvGP activation upon receptor binding or acidification
with interactions of the cytosolic tail of E2 with the nucleocapsid. Budding results in the formation of icosahedral-enveloped particles of triangulation $T = 4$, containing 80 trimeric E2/E1 spikes.

By contrast, flavivirus particles bud into the endoplasmic reticulum as immature virions formed by 60 trimers of prM–E. The immature particles have an organization similar to that of mature alphaviruses, with each trimer forming a

of the endosomal compartment, exposes the fusion peptide that is projected toward the top of the glycoprotein, allowing the initial interaction with the target membrane. The second step is the folding back of the C-terminal region onto a trimeric N-terminal region that leads to the formation of a postfusion protein structure. The third and final step also requires further refolding of the membrane proximal and transmembrane regions in order to obtain a full-length postfusion structure where both membrane anchors (fusion peptide and tm domains) are present in the same membrane. Three different classes of fusion have been identified so far based on common structural motives. (A) The class I fusion proteins are characterized by trimers of hairpins containing a central alpha-helical coiled-coil structure. For retroviruses, receptor binding induces the movement of the SU, allowing a loop-to-helix transition of a polypeptide segment of TM that was previously buried underneath the SU heads, projecting the fusion peptide \( \sim 100 \, \text{Å} \) toward the target membrane, where it inserts irreversibly. This occurs by a “spring-loaded” mechanism. The HR2 C-terminal end (green) of the long TM \( \alpha \)-helix jackknifes back, reversing the direction of the viral-membrane-proximal segment of TM, which then interacts in an antiparallel fashion with the groove formed by the N-terminal HR1 (blue) trimeric coiled coil. The final postfusion conformation of TM is, therefore, a highly stable rod with the TM and fusion-peptide segments together at the same end of the molecule, a structure termed a “trimer of hairpins” or helix budle (HB). (B) Class II fusion proteins are characterized by trimers of hairpins composed of beta structures. The red, yellow, and blue parts of each subunit correspond, respectively, to domains I, II, and III of the ectodomain. The fusion loop is at the tip of domain II. Monomeric transition between the prefusion dimer and the trimeric-extended intermediate is shown. After exposure to the low pH of the endosomes, domains I and II swing outward, while domain III and the stem remain oriented against the membrane roughly similar to the prefusion state. The fusion loop, at the top of the diagram, interacts with the target bilayer. Domains I and II associate into the trimeric core of the postfusion conformation, and domain III must then zip back along the trimer core, thus reorientating the domain III. (C) A third class of fusion proteins has been described recently, which also forms trimers of hairpins by combining the two structural elements alpha-helix and beta-sheet structures. Class III fusion proteins are composed of five domains that give rise to a molecular architecture very distinct from any reported class I or class II fusion proteins. Interestingly, the ectodomain of G has been crystallized in its pre and postfusion (low-pH) state. During the conformational change that occurs upon low pH exposure, the domains of G radically change their position and orientation as a result of rearrangements that occur in the linker regions. Domain I (yellow), carrying the fusion loops, and the transmembrane domain move 16 nm from one end of the molecule to the opposite (Backovic and Jardetzky, 2009). Only domain III (blue) undergoes significant refolding with extension of the central helix F. To complete the process, the C-terminal helices of domain IV (red) insert into crevices formed by two other protomers in the postfusion form, reminiscent of the structural changes observed during refolding events of class I fusion proteins with HB formation.
spike in which prM covers the fusion protein E. The newly formed virions are then transported to the external milieu through the exocytic pathway. Processing of prM generates the mature M protein with a short (~40 residues) ectodomain (Kuhn et al., 2002; Zhang et al., 2003b). Presumably because of the removal of a large portion of the prM ectodomain, the flavivirus surface dramatically reorganizes after processing to give 90 E–E homodimers arranged with icosahedral symmetry. The mature flavivirus particles display a smooth, spikeless surface, with E dimers ordered in a characteristic “herring bone” pattern.

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**Table 4.2. Classification of Fusion Proteins Based on Their Family, Class, and Activation Mechanism**

| Virus family | Virus species | Fusion proteins | Fusion pH for activation |
|--------------|---------------|-----------------|--------------------------|
| **Class I**  |               |                 |                          |
| Orthomyxoviridae | Influenza A virus | HA2             | Low                      |
|               | Influenza C virus | HEF             | Low                      |
| Paramyxoviridae | Simian parainfluenza virus 5 | F (F2–F1) | Neutral                  |
|               | Newcastle disease virus | F              | Neutral                  |
|               | Respiratory syncytial | F              | Neutral                  |
| Filoviridae   | Ebola virus    | Gp1-Gp2         | Low (for cathepsin cleavage) |
| Retroviridae  | Moloney Murine leukemia virus | TM (gp21) | Neutral                  |
|               | Human immunodeficiency virus 1 | gp41          | Neutral                  |
|               | Simian immunodeficiency virus | gp41          | Neutral                  |
|               | Human T cell leukemia virus 1 | gp21          | Neutral                  |
|               | Human syncytin-2 | TM              | Neutral                  |
|               | Visna virus     | TM              | Neutral                  |
| Coronaviridae | Mouse hepatitis virus | S2             | Low (for cathepsin cleavage) |
|               | Sars corona virus | E2              | Low (for cathepsin cleavage) |
| **Class II**  |               |                 |                          |
| Flaviviridae  | Tick-borne encephalitis virus | E              | Low                      |
|               | Dengue virus   | E               | Low                      |
| Togaviridae   | Semliki forest virus | E1             | Low                      |
| **Class III**|               |                 |                          |
| Rhabdoviridae | Vesicular stomatitis virus | G              | Low                      |
| Herpesviridae | Herpes simplex virus | gB            | Neutral                  |
|               | Eptsein Barr virus | gB            | Neutral                  |

There is no correlation between these criteria for class I and class III fusion proteins, but all the viruses harboring class II fusion proteins are pH-dependent.
Finally, the four class III fusion proteins resolved (VSV-G, gB of HSV1 and EBV and baculovirus gp64) in spite of their homology of trimeric structure have some major functional differences arising from their difference of biosynthesis. G protein is the only class III fusion protein whose prefusion structure is known. The proteins of class fusion III have a combination of the two structural elements, alpha helix like class I and beta sheet like class II (Backovic et al., 2009; Heldwein et al., 2006; Kadlec et al., 2008; Roche et al., 2006, 2007) (Fig. 4.2C). The trimers are maintained by interaction between central alpha helixes, but each domain of fusion exposes two buckles of internal fusion peptide placed at the extremity of a long beta sheet. The G protein is the only protein responsible for the binding and the entry of the VSV. The rhabdovirus VSV possesses 1200 molecules of the G protein on its surface and forms 400 trimers. In contrast, the HSV-1 virus incorporates 12 different EnvGP on its surface, of which 4 are essential for the entry step (gD, gB, and gH/gL) (Turner et al., 1998). During their biosynthesis, neither the G protein of VSV nor gB of HSV-1 are cleaved, and they present internal fusion peptides. However, whereas the G is functional by itself, the gB envelope glycoprotein alone is not sufficient to induce the entry of the virus or the membrane fusion. Nevertheless, currently, no precise interaction between gD, gB, and gH/gL has been identified, even though the gD ectodomain has been shown to allow the entry of engineered HSV-1 virus particles that lack gD (that is gD-null mutants; Cocchi et al., 2004). Gp64 is the major component of the viral envelope, and the sole fusogenic proteins that are triggered to induce the fusion in the low pH environment of endosomes for baculovirus. Interestingly, the distinguishing feature between G and gp64 and any other fusion protein is that they can undergo a reversible conformational change, unlike class I and most class II fusion proteins, for which the postfusion conformation is thermodynamically more stable at all pH values, and the conformational rearrangement is effectively irreversible. VSV or gp64 exposure to low pH inactivates the virus, but the fusion activity can be fully recovered when the pH is raised. It has been proposed that the reversibility of the conformational change allows G to avoid unspecific activation during transport through the acidic Golgi vesicles.

As seen in the previous sections, though there is much described variation in the manner of activation of the fusion proteins and additional mechanisms await discovery, only three classes of fusion proteins have been defined so far (Weissenhorn et al., 2007). As previously described, based on the main structural organization of their EnvGP, the viruses are now assigned to the class I, II, or III. In parallel, based on the activation mechanism of the fusion protein, viruses have been classified as pH-dependent or -independent. Interestingly, the relationship between the mechanism of activation of the fusion and the class of protein of fusion is not correlated. The HIV or Influenza EnvGP are both class I and yet they have a process of activation that is pH-independent and
-dependent, respectively, for the entry of the virus. Similarly, the class III fusion proteins enclose the Herpes virus simplex 1 (HSV-1) gB protein and the rhabdovirus VSV-G protein that are pH-independent and pH-dependent, respectively, for their activation. So far, all the class II fusion proteins have been pH-dependent for their activation.

E. Common refolding process

Although there are notable differences between the activation processes, the structural motives used, and the initial oligomeric states of the fusion proteins (the native trimeric conformation of class I and III proteins in opposition to the homo- or heterodimers of the class II fusion proteins), the common features of the final structures obtained after fusion seem to suggest some generic mechanism of conformational changes common to all EnvGP of enveloped viruses (see Fig. 4.2).

First, the activation of the fusion protein following the interaction with the cellular receptor(s), coupled or not to the exposure to the acid environment of the endosomes, exposes the fusion peptide that is projected toward the top of the glycoprotein, allowing the initial interaction with the cellular target membrane. For class I fusion proteins, the proposed model indicates that the transition of conformation requires the transformation of a part of the molecule in alpha helix and the association of this in three helixes bundle (named the “coiled coil”; Weissenhorn et al., 2007). For retroviruses, this movement allows a loop-to-helix transition of a polypeptide segment of TM that was previously buried underneath the SU heads, projecting the fusion peptide toward the target membrane, where it inserts irreversibly. In the case of class I fusion proteins like retroviruses, this occurs by a “spring-loaded” mechanism. This initial change is proposed to result in a “prehairpin intermediate,” an extended structure that is anchored both in the target membrane by the fusion peptide and in the virus membrane by the TM transmembrane segment. For the class II fusion proteins, the projection of the fusion peptide requires the dissociation of the hetero- or homodimers and modifications in the “hinge region” unstructured before conformational changes (Stiasny and Heinz, 2006). For the class III (Roche et al., 2008), similarly to the fusion proteins of class I, the exhibition of the fusion peptide requires a rearrangement of the domains mediated by modification of the central helixes that do remain parallels.

Second, the folding back of the region including the fusion peptide onto a trimeric C-terminal region leads to the formation of a postfusion protein structure with the outer regions zipped up against an inner trimeric core. Interestingly, it has been described that all the class I, II, and III peptides can inhibit this step by competing with the interaction of EnvGP-specific domain for the formation of this structure.
Third, the final steps require further refolding of the juxtamembrane and transmembrane regions to obtain a stable postfusion structure with the fusion peptides and the transmembrane domains at the same extremity of a stable stem of a protein complex anchored in the target membrane. This structure brings the two membranes proximal and provides free energy to overcome the barrier of membrane merging (Melikyan, 2008). Membrane fusion occurs, which leads to pore formation and release of the viral genome into the cytoplasm.

The refolding of class II fusion proteins generates trimers from monomeric intermediate. The existence of monomeric intermediates for class I and III is not well known. However, if the steps that led to exposition of the fusion peptide and its interaction with the membrane targets maintain a three-order symmetry, the refolding of the C-terminal region requires the destruction of the trimer at least to the juxtamembrane region. Moreover, from the differences observed between the amino acids involved in the interface of the reconstituted resolved trimers in their pre- and postfusion conformations, it is probable that the conformational changes are going through a monomeric intermediate for the class III G envelope glycoprotein of VSV and the class I F protein of paramyxovirus. On the contrary, the interface of the class I HA2 subunit of the HA envelope glycoprotein trimer is very similar between the pre- and postfusion conformation, shedding doubt on the existence of monomeric intermediates. In contrast, when fusion is initiated at low pH, the dimers of the class II fusion proteins are broken, freeing monomers that reassociate in trimers.

Finally, precise structural information of the native metastable conformation (prefusion) and the final stable conformation (postfusion) is available only for a limited number of viruses (for envelope glycoprotein of influenza virus, SFV, TBEV, VSV, and the parainfluenza 5 F protein). The structural conversion of the native metastable conformation in a final stable conformation is not precisely known and is highly speculative for most viruses, and the envelope glycoprotein domains implied in these molecular rearrangements are little-referenced. Clearly, more studies are necessary to identify the intermediaries of envelope glycoprotein conformations. These intermediates would identify the domains that interact during the conformational changes which will highlight ways to generate inhibitory peptides.

**F. Domain organizations/fusion domains**

1. Acquisition of fusion competence: Priming by cleavage

During virus production, the host cell is basically preserved, since the expression of fusogenic competent glycoproteins is highly controlled for most viruses. However, for some viruses, the EnvGP induce a cytopathic effect that leads to
the generation of multinucleated cells, called syncytia, which are induced by the fusion of cells that express the envelope glycoprotein and receptors. Abundant glycoprotein at the surface of the cell could induce cellular death by syncytia formation, toxicity via receptor interaction, or immune recognition. For these reasons, the localization and the amount of the oligomerized envelope glycoprotein at the host cellular surface are highly modulated by fine trafficking and sequestration mechanisms. The receptor interference mechanism (either by saturation of binding site on receptor by envelope glycoprotein or by internalization of receptor by different viral proteins, as it is achieved by some retroviruses; Hunter, 1997) can also limit the amount of receptors available for fusion between infected cells. The control of the cleavage of the EnvGP to free the fusion peptide is also a regulation process of the fusion protein fusogenicity (Labonte and Seidah, 2008). Finally, EnvGP fusion competency may be a late event that occurs during virus budding, as described for MuLV retroviruses (Rein et al., 1994).

Proteolytic priming is a common method of controlling the activation of membrane fusion mediated by viral glycoproteins. The members of the proprotein convertase (PC) family play a central role in the processing and/or activation of various protein precursors involved in many physiological processes and various pathologies such as neurodegenerative pathology, cancer bacterial toxins activation, and viral infections. The proteolysis of these precursors that occurs at basic residues within the general motif (K/R)-(X)-(K/R) is mediated by the proprotein convertases PC1/3, PC2, Furin, PACE4, PC4, PC5 (also called PC6), and PC7 (also called PC8, LPC, or SPC7), whereas the proteolysis of precursors within hydrophobic residues performed by the convertase S1P/SKI-1 and the convertase NARC-1/PCSK9 seems to prefer to cleave at a LVFAQSIP motif (Lahlil et al., 2009). The seven PCs have different, albeit partly, overlapping expression patterns and subcellular localization. They have conserved aminotermini with highest homology in the subtilisin-like catalytic domain.

Data on various infectious viruses revealed that the cleavage of their envelope glycoprotein precursors by one or more PCs is a required step for the acquisition of the infectious capacity of viral particles. Indeed, various studies have demonstrated the capacity of the PCs to correctly cleave a variety of viral surface glycoproteins. These include the HIV-1 gp160 (Decroly et al., 1996) and surface glycoproteins of Hong Kong, Ebola virus, the severe acute respiratory syndrome coronavirus and chikungunya virus (Basak et al., 2001; Bergeron et al., 2005; Ozden et al., 2008). In parallel, other studies revealed that the inhibition of processing of these viral surface glycoproteins by the PC inhibitors such as dec-R-V-K-R-CMK completely abrogated the virus-induced cellular cytopathicity. The surface glycoproteins of other viruses, particularly the hemorrhagic fever viruses ( Arenaviridae family), such as Lassa (Basak et al., 2002; Lenz et al., 2001), Crimean Congo hemorrhagic fever (Vincent et al., 2003), and lymphocytic choriomeningitis (Beyer et al., 2003), were shown to be cleaved by the
convertase SKI-1 that cleaves at hydrophobic residues. Similarly, blocking of SKI-1 activity by a specific inhibitor has also shown to affect the processing and the stability of the glycoproteins of these viruses (Pullikotil et al., 2004).

For all highly pathogenic avian influenza (HPAI) viruses of subtypes H5 and H7 known to date, the cleavage of HA occurs at the C-terminal R residue in the consensus multibasic motifs, such as R-X-K/R-R with R at position P4 and K-K/R-K/T-R with K at P4, and leads to systemic infection. Early studies demonstrated that the ubiquitously expressed furin and proprotein convertases (PCs 5 and 6) are activating proteases for HPAI viruses (Basak et al., 2001; Stieneke-Grober et al., 1992). Recently, ubiquitous type II transmembrane serine proteases, MSPL and its splice variant TMPRSS13, have been proposed as novel candidates for proteases processing HA proteins of HPAI (Okumura et al., 2010).

Is it interesting to note that viral receptors can also be modified by proprotein convertase. Indeed, PCSK9 impedes hepatitis C virus infection in vitro, modulates liver CD81 expression, and enhances the degradation of the low-density lipoprotein receptor (LDLR) (Labonte et al., 2009), bestowing on the proprotein convertase an additional role in controlling the fusogenicity of the envelope glycoprotein.

2. Fusion peptide

The exhibition and insertion of a hydrophobic fragment of 10–30 residues in the membrane, named “fusion peptide” or “fusion loop” is a crucial step of the fusion process (Epand, 2003). The fusion peptides in an N-terminal position (such as for the retrovirus or the influenza virus) is liberated for most viruses after envelope glycoprotein cleavage, and it can insert into the external layer of the membrane in an oblique manner, whereas the fusion loop (for the class II and III viruses) remains probably more superficial (see Table 4.3). The fusion peptide of the class I and II proteins is initially buried in the envelope glycoprotein trimer or dimer, respectively. For the class III, the fusion loop is present outside of the structure, most likely because the fusion peptide of class III fusion proteins is weakly hydrophobic and probably requires a cooperation between several loops to be functional and efficient. The simple picture of a viral fusion protein acting on cell and viral membranes by means of only two restricted segments, that is to say, the fusion peptide and the transmembrane domain, is too simplistic. Instead, a more complex concerted action of different membranotropic segments of the fusion proteins is necessary. More conformational changes are required to achieve a complete fusion of the two lipid bilayers. As described previously, the class I–III fusion proteins roughly share common refolding processes and formation of intermediates. Several regions of the fusion protein complex indirectly aid the fusion process, as for example, the “stem” regions (see below).
Contrary to the relatively simple and canonical organization of the fusion peptides for the influenza virus or the flavivirus E protein, the recently resolved structures of the gB glycoprotein of the simplex herpes type 1 virus (Heldwein et al., 2006) and G protein (Roche et al., 2006, 2007) of the vesicular stomatitis virus (VSV) indicated a bipartite fusion peptide composed of two hydrophobic loops, each loop being relatively nonpolar or very weakly hydrophobic (which rarely leads to the identification of a fusion peptide by fusion peptide prediction based on hydrophobic domain identification). These differences in the organization of the fusion peptides suggest differences of action of the fusion proteins, notably in the number required. According to experiments using neutralization assays, it seems that one HIV envelope glycoprotein is capable of inducing membrane fusion. However, it has been shown that the induced fusion by HA requires a collaboration of several complexes of envelope glycoprotein (8–9 trimers). In the same way, different networks of class II fusion proteins have been proposed, such as the hexagonal organization observed on the surface of liposomes or an association of five trimers in a structure similar to a volcano based on structure predictions (Stiasny and Heinz, 2006). Concerning the fusion protein of class III, a hexagonal structure has been proposed for rabies virus envelope glycoprotein and recently for VSV-G according to a modeling using its structure. The requirement for multiple fusion peptides may be compared to the

| Fusion peptide          | Class I | Class II | Class III                                      |
|------------------------|---------|----------|-----------------------------------------------|
| Initial situation      | Buried in trimer interface | Buried in the dimer interface | Buried in the interface between different trimers |
| Localization           | N-term (HIV, HA2, etc.) or internal (RSV, Ebola, etc.) | Internal loop embedded between 2 beta-strands | 2 internal loop (segmented fusion peptide; nonobvious on primary sequence) |
| Structure flexibility   | Alpha helix ↔ random coil/turn | Stable random ↔ coil and turn | Alpha helix ↔ random coil/turn |
| Interaction with membrane | Insert into one bilayer leaflet | Stay at the membrane surface (insert into hydrocarbon chains of the outer leaflet) | Stay at the membrane surface (insert into hydrocarbon chains of the outer leaflet) |
| Maturation to prefusion state through Activated in the glycoprotein complex | Proteolytic processing of fusion protein (except Ebola, Sars) | Proteolytic processing of companion protein | No proteolytic process |
|                         | 2 proteins (PIV 5) | 2 identical or different proteins (SFV, TBEV) | 1 uncleaved protein (G) |
|                         | 1 cleaved protein (HA) |                                       | 3 proteins (gB with gH/gL) |
|                         | 1 uncleaved (Ebo, SARS) |                                       |                             |

Table 4.3. Fusion Peptide Characteristics from the Fusion Protein from Different Classes
number of receptors required. The assembly of a complex containing several receptors may be a prerequisite for the membrane fusion steps that require multiple EnvGP molecules to cooperatively participate in this process. For example, in the case of HIV-1, the presence of more than one CD4 in contact with the virus enhances the infectivity dramatically and reduces the concentration of coreceptors needed for infection (Platt et al., 1998). Further investigation of this system has implied that a critical complex containing approximately four to six coreceptors is a requirement for infection, although it is not known whether this complex performs a transient role and then disperses or is maintained throughout the membrane fusion process (Kuhmann et al., 2000). Despite some uncertainties, several lines of evidence have suggested that three to six hemagglutinin trimers may cooperatively participate in the influenza A virus-mediated membrane fusion reaction (Blumenthal et al., 1996; Boulay et al., 1988) and that multiple envelope glycoprotein trimers are required for rabies virus-mediated membrane fusion (Roche and Gaudin, 2002). See Table 4.3.

3. The role of cytoplasmic tail in fusion and influence of its length

The cytoplasmic tails of envelope viruses harbor different motifs that are responsible for its trafficking and are variable in length. It is surprising to see that the cytoplasmic tails of fusion proteins are not exchangeable. For example, when the HCV E1 and E2 cytoplasmic tails or the F cytoplasmic tails of HRSV (Human Respiratory Syncytial virus) are substituted for that of VSV-G envelope glycoprotein (or CD4), the fusogenicity of these envelopes in cell–cell fusion assays and virus–cell assay (infection) is destroyed (Buonocore et al., 2002; Oomens et al., 2006). Similarly, when the HA glycoprotein is anchored by a GPI, the entry process is stopped at the hemifusion step (Kemble et al., 1994; Markosyan et al., 2000). The cellular localizations (e.g., cholesterol-rich microdomains) are sometimes modified, and biochemical modifications (glycosylation, oligomerization) can affect the properties of the VSV-G EnvGP (Kemble et al., 1994). Nevertheless, in their initial context, these cytoplasmic tails allow fusion.

For some viruses, regulation of fusion is mediated by the cleavage of the cytoplasmic tail. For γ-retrovirus, in addition to the SU–TM cleavage, a significant fraction of virion-associated TM is further processed by the viral protease removing the C-terminal 16 amino acids of the cytoplasmic domain, the R peptide. γ-Retrovirus virions assemble and bud from infected cells as immature particles that must undergo an additional proteolytic maturation to become infectious (Green et al., 1981; Lavillette et al., 1998, 2002; Rein et al., 1994). This maturation concerns the viral protease-dependent cleavage of the so-called peptide R at the C-terminus of the cytoplasmic tail. The R peptide inhibits fusion, and different hypothesis have been proposed. First, the R peptide contains
an Y-X-X-internalization motif, and the removal of this motif following the cleavage of the R peptide might result in a higher amount of envelope at the surface membrane and thus more fusion (Song et al., 2003). Second, following the R peptide cleavage, the remaining cyt tail forms a membrane-embedded amphiphilic alpha-helix domain that destabilizes the membrane (Rozenberg-Adler et al., 2008; Zhao et al., 1998). Third, it has been proposed that as the R peptide contains a palmitoylation, its removal induces the close trimerization of the cyt tail and drastic conformational changes in the ectodomain of Env (Aguilar et al., 2003) which might influence Env fusogenicity by destabilizing SU–TM complexes. These conformational changes are necessary for the isomerization of the SU–TM disulfide MLV Env (Loving et al., 2008). This R peptide cleavage is the last step leading to a fusion competent infectious MLV retrovirus, but this final modification does not exist in lentiviruses which harbor a long cytoplasmic tail. However, truncations of the long cytoplasmic domains of lentiviral Env proteins occur under certain culture conditions (Chakrabarti et al., 1989) and increase Env fusogenicity in a similar way to mutated truncated versions of SIV, HIV-1, and HIV-2 Envs (Mulligan et al., 1992; Spies et al., 1994; Wilk et al., 1992). This regulation is a hallmark of adaptation of endogenous retroviruses, as this cleavage is fulfilled by a cellular protease that activates the endogenous EnvGP HERV-W in relevant tissues involved in placenta development. It is interesting to note that for most viruses (and not only the γ-retrovirus), the truncation of the cytoplasmic tail increases the fusogenicity of the EnvGP, as is seen in paramyxoviruses (Moll et al., 2002). In many cases, this truncation seems to increase the cell surface expression (since cellular trafficking signals in the cytoplasmic tails are modified), which may explain the increase of fusogenicity. For certain other Env, the truncation leads to conformational changes in the ectodomain which lowers the activation threshold for fusion, resulting in enhanced Env fusion activity and kinetics (Aguilar et al., 2003; Cote et al., 2008; Spies et al., 1994; Zhao et al., 1998). Finally, the cleavage of the cytoplasmic tail sometimes allows, at least partially, a cell–cell fusion at neutral pH (pH-independent), although the entry of the virus remains pH-dependent. The notion of pH-dependence, seems in this case, to be due to a specific conformation or a particular density of envelope glycoprotein (Cote et al., 2008).

4. The transmembrane proximal region

Numerous studies on several viruses have highlighted the critical role of the pretransmembrane sequence (PTM), also called the membrane proximale region or the juxtamembrane domain (JMD), which is rich in aromatic amino acids. The class I fusion glycoproteins of coronavirus, lentivirus (HIV, SIV, FIV), ebola virus (Munoz-Barroso et al., 1999; Saez-Cirion et al., 2003), and many other
viruses contain this short JMD region in the ectodomain between the end of HR-2 and the beginning of the transmembrane (TM) domain (Salzwedel et al., 1999). The class II (SFV, dengue, TEBV) and class III (VSV and the herpes virus) fusion proteins also possess these regions, although they are less rich in tryptophan than the class I JMD (Jeetendra et al., 2002, 2003; Roche et al., 2008). Although the JMDs of fusion proteins of enveloped viruses are rich in aromatic amino acids, the number, spacing, and sequence of the aromatic amino acids are quite variable; however, the function remains the same. These JMDs contribute to the conformational changes that occur during membrane fusion, interact with membranes, induce membrane destabilization, and/or facilitate membrane fusion (Munoz-Barroso et al., 1999). Therefore, the JMD of viral fusion proteins is a potential target for viral inhibitors. Entry inhibitors that target the JMD of class I fusion proteins include monoclonal antibodies that bind the JMD of gp41 to the FIV and to the HIV (Lorizate et al., 2006; Purtscher et al., 1994). Some peptides that mimic the JMD have been designed for EnvGP of FIV (Giannechi et al., 2004), HIV (Moreno et al., 2006), Ebola virus (Saez-Cirion et al., 2003), and the SARS virus (Howard et al., 2008) and inhibit viral entry. This strategy has been also broadly used against many other paramyxoviruses such as the Sendai virus (Joshi et al., 1998; Rapaport et al., 1995), the Newcastle disease virus (Young et al., 1999), the human parainfluenza type 3 (HPIV-3) (Yao and Compans, 1996), the respiratory syncytial virus (RSV), and the measles virus (MV) (Lambert et al., 1996). In the same way, peptides that mimic the JMD from class II and III fusion proteins have also been developed against infection by Dengue virus (Hrobowski et al., 2005) and CMV (English et al., 2006; Lopper and Compton, 2004). In the case of HCV, some juxtamembrane domains have been proposed in E1 and E2 (Drummer and Poumbourios, 2004; Drummer et al., 2007). High-throughput screening (HTS) of peptides derived from E1 and E2 sequences has identified inhibitory peptides close to the transmembrane domain of E2, though they are not among the most inhibitory (Cheng et al., 2008). However, these strategies suffer from certain limitations. The derived peptides of these JMD often have the capacity to oligomerize, which inactivates them, and some stratagems need to be implemented to make the peptides more bioreactive against viruses. Moreover, these peptides that prevent the correct conformational change of the envelope glycoprotein, must act in a certain window of time and in a particular compartment compatible with their active structure. Indeed, the acid pH of some compartment is not compatible with the bioactive structure of the peptide. For the SARS-CoV virus, the peptide is able to inhibit the entry of the virus in the presence of protease at the cellular surface, but the peptide has little effect on the entry of the virus by endocytosis or on the activation by cathepsin L in the acid conditions of the endosome (Ujike et al., 2008). In the case of Influenza, it has not been possible to develop such inhibitory peptides targeting the juxtamembrane domain. Reasons suggested for this include the
entry through acid compartments which modify protein structures, the entry by different pathways of endocytosis, and the rapidity of the conformation changes of HA. Some modifications can be made to these peptides to increase their efficiency for viruses that fuse at the plasma membrane. For three paramyxoviruses, HPIV-3, a major cause of lower respiratory tract diseases in infants, and the emerging zoonotic viruses Hendra virus (HeV) and NiV, which cause lethal central nervous system (CNS) diseases, the addition of cholesterol to a paramyxovirus HRC-derived peptide (derived from the heptad repeat immediately preceding the transmembrane domain) increased antiviral potency by 2 log units (Porotto et al., 2010). This enhanced activity is the result of the targeting of the peptide to the plasma membrane. The cholesterol-tagged peptides on the cell surface create a protective antiviral shield, target the F protein directly at its site of action, and expand the potential utility of inhibitory peptides for paramyxoviruses.

III. HOW VIRUSES SUBVERT DIFFERENT CELL PROTEINS FOR ENTRY?

A. Definition of receptors, adsorption molecules, and cofactors

The definition of a receptor is very complicated and has limitations. It is sometimes difficult to distinguish between “simple” receptors that mediate adsorption or binding and that may not even initiate conformational changes, and “critical” receptors for fusion which, upon binding, will generate the conformational changes that will allow the exposure of the fusion peptide and lead to membrane fusion. Some cellular molecules are also involved in the localization or trafficking of viral receptors and these are important cofactors of entry. Another ambiguity is the role of enzymatic activities. Some are necessary to process the fusion protein inside producer cells, or inside the endosomes of target cells, and they are necessary to activate the potential of the EnvGP for membrane fusion. While it is inaccurate to consider them as “receptors”, they are certainly critical cofactors.

1. Virus adsorption

All viruses likely bind at least weakly to multiple cell surface components such as heparan sulfate proteoglycans, DC-SIGN, integrins, or glycolipids (Bounou et al., 2002; Cantin et al., 1997; Fortin et al., 1997; Jinno-Oue et al., 2001; Mondor et al., 1998; Saphire et al., 1999, 2001). Although such binding substances probably do not induce conformational changes in EnvGP that are necessary for membrane fusion, they can enhance viral adsorption and substantially increase efficiencies
of infections, thus contributing to pathogenesis (Alvarez et al., 2002; Bounou et al., 2002; Geijtenbeek et al., 2000; Jinno-Oue et al., 2001; Saphire et al., 2001). Because such binding proteins contribute to infections, it can be difficult to unambiguously distinguish them from receptors that directly mediate the membrane fusion process, especially for viruses that bind to their authentic receptors only weakly (e.g., for retroviruses, in the cases of FeLV-T or polytropic MuLVs; Anderson et al., 2000; Marin et al., 1999; Temin, 1988). We emphasize this because pathogenic variants of different animal viruses have often been associated with abilities to bind to apparently novel cell surface components, and it has sometimes been inferred that the viruses have switched their receptor specificities. In these instances, it has generally not been established that the cell surface binding components are receptors that directly mediate infections. In the case of Ebola, for example, the receptor(s) that mediates its entry has yet to be definitively identified. C-type lectins such as DC-SIGN and DC-SIGNR are thought to serve as adherence factors for Ebola or Marburg virus (Marzi et al., 2006; Matsuno et al., 2010). Other plasma membrane-associated proteins have been implicated in EBOV uptake, including fólate receptor alpha and the tyrosine kinase receptor Axl (Chan et al., 2001; Shimojima et al., 2006, 2007; Sinn et al., 2003), but the physical interaction of EBOV GP and these proteins has not been demonstrated, and cells that do not express these proteins are permissive for EBOV GP-mediated virion uptake. Some previous studies have implicated the actin cytoskeleton in EBOV entry, where agents such as cytochalasin D and swinholide A that impair microfilament function, inhibited GP-mediated entry (Yonezawa et al., 2005). Similarly, VSV was shown to bind ubiquitously to cells via phosphatidylserine (PS) (Schlegel et al., 1983). However, a more recent study reports that PS is not a receptor for VSV, as no correlation was found between cell surface PS levels and VSV infection, and annexin V, which specifically binds PS, did not inhibit infection of VSV (Coil and Miller, 2004). Therefore, the cell surface receptors for VSV have not been identified, but it is generally thought that binding via the G-protein is rather unspecific and involves negative charges on the plasma membrane (Carneiro et al., 2006; Coil and Miller, 2005). Adsorption of viruses onto cultured cells from the medium is usually a very slow and inefficient process, principally because of the slow rates of their diffusion into contact with the cell surfaces (Allison and Valentine, 1960; Andreadis et al., 2000). In general, the rate of contact cannot be significantly enhanced by mixing or stirring, because the boundary layer of the relatively stationary fluid that surrounds walls or other large objects (e.g., cells) in flowing liquids is substantial compared with the rate of virus diffusion, hence stirring does not increase the concentration of virus surrounding this boundary zone (Allison and Valentine, 1960). In the case of retroviruses, it has become especially clear that adsorption is a severely limiting step in infection of cultured cells. In classic studies in which virus samples were incubated with cells for several hours before
washing with fresh medium and subsequently detecting the foci of infection, it was estimated that only 1/1000 or fewer of the virions in the medium were infectious. In contrast to previous interpretations, studies suggest that this low infectivity-to-virion ratio is principally caused by the inefficiency of adsorption (Andreadis et al., 2000). Accordingly, serial incubation of a virus-containing medium for 2-h periods with sequential cell cultures results in the same titers in each of the cultures after correction for spontaneous viral decay (Kabat et al., 1994). Furthermore, centrifuging the virus down onto the cultured cells (i.e., spinoculation) often increases retroviral titers by 1–2 log orders of magnitude (Bahnson et al., 1995).

Studies aiming to count retrovirions adsorbed onto cell surfaces by confocal immunofluorescence microscopy or by quantitative PCR methods (Marechal et al., 2001; Pizzato et al., 1999, 2001) have demonstrated that receptors for viral entry are irrelevant for initial adsorption of retrovirions onto surfaces of most cells (Pizzato et al., 1999, 2001). On the contrary, the initial steps of virus attachment seem to more critically depend on accessory cellular binding substances, such as heparin sulfates, integrins, or lectins, including DC-SIGN (Bounou et al., 2002; Guibinga et al., 2002; Jinno-Oue et al., 2001; Mondor et al., 1998; Saphire et al., 2001). By forming multivalent weak reversible bonds with such abundant cell surface components, a virus would become efficiently bound in a manner that would allow it to “graze” until it makes appropriate contact with a true receptor (Haywood, 1994; Park et al., 2000).

2. S–S shuffling

It is well known that for several viruses (Rubella togavirus, BVDV pestivirus, Newcastle disease paramyxovirus, HIV lentivirus, mouse hepatitis coronavirus (MHV)), rearrangements of the thiol content and of the disulfide bridges, induced by thioredoxin or protein disulfide isomerase (PDI), are essential to induce some big conformational changes necessary for the membrane fusion (Fenouillet et al., 2007).

Interestingly, the MLV and HTLV retroviruses developed an “internal” oxdoreduction activity by adapting a catalytic motif involved in disulphide bridge isomerization. The two SU and TM subunits can be linked in either a covalent or noncovalent manner. For HIV-1, the existence of the soluble gp120 protein indicates a noncovalent link between SU and TM (Kowalski et al., 1987). However, for most other retroviruses, a covalent link was described at one stage. In all cases, with the exception of MMTV and JSRV, a disulfide bond between the SU and the TM is formed between the highly conserved CX6CC motif of the TM and the CXXC of the SU (Pinter et al., 1997; Schulz et al., 1992; Sitbon et al., 1991). This CXXC motif is extremely rare in cellular proteins and is
similar to a motif found in the catalytic site of enzymes involved in thiol isomerization, like PDI or thioredoxin (Pinter et al., 1997; Sanders, 2000). This motif in the SU has been shown to be part of an autocatalytic isomerization function of SU to destroy the initial bond between SU and TM generated during Env synthesis and create an intra-SU bond inside the CXXC motif (Li et al., 2008; Wallin et al., 2004). This disulfide bond isomerization is crucial for the fusogenicity of γ-retrovirus (MLV) (Fenouillet et al., 2007).

3. Fusion activation by proteases

Some viruses use the protease activity of particular cellular enzymes localized in the endosomes or at the cellular surface for activating their EnvGP. The Ebola filovirus (for which the GP1–GP2 envelope glycoprotein is cleaved by the furin in the producer cells) (Chandran et al., 2005; Schornberg et al., 2006), the HeV (Pager and Dutch, 2005), NiV (Diederich et al., 2009), or the SARS-CoV and MHV-2 coronaviruses (for which the S spikes is not cleaved in the producer cells) (Huang et al., 2006; Qiu et al., 2006; Simmons et al., 2005) require the cysteins lysosomal proteases, the L or B cathepsine (CatL or CatB) for their entry process. After virus uptake following angiotensin-converting enzyme 2 receptor binding, cathepsin L-mediated proteolysis induces conformational changes in the SARS-CoV S glycoprotein to trigger the endosomal membrane fusion process (Simmons et al., 2005). Likewise, cleavage of the Ebola glycoprotein by CatL cleavage removes a glycosylated glycan cap and mucin-like domain (MUC domain) and exposes the conserved core residues implicated in receptor binding (Chandran et al., 2005; Hood et al., 2010; Lee et al., 2008; Schornberg et al., 2006). Entry of this virus is pH-dependent and associated with the cleavage of GP by proteases, including CatL and/or CatB, in the endosome or cell membrane, which is required for entry into the host cell. However, the precise role of the cleavage of Ebola envelope glycoprotein, which is already cleaved intracellularly during its exit, is uncertain. The cleavage of the GP to a stable form of 18 kDa of GP1 may increase binding, suggesting that the cleavage facilitates the interaction with a cellular receptor (Dube et al., 2009; Kaletsky et al., 2007). Another possibility is that the CatB cleavage is required to facilitate the triggering of viral membrane fusion by destabilizing the prefusion conformation of Ebola envelope glycoprotein (Wong et al., 2010).

The cathepsins comprise a family of lysosomal protease enzymes whose primary function (i.e., protein degradation) plays a critical role in normal cellular homeostasis. Cathepsin L is one of the 11 members of human lysosomal cysteine proteases (i.e., B, C, F, H, K, L, O, S, V, W, and X) that fall in the C1 family (papain family) of the CA clan (Rossi et al., 2004). These enzymes were traditionally linked to nonspecific proteolytic activity within lysosomes. More
recently, cathepsin L has been implicated in regulatory events relating to cancer, diabetes, immunological responses, degradation of the articular cartilage matrix, and other pathological processes (Vasiljeva et al., 2007), including osteoporosis, rheumatoid arthritis, and tumor metastasis (Palermo and Joyce, 2008).

Interestingly, several host cell proteases appear to be able to prime fusion activation in the case of SARS-CoV, including cathepsin L, trypsin, factor Xa, thermolysin, plasmin, TMPRSS11a, and elastase. The proteolytic cleavage events in SARS-CoV S that lead to membrane fusion occur both at the S1/S2 boundary and adjacent to a fusion peptide in the S2 domain (Belouzard et al., 2009). Elastase-mediated activation of SARS-CoV was originally reported by Taguchi and coworkers, and it has been proposed that elastase may have important implications for viral pathogenesis (Matsuyama et al., 2005). Elastase is known to be secreted by neutrophils as part of an inflammatory response to a viral infection and is also produced by opportunistic bacteria (e.g., *Pseudomonas aeruginosa*) that can colonize virally infected respiratory tissue. As such, it has been considered that elastase-mediated activation of SARS-CoV might be an important factor in the severe pneumonia seen in SARS-CoV-infected patients (Belouzard et al., 2010).

In conclusion, in order to better classify the receptors, the receptors must be differentiated according to their precise function: those that permit a nonspecific adsorption, such as the glycosaminoglycans (used by Dengue, TBE, HSV-1), the type C lectin receptors, such as L-SIGN, DC-SIGN, the hMGL, the LSECtin, and the asialoglycoprotein receptor (used by HCV, Ebola, Marburg); those that permit a specific adsorption receptor (binding receptor) allowing the sorting (toward particular endosomes and intracytoplasmic compartment) and the initial conformational changes (such as the CD4 to HIV, the integrins or the laminin receptor for the Dengue, Sindbis, or Lassa viruses); and finally, those that permit the exposition of the domains implicated in the destabilization of the membrane (like the fusion peptide) and are the latest receptors to act (such as the HIV coreceptors). Therefore, studying the kinetics of the conformation changes of the EnvGP and the kinetics of action and utilization of the receptors is essential to accurately categorize the receptors (nonspecific adsorption receptor, receptor of binding, receptor of fusion).

All the cellular proteins that allow the exposition, localization, and the trafficking of these receptors and/or endosomes should be considered as cofactors. With all the siRNA screens that are coming out, the list of such cofactors is dramatically increasing.

**B. Use of multiple receptors—Receptor switch**

The use of different receptors often correlates with the need of a virus to overcome barriers existing in the cell type or tissue that they infect. One well-studied example is the binding of Coxsackievirus B to decay-accelerating factor
(DAF) in the apical surface of epithelial cells, and subsequently to the Coxsackievirus and adenovirus receptor (CAR), which is localized in the tight junction region. DAF helps to bring the virus to the tight junctions, and CAR induces a conformational change and promotes endocytosis (Coyne and Bergelson, 2006).

Almost all animal viruses use receptors exclusively containing a single TM sequence (see Table 4.1). In striking contrast, cell surface receptors for \(\gamma\)-retroviruses have multiple transmembrane (TM) sequences, compatible with their identification in known instances as transporters for important solutes. Similarly, hepatitis C virus, in addition to LDL receptor, exclusively uses multitransmembrane receptors: the Scavenger receptor class B type 1 (SRB1 with two transmembrane domains), the tetraspanin receptors CD81 and Claudins (4 TMs), and another tight junction protein Occludin-1 (4 transmembrane domains; Ploss and Rice, 2009).

Another surprise is that some viruses, including many \(\gamma\)-retroviruses, use not just one receptor but pairs of closely related receptors as alternatives (Tailor et al., 2003). This appears to have enhanced viral survival by severely limiting the likelihood of host escape mutations. All of the receptors used by \(\gamma\)-retroviruses contain hypervariable regions that are often heavily glycosylated and that control the viral host range properties, consistent with the idea that these sequences are battlegrounds of virus–host coevolution. However, in contrast to previous assumptions, it is probable that \(\gamma\)-retroviruses have adapted to recognize conserved sites that are important for the receptor’s natural function and that the hypervariable sequences have been elaborated by the hosts as defense bulwarks surrounding the conserved viral attachment sites.

The fact that all virus groups have been severely limited throughout evolution in the types of receptors they can employ, may initially appear inconsistent with evidence that some viruses can switch their receptor specificities with apparent ease. This has been most dramatically suggested by shifts of influenza A viruses between animal reservoirs, which involve single amino acid changes in the viral hemagglutinin, enabling recognition of different sialic acid structures (e.g., \(N\)-acetyl or \(N\)-glycolyl neuraminic acids in \(\alpha2,6\) or \(\alpha2,3\) linkages to galactose) that predominate in the different host species (Baranowski et al., 2001; Gambaryan et al., 2005; Skehel and Wiley, 2000). Similarly, slight changes in specificity for receptors accompanied the emergence, in 1978, of the canine parvovirus (Parker et al., 2001). However, these are small shifts in receptor specificities rather than global jumps to dissimilar receptors. Similar slight shifts are involved in the change from the CCR5 to CXCR4 coreceptor usage during AIDS progression (Scarlatti et al., 1997). Small shifts in usages of highly similar receptors have also been reported during cell culture selections of subgroup B, D, and E avian leukosis viruses that all use polymorphic variants of the same TVB receptor (Taplitz and Coffin, 1997) and during cell culture selections of HIV-1 variants (Platt et al., 1998). Therefore, despite the rarity of receptor repertoire
expansions throughout millions of years of retrovirus evolution, limited switches can occur within single infected animals. For example, there is an evolution of coreceptor usage in HIV-1/AIDS, some in vivo adaptations of ecotropic MuLVs and formation of polytropic MuLVs, and some evolution of altered receptor usages in domestic cats infected with FeLV-A (Tailor et al., 2003).

Several viruses have been reported to use multiple alternative receptors or even alternative pathways for infection of cells. For example, MV isolates appear to be capable of using CD46 or SLAM, which both contain single TM domains (Baranowski et al., 2001; Oldstone et al., 1999; Tatsuo et al., 2000). Complex viruses such as herpesviruses that contain several distinct EnvGP are also typically able to bind to several cell surface components (Baranowski et al., 2001; Borza and Hutt-Fletcher, 2002). The foot-and-mouth disease picornavirus (FMDV) may also use multiple receptors, including heparan sulfates and integrins, and may, in addition, be able to invade cells via immunoglobulin Fc receptors when the virus is coated with antibodies (Baranowski et al., 2001; Mason et al., 1994). This alternative entry route is also used by the dengue flavivirus, which may explain the extremely strong pathogenesis that occurs when it reinfects previously exposed individuals (Baranowski et al., 2001). In the case of FMDV, it has not been established whether heparin sulfate is a true receptor that directly mediates infection or merely a binding factor that influences infection indirectly by enhancing virus adsorption. HIV-1 infections are also strongly stimulated by accessory cell surface binding components including heparan sulfates, glycolipids, and DC-SIGN (Bounou et al., 2002; Geijtenbeek et al., 2000; Pohlmann et al., 2001). Similarly, a paralysis-inducing neurotropic variant of Friend MuLV binds more strongly than the parental virus to heparan sulfate, and thereby becomes more infectious for brain capillary endothelial cells while still remaining dependent on the CAT1 receptor (Jinno-Oue et al., 2001). These examples illustrate how changes in affinities for accessory binding substances can dramatically alter cellular tropisms and pathogenesis of viruses, and why it has often been difficult to distinguish such accessory binding factors from true receptors or coreceptors that are essential for infections. On the basis of these considerations, we believe that the available evidence strongly supports our proposal that all virus groups have been severely constrained in the types of receptors they can employ for infection of cells. However, some viruses have evolved several pathways for infection, and viruses such as HIV-1 have evolved distinct sites in a single SU glycoprotein for recognition of dissimilar receptors and coreceptors.

C. Separation of the binding and fusion functions

The complexity of EnvGP is variable. An example of a very simple fusion protein is the FAST proteins of Orthoreovirus that do not belong, however, to the family of the enveloped virus (Barry et al., 2010; Shmulevitz and Duncan, 2000). The
Orthoreovirus genus includes some no-enveloped viruses that cause the cell–cell fusion of infected cells. This fusion activity is due to the small no-structural membrane viral proteins named FAST proteins (fusion-associated small transmembrane protein), in size ranging between \( \sim 10 \) and \( 15 \) kDa. The FAST protein ectodomains are very small, with extreme cases of only 20 residues, and contain hydrophobic short regions with/without acid properties, and a myristoylation site. Though their small size challenges their ability to form a hairpin structure, these FAST proteins expressed alone are sufficient to induce the membrane fusion (Barry et al., 2010; Shmulevitz and Duncan, 2000). Surprisingly, they are able to traffic as far as the plasma membrane without inducing intracellular disorder. Indeed, by opposition, the expression of retroviral TM or orthomyxovirus HA2 alone does not lead to cell surface membrane expression, and these subunits are blocked intracellularly. The fusion induced by the FAST protein is very broad, which questions their use of a particular protein receptor. Thus, some fusion proteins are simple and possess solely the fusion function, and others comprise several domains in addition to the domain involved in fusion. The function of some of these domains has remained more or less independent from the fusion domain, and sometimes, they can be naturally separated in different proteins (i.e., H and F from paramyxoviruses) or experimentally on different fragments, as explain below. Such is the case of the \( \gamma \)-retroviruses for which the function of binding to the receptor can be separated from the function of fusion. This line of inquiry was initiated by the studies of Bae et al. (1997) relating to a conserved PHQ motif that occurs near the amino-terminal ends of SU glycoproteins in all \( \gamma \)-retroviruses. Mutation of this PHQ motif blocked membrane fusion but had no effect on receptor attachment. Subsequently, we discovered that noninfectious \( \gamma \)-retrovirions lacking this histidine could be transactivated by addition of a soluble SU or an amino-terminal fragment of SU, called the receptor-binding domain (RBD), to the cultured cells (Lavillette et al., 2000). Interestingly, studies by Overbaugh and coworkers have demonstrated that similar transactivation processes can occur in natural infections by \( \gamma \)-retroviruses. Specifically, they found that infections by the immunosuppressive FeLV-T virus, which has a Pro in place of His in its PHQ motif, require transactivation either by a soluble FeLV-B-related SU glycoprotein termed FELIX that is endogenously expressed in cat T cells or by an FeLV-B SU glycoprotein (Anderson et al., 2000). This activating process parallels that of herpes simplex virus for the transmission of a fusogenic signal among the EnvGP of the herpes simplex virus on receptor binding by glycoprotein gD. The soluble gD ectodomain has been shown to allow entry of engineered HSV-1 virus particles that lack gD (i.e., gD-null mutants; Cocchi et al., 2004). The evidence reviewed provides very strong support for the hypothesis that attachment of viruses to their receptors initiates a pathway that obligatorily contains intermediate steps. These intermediate steps very likely include viral association with multiple receptors,
cooperative conformational changes within Env glycoprotein, and cross-talk between Env on the viral surfaces. In the case of the retroviruses, this evidence suggests that virus binding to receptors does not directly induce irreversible structural changes in SU–TM complexes as was previously believed. Rather, it implies that the binding to receptors induces SU–SU interactions that are pre-requisites for later steps in a highly coordinated membrane fusion pathway. We anticipate that similar intermediate steps are likely to be involved in infections by other groups of retroviruses and perhaps in infections by other membrane-enveloped viruses.

Other viruses can be activated by soluble receptors that, by interacting with the envelope glycoprotein, induce some of the conformational changes necessary to trigger fusion. This example indicates that binding and fusion steps can be separated and can take place at different locations. In these cases, MHV, avian retrovirus ASLV, and the herpes simplex 1 (HSV-1) can infect some cells that do not harbor binding receptors provided that the soluble form is present in the infection media (the soluble CEACAM1 receptor for MHVR, the soluble Tva receptor for ASLV, and the soluble nectin-1 for HSV-1; Kwon et al., 2006; Matsuyama and Taguchi, 2002; Mothes et al., 2000).

IV. BASIC AND TRANSLATIONAL RESEARCH EXPLOITING ENTRY PROPERTIES OF VIRUSES

A. Tropism properties and use of pseudoparticles in gene therapy

Vectors derived from retroviruses such as lentiviruses and oncoretroviruses are probably among the most suitable tools to achieve long-term gene transfer, since they allow stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors should be the preferred gene-delivery vehicles over vectors derived from oncoretroviruses (MLV) since, in contrast to the latter, they can transduce nonproliferating target cells. Moreover, lentiviral vectors that have the capacity to deliver transgenes into specific tissues are expected to be of great value for various gene transfer approaches in vivo (Frecha et al., 2008b). To achieve such in vivo gene transfer, innovative approaches have been developed to upgrade lentiviral vectors for tissue or cell targeting and which have potential for in vivo gene delivery. One strategy is to develop vectors that harbor EnvGP with selective tropisms. Vectors derived from retroviruses offer particularly flexible properties in gene transfer applications, given the numerous possible associations of various viral surface glycoproteins (determining cell tropism) with viral cores. Selective tropisms were achieved by taking advantage of the natural tropisms of EnvGP from other membrane-enveloped viruses. For instance, the use of surface glycoproteins derived from viruses that cause lung infection and
infect via the airway epithelia, such as Ebola virus or Influenza virus, may prove useful for gene therapy of the human airway (Kobinger et al., 2001). Exclusive transduction of retinal pigmented epithelium could be achieved following sub-retinal inoculations of some vector pseudotypes in rat eyes (Duisit et al., 2002). High transgene expression was detected in dermal fibroblasts transduced with VSV-G-, EboZ-, or MuLV-pseudotyped HIV vector and effectively targeted quiescent epidermal stem cells which underwent terminal differentiation resulting in transgene expression in their progenies (Hachiya et al., 2007). Importantly, several viral EnvGP target lentiviral vector to the CNS such as rabies (Wong et al., 2004), mokola (Watson et al., 2002; Wong et al., 2004), lymphocytic choriomeningitis virus envelope (LCMV) (Miletic et al., 2004; Stein et al., 2005), or Ross River (Kang et al., 2002) viral EnvGP that even permit transduction of specific cell types within the CNS. Likewise, screening of a large panel of pseudotyped vectors established the superiority of the gibbon ape leukemia virus (GALV) (Sandrin et al., 2002; Stitz et al., 2000) and the cat endogenous retroviral-modified glycoproteins (RD114) (Sandrin et al., 2002) for transduction of progenitor and differentiated hematopoietic cells. Recently, a new LV carrying the MV EnvGP on its surface was able to overcome vector restrictions in both quiescent T and B cells (Frecha et al., 2008a, 2009). Importantly, naive as well as memory T and B cells were efficiently transduced, while no apparent activation, cell-cycle entry, or phenotypic switching were detected, opening the door to a multitude of gene therapy and immunotherapy applications. Vectors derived from HIV pseudotyped with Sendai virus fusion protein F (Kowolik and Yee, 2002) or E1E2 from hepatitis C virus (Bartosch et al., 2003), and such vectors are able to transduce human hepatoma cells and primary human hepatocytes efficiently, although they are unable to enter nonliver cells. The GP64 glycoprotein from baculovirus Autographa californica multinuclear polyhedrosis virus pseudotyped FIV efficiently and also showed excellent hepatocyte tropism (Kang et al., 2005).

B. Identification of viral cell entry receptors using pseudoparticles

The screening of cDNA libraries has emerged as a powerful tool to identify and clone viral entry receptors. It is an alternative to the use of human/Chinese hamster radiation hybrid panels of cells. In order to clone an unknown receptor by complementation screens, cDNA from a cell permissive to infection by a certain virus is introduced into a nonpermissive cell. As some recessive cell lines are poorly transfectable, the use of pseudoparticles provides a good tool to transduce the cDNA library for this cloning strategy. A retroviral cDNA library approach, involving transfer and expression of cDNAs from highly infectable cells to nonpermissive cells, has been used to clone and identify the MuLV polytropic X-receptor (Battini et al., 1999; Tailor et al., 1999a; Yang et al., 1999),
the RD114 ASCT2 receptor (Rasko et al., 1999; Tailor et al., 1999b), FeLV-C FLVCR1 receptor from human and domestic cat cDNA libraries (Quigley et al., 2000; Tailor et al., 1999c), and two closely related human proteins, PHuR-A1 and PHuR-A2, that function as receptors for PERV-A (Ericsson et al., 2003). Briefly, when a cell line that is not susceptible to a particular pseudotype retrovirus vector harboring an envelope for which the receptor is not known, a cDNA library from a cell line highly susceptible to transduction, was constructed by cloning the cDNA into a retroviral expression vector. Afterward, the cDNA retroviral expression library was transduced into nonsusceptible cells by infection at a relatively low multiplicity of infection so that the majority of infectants would contain single-copy provirus inserts. The library-containing cells were then screened for susceptibility to pseudotype vector transduction through selection of drug-resistant cells after exposure to the vector carrying a resistance gene. Of drug-resistant clones obtained from the primary screen and using PCR primers specific for vector sequences, cDNA products from clones with conferred susceptibility were identified after nested PCR was performed on DNA extracted from reinfectable clones.

However, for many viruses, initial attempts using a retroviral cDNA library were unsuccessful due to an inherent background of nonspecific infection with pseudoparticles. In fact, no cell line was completely nonpermissive to even “no envelope” pseudoparticles bearing no viral envelope proteins, indicating the existence of nonspecific uptake mechanisms. In the screens, this resulted in a high background of drug-resistant colonies, independent of glycoprotein-mediated cell entry. Thus, unless the entry factor cDNA was highly represented in the library, a single round of transduction/challenge would not suffice. To deal with the high background observed in screens, methods that would allow multiple rounds of selection and enrichment have been developed. Recently, the use of a cyclic packaging rescue (CPR) system using non-self-inactivating vectors has been shown to increase the efficiency of receptor cloning with powerful iterative screening methods (Evans et al., 2007; Ploss et al., 2009). Most retroviral vectors commonly used for gene-delivery applications are self-inactivating vectors that contain deletions in the long terminal repeat (LTR) elements. No packaging competent retroviral RNA transcripts are generated from such integrated proviruses; instead, transgene expression is driven by an internal nonretroviral promoter. In contrast, if the cDNA library is constructed in a provirus that retains the complete LTR elements, the retroviral promoter is active in transduced cells and a full-length viral RNA is expressed. Expression of the packaging components, gag-pol and vesicular stomatitis virus VSV-G envelope (that will efficiently infect recessive cell lines), in these cells allows packaging of the RNA into pseudoparticles capable of transducing naive cells. This approach, termed CPR (Bhattacharya et al., 2002; Koh et al., 2002), allows retrieval of the library after selection has been performed, followed by
transduction of a naive cell population, concluded by a new round of selection. This process can be repeated sequentially for an unlimited number of selection/enrichment steps.

For an additional level of selection, different challenge virus genomes can be used, each encoding a different drug-selectable marker (e.g., puromycin (PuroR) or zeocin (ZeoR) resistance), in self-inactivating retroviruses. Thus, after challenge and selection of a library-transduced population with one pseudoparticle-packaged selection cassette (e.g., PuroR), the population can be pooled and rechallenged with the second-selectable pseudovirus (e.g., ZeoR). Then, during CPR, only the full-length retroviral transcripts from the non-self-inactivating provirus that encodes the library but not the self-inactivating challenge virus genomes are repackaged and transferred to the naive cell population. This enables researchers to perform multiple rounds of selection, thereby overcoming the background of nonspecific pseudoparticle uptake. Moreover, using this scheme, underrepresented cDNA will be enriched. Once the final round of selection of pseudotype-susceptible cells has been achieved, genomic DNA can be prepared from selected clones and used as a template in a PCR across the provirus cDNA-cloning site.

The nonpermissive target cell line for the cDNA screen adheres to several stringent criteria. As stated above, the primary requirement is that (1) to minimize nonspecific background, cell lines with minimal uptake of pseudoparticle of interest and “no envelope” pseudoparticles were preferred. (2) To ensure that nonpermissiveness was a phenotype due to the lack of a pseudoparticle-specific entry factor(s) rather than poor infection by pseudotypes in general, chosen cell lines should be highly permissive to an unrelated pseudoparticle (VSVGpp, rhabdovirus) infection. In addition, this also ensures that the target cell line would be easily transduced with the cDNA library. (3) For selection, candidate cell lines also had to be susceptible to the desired drug selections. (4) To perform multiple rounds of screening involving CPR, the ideal cell line had to demonstrate this method well and be highly transfectable. (5) Finally, to facilitate the screen, the chosen cell line needed to be relatively fast growing and clone efficiently.

C. Determining the endocytosis pathway of entry and the different cell proteins involved in entry by RNA interference screens

To gain insights into virus entry, it is necessary to examine several inhibitors of pathway-mediated endocytosis in terms of their role in blocking infection mediated by pseudotypes with different EnvGP. An advantage of pseudoparticles is that the entry process of pathogens of BSL4 can be studied in BSL2 conditions. For example, to gain insights into Ebola virus entry, inhibitors against different endocytosis pathways have been examined for their ability to block infection.
mediated by HIV pseudotyped with the Ebola EnvGP (Bhattacharyya et al., 2010). The use of control pseudoparticles (e.g., pseudotyped with Vesicular Stomatitis Virus EnvGP (VSV G)) can be used as controls to assess cell viability and specificity of inhibition. Inhibition of clathrin function traditionally relied on three principal approaches: drugs that inhibit acidification of endosomes (such as BafA1, a specific, nonreversible endosomal proton pump inhibitor), as well as commonly used lysosomotropic agents (such as ammonium chloride (NH₄Cl) and chloroquine); potassium depletion; and finally, treatment of cells with brefeldin A (BFA) or chlorpromazine (Sieczkarski and Whittaker, 2002). As such, these drugs have multiple effects on cell function, and their use to inhibit virus infection should be treated with some caution. Thanks to pseudotypes, some other studies have implicated the actin cytoskeleton in Ebola virus entry, where agents such as cytochalasin D and swinholide A that impair microfilament function were shown to inhibit EnvGP-mediated entry (Yonezawa et al., 2005). Ebola enters cells through a low-pH-dependent, endocytosis-mediated process. A large body of evidence indicates that Ebola viruses enter cells by clathrin-mediated endocytosis (Sanchez, 2007), but lipid raft-associated, caveolin-mediated endocytosis has also been proposed as an alternative mechanism of Ebola virus uptake (Empig and Goldsmith, 2002). Low-pH events lead to cathepsin-dependent cleavage of Ebola virus EnvGP that is required for productive uptake of the virus (Chandran et al., 2005; Kaletsky et al., 2007; Schornberg et al., 2006). Other low-pH-dependent events have been postulated to be required as well (Schornberg et al., 2006). Furthermore, Ebola virus likely uses a Rho-mediated pathway, as is seen in VSV virus, suggesting that this may be a route of entry utilized by many different viruses (Quinn et al., 2009).

More recently, proteins interfering with endocytosis, such as the use of dominant-negative Eps15, or RNA interference (RNAi) have been developed, and such approaches target the different pathways with higher specificity (Mercer and Helenius, 2009; Mercer et al., 2010b). The RNAi approach allows researchers to perform screens to identify previously unrecognized host factors that are required for viral replication. RNAi screens rely on either short interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) to knock down the function of a particular gene in a cell. Researchers can then infect the cells with specific viruses and monitor levels of viral replication. If viral replication is reduced, then the knocked-down gene might be necessary for the virus to replicate itself or function within the host cell.

Some small-scale screens have been developed using wild-type viruses (Kolokoltsov et al., 2007) or pseudoparticles (Trotard et al., 2009). Pseudoparticles can be exploited to focus a siRNA screen specifically on the entry step of a virus. Indeed, only the entry steps are governed by the EnvGP, whereas all the uncoating, integration, and expression steps of the transgene depend on
retroviral proteins. Having said that, this suffers an inherent drawback in that some steps are dependent on retroviral proteins and therefore limits the scope of the screen. One limitation to the siRNA screen is specificity and cell toxicity, which can be overcome by the use of pseudotypes. Indeed, a siRNA screen can be done with different pseudotypes in parallel. If, contrary to the pseudotypes of interest, some pseudotypes are not affected by siRNA, this indicates that the siRNA specifically inhibits the virus of interest and, moreover, that the siRNA is not toxic. To better characterize the entry pathway of the hepatitis C virus, a small interfering RNA library dedicated to membrane trafficking and remodeling was screened in the context of the Huh-7.5.1 liver cell line cells infected by HCV pseudoparticles (HCVpp) (Trotard et al., 2009). Results showed that the downregulation of different factors implicated in clathrin-mediated endocytosis inhibit HCVpp cell infection. In addition, knockdown of the phosphatidylinositol 4-kinase type III-alpha (PI4KIIIalpha) prevented infection by HCVpp, and the presence of PI4KIIIbeta in the host cells influenced their susceptibility to HCVpp infection. This library screening using pseudoparticles identified two kinases, PI4KIIIalpha and beta, as being involved in the HCV life cycle. These results have been confirmed in published works of the identification of cellular factors required for the HCV life cycle using siRNA libraries screening either over 4500 drugable genes (Ng et al., 2007; Vaillancourt et al., 2009), 140 cellular membrane-trafficking genes (Berger et al., 2009; Coller et al., 2009), kinome (Supekova et al., 2008), or the entire genome (Li et al., 2009; Tai et al., 2009). These works are much more time- and cost-consuming, and even though pseudoparticles may appear to be an overly simple model to study and identify host factors necessary for viral infection, they are also powerful and flexible tools that have led to major discoveries, as these examples have shown.

Recently, however, in order to identify host factors necessary for viral infections, researchers are turning to genome-wide genetic screens. Indeed, the sequencing of the human genome and the emergence of technologies that allow the silencing of individual genes in cells one by one using siRNA, when combined with the use of genome-wide siRNA libraries and automated HTS methodology, allows molecular information to be gained about virtually every critical intracellular event occurring during virus infection. Within the last few years, there have been several publications describing such genome-wide siRNA screens that have been applied to virus infections in tissue-cultured cells. RNAi screens have now been preformed for several viruses, including HIV (Brass et al., 2008; Konig et al., 2008; Yeung et al., 2009; Zhou et al., 2008), West Nile Virus (Krishnan et al., 2008), Influenza (Karlas et al., 2010; Konig et al., 2008), and, in drosophila cells, Dengue (Sessions et al., 2009) and vaccinia virus (Moser et al., 2010). From the hundreds of cellular factors that have been implicated in the outcome of infection, some were already known from other studies, but the majority are entirely novel, and the validation and analysis of data is ongoing.
in many laboratories. However, a poor overlap between results from different screens published on the same viruses has been observed. The differences are probably linked not only to technical variations but also to the inherent risk of false positives through off-target effects and false negatives due to inefficient silencing. Cell toxicity is a common complication, as well as cell-type specificity. Following-up such screens with pseudoparticle studies is useful to validate the cell proteins that are involved in the entry step only and facilitate the analysis, compared to the use of a replication competent virus.

**D. Screening and development of entry inhibitors using pseudoparticles**

Resistance to individual antivirals is likely to develop for most specific therapeutics targeting particular viral proteins, thus making therapy consisting of a combination of drugs targeting different stages of the viral life cycle highly desirable. The entry process represents another series of potential targets for therapeutic intervention. It has not been extensively explored due to high-throughput experimental limitations for some viruses that require biosafety level 3 or 4 or for the viruses with no robust infection system.

Pseudoparticle infection systems, utilizing different reporter genes or proteins (i.e., firefly luciferase or GFP), can be developed for HTS of small molecule libraries, peptide libraries, or neutralizing antibodies for their entry inhibitor properties. In order to facilitate the screen, assay performance can be improved by modifying the properties of both the parental host cell line and the pseudovirus. For example, cells with improved pseudoparticle entry and cell spreading can be selected. Pseudoparticles can be improved by using EnvGP that increase infectivity either by selecting a specific variant or by expressing a human codon-optimized envelope glycoprotein sequence. Finally, the pseudoviruses can be engineered to express a human codon-optimized reporter to improve the sensitivity of the assay. Using these modifications, HTS can be developed using 96- or 384-well plates. Compounds found to inhibit pseudoparticle infection can then be counterscreened against pseudoparticles containing other variants to characterize the cross-reactivity of the molecules in a virus family. Moreover, molecules can then be counterscreened against pseudoparticles harboring EnvGP from other virus families to evaluate the specificity of the inhibitor or its large spectrum.

A wide variety of entry inhibitors, namely, peptides, chemical compounds, or antibodies, exist. They can interfere with the different steps of the entry process, such as the binding to receptors or the conformational changes of the fusion proteins, by acting on the envelope protein itself, on the acidification of the endosomes, if necessary, or on the endocytosis.
For HIV, most marketed drugs for treating AIDS are inhibitors of HIV-1 reverse transcriptase or protease enzymes, but new targets include the integrase enzyme, cell–surface interactions, membrane fusion, and also virus particle maturation and assembly (Kaushik-Basu et al., 2008). Entry inhibition entails preventing HIV-1 breaching the cell, either as a strategy to prevent infection altogether or to curtail infection of new cells in an HIV-positive individual. Several strategies have proved effective in HIV-1 entry inhibition either in vitro (using pseudoparticles or replication competent viruses) or in vivo: binding to viral surface proteins gp120 and gp41, binding to human cell surface receptor CD4, and binding to human cell surface coreceptors, CCR5 and CXCR4 (Leonard and Roy, 2006; Liu et al., 2007). In particular, the synthesized peptide T-20 is believed to act by binding to gp41 (Wild et al., 1993, 1994) and is currently in clinical use. Yet, several more recent studies have revealed that T-20 does not block the six-helix bundle prehairpin formation (Liu et al., 2005). Another peptide, C37, derived from the C-terminus of gp41 (and nearly identical to the widely reported C-peptide C34; Liu et al., 2005; Root et al., 2001), is also described as having strong anti-HIV entry activity due to the tight binding of the gp41 N-terminal helices. Maraviroc is a small antiretroviral compound known to be a CCR5 antagonist, which blocks R5-tropic HIV entry into CD4 cells (Hunt and Romanelli, 2009). Several studies have established that synergy can occasionally be observed when two fusion inhibitors are combined in a viral assay or in vitro fusion assay. For example, some synergy is observed in the combination of CCR5 antibodies with T-20 (Ji et al., 2007; Murga et al., 2006), the combination of small molecular antagonists of coreceptors with T-20 (Tremblay et al., 2000, 2002), the combination of small molecular antagonists of CCR5 with CCR5 antibodies (Ji et al., 2007; Murga et al., 2006), and the combination of small molecular antagonists of CCR5 with chemokines (Murga et al., 2006; Tremblay et al., 2002).

In the case of HCV, entry into hepatocytes is a multistep process, involving at least four cellular receptors, leading to virion endocytosis and fusion of the viral and cellular membranes. Unlike the HCV replication process, these steps have not been thoroughly exploited as targets for antiviral intervention. Recently, with the development of HCVpp and the JFH1 infectious molecular clone, it has become possible to test drugs against entry. In vitro, proof-of-concept studies for inhibiting the HCV entry process have been demonstrated using cyanovirin-N that targets the N-linked glycans of the viral envelope proteins and prevents E2–CD81 interaction (Helle et al., 2006), neutralizing antibodies directed against the HCV E1 and E2 proteins (Broering et al., 2009; Habersetzer et al., 1998; Keck et al., 2008; Law et al., 2008; Owsianka et al., 2005; Perotti et al., 2008; Schofield et al., 2005; Yu et al., 2004), antibodies against cellular receptors CD81 (Bartosch et al., 2003; Cormier et al., 2004; Lavillette et al., 2005; Molina et al., 2008), SR-BI (Catanese et al., 2007; Zeisel et al., 2007), Claudin-1
(Krieger et al., 2010), and agents that block endosomal acidification (Bartosch et al., 2003; Hsu et al., 2003; Koutsoudakis et al., 2006; Lavillette et al., 2005). In vivo studies using humanized trimera mice model or human liver-u-PA-SCID mice have also demonstrated prophylactic efficacy of monoclonal anti-E2 (Eren et al., 2006) and anti-CD81 antibodies (Meuleman et al., 2008), respectively. Some broad-spectrum antiviral drugs have also been tested (Pecheur et al., 2007), but more recent studies have used the HCVpp system in order to undertake a screening campaign that led to the discovery of a class of small molecule HCV-specific inhibitors consisting of several structurally related compounds defined by a common triazine core (Baldick et al., 2010). Inhibition of entry was confirmed by using time-of-addition experiments to demonstrate that inhibitor activity is confined to the first 3 h of infection, with inhibition occurring postattachment and closely linked to the inhibition kinetics of the endosomal acidification inhibitor bafilomycin.

Pseudoparticles can also be used to identify the mode of action of molecules identified using a replication competent virus in a cell-based screening system involving multiple rounds of infection in a 96-well format. After analysis of a publicly available library of 446 clinically approved drugs, the impact of 33 identified compounds on viral entry was tested using HCVpp infection to recapitulate HCV particle adsorption, internalization, and viral envelope-mediated fusion (Gastaminza et al., 2010). Many of the candidates were lysosomotropic compounds that inhibited HCV entry with differential efficacy.

Both pseudotype (Larson et al., 2008) and infectious virus screening (Bolken et al., 2006) identified broadly active arenavirus entry inhibitors. Isolation and mapping of resistant viruses, as well as chimeras between sensitive and resistant strains, mapped the target of activity to the GP2 subunit of the G envelope protein complex, specifically to the interface between the C-terminal stem and TMD domains. Mechanistic studies showed that these arenavirus inhibitors prevented low-pH-induced fusion by blocking reorganization between the GP2 stem with N-terminal domains of the G protein complex (York et al., 2008).

Another alternative for developing entry inhibitor compounds is to target endosomal protease necessary for entry of certain viruses. Inhibitors of cathepsin L block viral entry of severe acute respiratory syndrome coronavirus (SARS-CoV) and Ebola virus and impair conversion of HeV glycoprotein into the mature, active form (Chandran et al., 2005; Pager and Dutch, 2005; Simmons et al., 2005). With respect to the development of antiviral agents, inhibitors of human cathepsin L are not subject to resistance arising from rapid mutations of the viral genome (Shah et al., 2010), making Cathepsin L an attractive target for drug development. HTS for cathepsin L inhibitors identified a novel thiocarbazate compound exhibiting potent inhibition against cathepsin L (Beavers et al., 2008; Myers et al., 2008; Shah et al., 2008). This compound prevented 293T cell
infection by the Ebola and SARS-CoV pseudotypes, respectively. In addition, the thiocarbazate inhibited in vitro propagation of malaria parasite *Plasmodium falciparum* and inhibited *Leishmania* major (Shah et al., 2008).

Finally, another natural entry inhibitor is provided by monoclonal neutralizing antibodies. Many monoclonal antibodies can be isolated from immortalized B-cells recovered from patients or from mice hybridomas following immunization. The antibodies can be selected by ELISA assay using soluble envelope proteins or pseudoparticles. The neutralizing potential of these antibodies can be easily screened using pseudoparticles with high-throughput infection assays, and the inhibitory effect can be verified with replicating viruses. In the case of HCV, using an antibody antigen-binding fragment phagedisplay library generated from a donor chronically infected with HCV, of 115 clones showing specific binding to HCV E2 glycoprotein, 5 monoclonal antibodies presented neutralizing activities against cell-culture HCV (HCVcc), JFH-1 virus, and a panel of HCVpp displaying E1–E2 from diverse genotypes (Law et al., 2008). Overall, neutralizing antibodies can inhibit the different steps of the entry process from binding to membrane fusion by targeting the domains involved in this step or by limiting the conformational changes of the envelope complex. Interestingly, one study has recently highlighted the feasibility of targeting short-lived envelope glycoprotein intermediates for inhibition of membrane fusion using monoclonal antibodies (York et al., 2010). This action is very similar to the peptides against HIV membrane fusion on the market. Such strategies to effectively target fusion peptide function in the endosome may lead to the discovery of novel classes of antiviral agents, and screens using pseudoparticles will provide an easily wielded system to identify such infrequent antibodies.

**V. CONCLUSIONS**

All viruses have developed varied mechanisms to reach the same goal. They vary greatly in structure, but all seem to have a common mechanism of action, in which a ligand-triggered large-scale conformational change in the fusion protein is coupled to apposition and merger of the two bilayers. In spite of the different mechanisms to activate the fusion peptide, fusion proteins are distributed into three classes based on their structural homologies. Future experiments must aim to elucidate the molecular mechanisms and the dynamics of the conformational changes driving virus entry. This will require the development of new approaches to study the rapid conformational changes of a small number of membrane interacting protein molecules and domains. A more realistic goal is the determination of all the structures of proteins that mediate the entry of all human viruses, either at a prebinding or postfusion stage.
Another challenge will be the identification of the cognate cellular receptors. Identification of all the cellular receptors for human viruses would be an important contribution to our understanding of virus tropism and pathogenesis. Once known, the role of receptors in entry, as a specific or nonspecific binding factor, or as receptors needed for conformational changes, or for routing the virus to the right compartment, will have to be established. Moreover, for most viruses, the EnvGP appear to function in an autonomous manner and can permit fusion without a requirement for receptors at acidic pH. Thus, the role of varied receptors remains enigmatic for many viruses. It is difficult to predict the roles of a receptor in fusion, sorting and routing the virus toward a particular favorable compartment in the pursuit of the infectious cycle based upon its family and its structure. The activation process of EnvGP and the postbinding events are early steps that are crucial to understand, as they could provide targets for the development of therapeutics. The better understanding of the envelope–receptor interaction also raises hopes for the possibility of designing entry machines that can deliver genes and other molecules to any cell of choice.

Recently, the use of whole genome siRNA screen has become more and more widely used for different viruses in order to identified factors important for entry. It should drastically increase our knowledge of the factors necessary for entry of viruses. Similarly, many high-throughput interactome studies will identify cellular proteins interacting with the different viral components. Altogether, the comparison of all these high-throughput screens should help us to identify cellular proteins and pathways common to different viruses which may help, with rational structure/mechanism-based design of entry inhibitors, to develop inhibitors that cross-react with different pathogens. In a similar fashion, the design of vaccine immunogens that are capable of eliciting potent, broadly neutralizing antibodies of known epitopes, is expected to contribute toward the development of vaccines. In parallel, the development of panels of human monoclonal antibodies against every entry-related protein from all pathogenic human viruses could accelerate our understanding of entry mechanisms and help to fight viral diseases. If research continues at the present pace, most of these goals could be accomplished within the next few decades.

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