CHAPTER 9

ENTRY OF RHABDOVIRUSES INTO ANIMAL CELLS

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Abstract: Entry is the first step in the infectious life cycle of a virus. In the case of rhabdoviruses, entry is facilitated exclusively by the envelope glycoprotein G and its interactions with the host cell. For vesicular stomatitis virus (VSV), attachment to the cell surface was thought to be facilitated by interactions with the lipid phosphatidylserine, however recent work suggests that it is in fact initiated by recognition of proteinaeous receptors. Clathrin-mediated endocytosis delivers the virions into endosomes where they have been proposed to traffic to multi-vesicular bodies. There, the viral envelope fuses with internal vesicles in a process mediated by glycoprotein G in a pH- and phosphatidylserine-dependent manner. A clear mechanistic understanding of glycoprotein G mediated fusion has yet to be obtained, however current data suggests that it is likely facilitated by events distinct from Class I or Class II fusion proteins of other viruses. Rhabdoviruses are also notable in that their fusion protein exists in a reversible pH-dependent equilibrium, which prevents irreversible preactivation during assembly, and may prove to be relevant in the mediation of cell-to-cell fusion - an alternate form of viral spread.

INTRODUCTION

The Rhabdovirus family comprises hundreds of viruses with a wide variety of hosts, comprising vertebrates, invertebrates and plants, which give rise to various diseases.[1] Virions are 100-430 nm long and 45-100 nm in diameter, with those infecting vertebrates possessing a bullet-shaped morphology. The virus consists of an envelope containing a single glycoprotein (G), a matrix protein (M) and a helical nucleocapsid of nonsegmented, negative-sense RNA and nucleocapsid (N) protein, together with the polymerase (L) and phosphoprotein (P). Here, we focus on the entry mechanism of two mammalian

Rhabdoviruses, vesicular stomatitis virus (VSV) the type species of the *Vesiculovirus* genus within the *Rhabdoviridae*, and Rabies virus (RABV) the type species of the *Lyssavirus* genus, as well describing some features of viral hematopoietic necrosis virus (VHSV) a member of the *Novirhabdovirus* genus that infects aquatic hosts. The entry pathway of VSV is summarized in Figure 1.
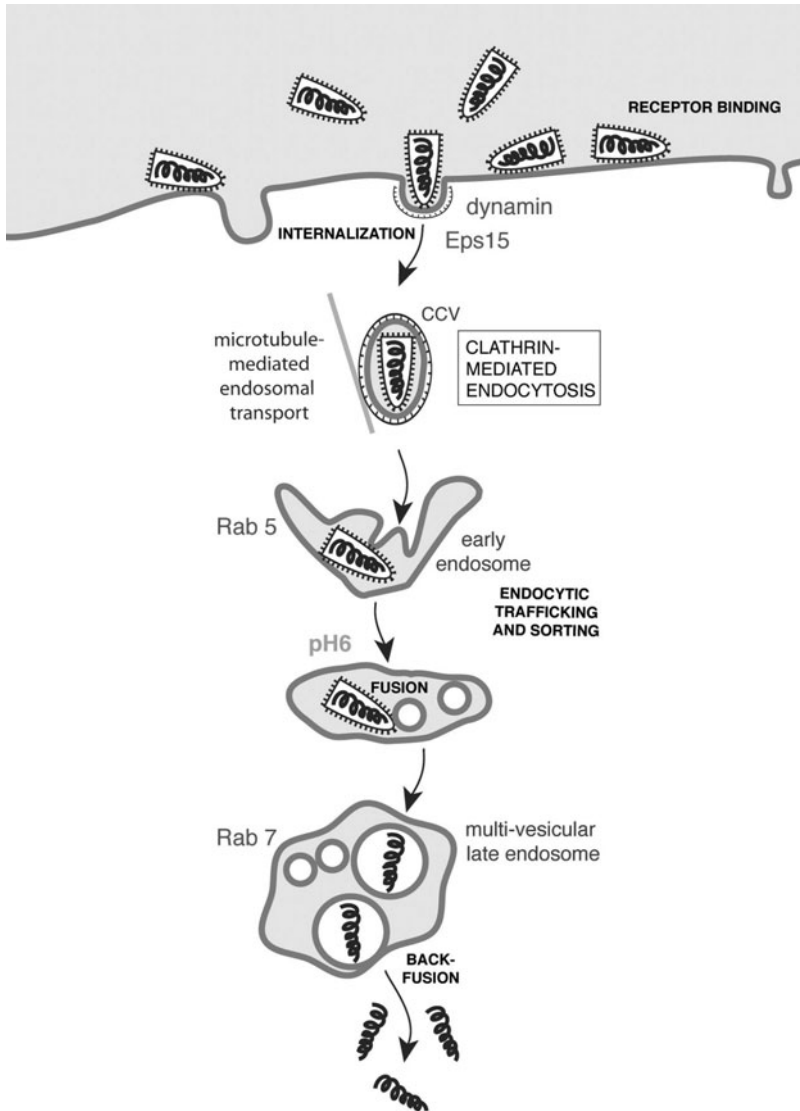
Figure 1. Route of Rhabdovirus entry into host cells. Following receptor binding, Rhabdoviruses are first internalized by clathrin-dependent endocytosis. Virions are trafficked in a microtubule-mediated manner through early endosomes, where low pH-dependent fusion is proposed to occur with internal vesicles of newly forming multi-vesicular endosomes. Back-fusion of these internal vesicles with the limiting late endosomal membrane then allows release of the virus core into the cytoplasm for replication.

VIRAL ATTACHMENT

Attachment to the cell surface represents the first step in entry of any virus, and for Rhabdoviruses this process is carried out by the envelope glycoprotein G. In recent years is has become apparent that the process of Rhabdovirus attachment is far more complex than initially reported. For some members of the Rhabdovirus family (e.g., RABV and VHSV) proteinaceous receptors have been identified,[2,3] while for others (e.g., the prototype VSV) the host cell receptor remains elusive.

Glycoprotein G

The Rhabdovirus glycoprotein G allows both viral attachment[4,5] and fusion.[6-8] Glycoprotein G is a type I transmembrane protein which varies in length between different Rhabdoviruses (VSV Indiana—511aa; RABV- 524aa; VHSV—507aa) and forms approximately 400 homotrimeric units on the surface of the viral envelope.[9] Each unit consists of noncovalently associated polypeptides which exist in a dynamic equilibrium between monomeric and trimeric states.[10-17] Among characterized isolates, glycoprotein G has been shown to contain between two and six potential N-linked glycosylation sites and twelve to sixteen conserved cysteine residues, which likely form internal disulfide bonds of structural significance.[9] No X-ray crystal structure for glycoprotein G has yet been reported, however electron microscopy shows that mature trimers project roughly 8nm from the surface of the viral envelope and consist of a globular head supported by a stalk region.[12]

Host Cell Receptors

The cellular receptors utilized by Rhabdoviruses have proven difficult to positively identify, in part due to the wide spectrum of cell tropism demonstrated by these viruses in vitro. Initial work with the prototype VSV confirmed the presence of at least one specific saturable binding site on the surface of Vero cells, suggesting the presence of a specific receptor.[18] Inhibition of infection was observed when VSV particles were preincubated with Vero cell membrane extracts, presumably by saturating receptor binding domains.[18-21] Inhibition was relieved upon preincubation of Vero cell membrane extracts with phospholipase C, but not by preincubation with neuraminidase, trypsin or heat,[22] leading to the assumption that the receptor was not proteinaceous, but in fact a phospholipid. Subsequently phosphatidylserine (PS) was shown to specifically inhibit VSV infection;[22] hydrophobic domains capable of binding PS, termed p2 peptides, were found in all Rhabdovirus isolates;[23,24] and a second 19 amino acid domain in VSV capable of binding tightly to PS was also identified.[25] Although these data strongly suggested that PS was the host cell receptor for VSV—and perhaps a general entry factor for all Rhabdoviruses—the sum of evidence was strictly indirect. Also complicating this model was the fact that in vivo most or all PS specifically localizes to the inner leaflet of the plasma membrane[26,27]—seemingly inaccessible and incapable of facilitating attachment.

In order to verify whether PS was in fact the host cell receptor for VSV, cell surface exposed PS was quantified by flow cytometry for a wide range of cell types permissive to VSV infection, and binding was shown to be independent of the amount of PS present.[28] In addition, saturating exposed PS with annexin V prior to incubation with VSV particles did not affect viral binding.[28] Although this demonstrates that PS is not the host cell

attachment factor for VSV, interactions between glycoprotein G and PS are strong and will be discussed later as they seem to play a downstream role in viral fusion[29]—a fact that likely affected initial characterization.

Although the VSV receptor remains undetermined, proteinaceous receptors for other Rhabdoviruses such as RABV and VHSV have been identified.[2,3] The current model of RABV entry proposes that virions are concentrated at neuromuscular junctions by binding to nicotinic acetylcholine receptor (nAChR).[30-34] This increases the chance of the virion binding to the neural cell adhesion molecule (NCAM) at the presynaptic membrane— which likely acts to facilitate internalization.[34,35] In the case of VHSV, antibodies directed towards fibronectin protected fish cells from infection, and fibronectin was shown to bind VHSV virions, strongly suggesting a role for this protein in viral entry.[2] Although a truly definitive understanding of host cell receptors has not yet been demonstrated for any Rhabdovirus, it is clear that proteinaceous receptors are utilized, that receptors vary between strains, and that attachment and internalization may involve a sequential series of events.

Endocytosis

As virus fusion is a pH-dependent process, Rhabdoviruses must be internalized into acidic endosomes for productive infection. Early studies of VSV entry relied heavily on morphological studies by electron microscopy. In some of these studies, the majority of incoming viruses were shown to be present in pits and vesicles with electron-dense coats, implying a dominant role for clathrin-mediated endocytosis; however noncoated vesicles were also observed.[36,37] In contrast, other investigators, also using electron microscopy, showed a preponderance of viruses in large noncoated vesicles, which were possibly macropinosomes.[38] More recently, the use of a dominant negative inhibitor of clathrin-mediated endocytosis (Eps15Δ95/295), combined with pharmacological approaches and knock-down of clathrin heavy chain using RNAi technology, has clearly shown that clathrin-mediated endocytosis is the predominant route of entry.[39] Additional information on the route of entry has come from studies where VSV has been used as model virus, or as a control virus in pseudotyping experiments. Such work has shown a role for COP-mediated endocytic trafficking, and endosomal Rab proteins,[40,41] and high-throughput RNAi screens have shown that VSV endocytosis is highly regulated by specific kinase families.[42] Additionally, VSV is used extensively as a model for studying trafficking in polarized cells, and such studies have found that the virus utilizes a specific route of endocytosis through the basolateral surface of polarized epithelial cells.[43,44]

MEMBRANE FUSION

Glycoprotein G carries out membrane fusion in a low pH-dependent manner,[45-49] and involves dramatic structural reorganization,[50,51] although a precise mechanistic understanding of this process remains unclear. The optimal pH of fusion for VSV and RABV occurs within a narrow range around 6.0,[6,50,52] but fusion activity may still be observed outside this range. One of the more interesting characteristics of the glycoprotein G low-pH induced conformational changes is that it is fully reversible,[53] in contrast to well characterized class I proteins of other viruses.[54]

Localization of Fusion

Based on the pH trigger for fusion, as well as existing data on endocytic trafficking, it was initially believed that VSV fuses from early endosomes.[49] However, recent work using live-cell imaging of individual viruses, combined with biochemical studies of virus penetration, has led to the novel concept that the release of the virus into the cytosol is actually a two-step event.[55] Virus-cell fusion occurs early in the endocytic pathway, based of the effects of nocodazole in dequenching assays of virus-cell fusion. Spatially however, this fusion event occurs with the internal vesicles present within sorting endosomes (which are proposed to be in excess to the limiting membrane facing the cytosol). Subsequent endosome trafficking is then followed by "back-fusion" of the VSV-containing internal vesicles with the limiting endosomal membrane. VSV infection is dependent on the molecular components involved in multivesicular endosome biogenesis,[56] however inhibiting the formation of the internal vesicles of these so-called multivesicular bodies (MVB) with the PI3-kinase inhibitor wortmannin actually led to a reproducible increase in virus entry; presumably in this case direct fusion with the limiting membrane occurred. It is presently unclear what the role of the two-step fusion process actually is. One suggestion is that this allows exposure of the virus core to cellular chaperones present within the internal MVB vesicles, which may have a role in virus uncoating and genome release.[57]

Glycoprotein G Fusion Domain

Class I viral fusion proteins show extensive α-helical structure and undergo dramatic conformational changes which result in the exposure of a hydrophobic fusion peptide that facilitates membrane fusion between two lipid bilayers.[54,58] Although glycoprotein G appears to form a conventional "spike" which is perpendicular to the viral envelope and thus shows certain features of a class I fusion protein, other features are notably absent including proteolytic cleavage and the presence of coiled coils.[59] Likewise, it shows only limited features of class II fusion protein, which typically lie flat to the vial envelope and are comprised predominantly of β-sheet.[60] Initial attempts to understand glycoprotein G mediated membrane fusion utilized hydrophobic photolabeling and demonstrated that VSV and RABV G was able to interact with the host cell membrane in response to low pH, and that residues 59-221 of VSV G and 103-179 of RV G were in close proximity to the membrane during this process.[61] Analysis of VSV G demonstrated that mutation of another highly conserved region, between residues 118-139, abolished fusion activity or modified the pH of fusion activation.[59,62,63] Other studies have also demonstrated that altering region 395 to 418 for VSV,[64] and 392 to 396 in RABV,[65] have an influence on glycoprotein G-mediated fusion. In addition, double mutants within region 118-139 and region 395-418 of VSV show an additive inhibition of fusion activity.[66] Overall, the generation of a definitive model for Rhabdovirus fusion has produced conflicting data that highlight distinct and separate regions of glycoprotein G as being important for facilitating this process. It seems likely that when a clear model has been established, current theories will be found to overlap, with multiple regions working in concert to facilitate fusion. Although the region between amino acids 118 and 139 is generally thought to represent a candidate internal fusion peptide,[58] data supporting this is limited and the possibility exists that existing data concerning this domain may be explained by modulation of fusion activity due to structural requirements elsewhere in the protein.

Recent studies have in fact identified region 145-164 of VSV G, termed the p2-like peptide, as being a pivotal domain in facilitating glycoprotein G mediated membrane fusion.[29,67] Although initially described as heptad-repeat sequences,[23,24] these differ from the heptad repeats found in class I fusion proteins of other viruses because they are not predicted to form coiled coils. As described earlier, the p2 peptide was originally identified in VHSV as a PS binding region;[23] p2-like peptides were subsequently found in all Rhabdovirus isolates.[24,68] In addition to binding PS, the p2 peptide has also been shown to mediate membrane fusion in a low pH- and PS-dependent manner with kinetics identical to that of Rhabdovirus particles,[67] and to insert into the membrane during fusion.[68] Also supporting this model is the finding that the low pH conformational changes of VSV G and membrane fusion have been shown to directly correlate to the PS content of the target membrane.[69] Further studies revealed that the p2-like domain of VSV was in fact capable of binding PS at acidic and neutral pH, the latter being an event specifically facilitated by electrostatic interactions between PS and two histidine residues within this region.[29] These histidines, which are conserved in the p2-peptides of all Rhabdoviruses, are proposed to become protonated when they are brought into extremely close proximity with the membrane surface, an event likely requiring a separate receptor interaction.[29] Substitution or modification of these histidines abolishes fusion activity[67] suggesting that the neutral pH interaction is required, and potentially represents the initial interaction between VSV G and the target membrane during fusion.

In addition to representing the putative fusion domain, the p2 domain of Rhabdoviruses may also be involved in the translocation of PS across the membrane. As previously mentioned, any model involving PS interactions is intrinsically flawed without an explanation of how PS, which specifically localizes to the inner leaflet of the lipid bilayer, is made available to bind glycoprotein G. Recent studies focused on VHSV G (where the p2 domain was first identified) have shown that protein fragments containing the p2 domain and its flanking region (termed p9) are capable of inducing fusion and translocating PS from the inner to the outer membrane leaflet.[70] This activity required a low pH reorganization of the fragment into a predominantly β-sheet structure and was reversible upon pH neutralization.[70] Region p9 alone does not bind to phospholipids within the membrane and therefore may represent the region responsible for facilitating close contact with the membrane through protein-receptor interactions. Homologous mechanisms likely exist within VSV and RABV, although this remains to be shown.

Further confounding these models is the finding that the membrane proximal domain (amino acids 421 to 461), along with the transmembrane domain itself, have also been shown to be essential for glycoprotein G-mediated membrane fusion.[71-75] In fact, synthetic peptides representing the transmembrane domain of VSV glycoprotein G are also capable of facilitating membrane fusion in a Ca^{2+}-dependent manner.[73]

pH-Dependent Reversibility

Low-pH induced reorganization of the prototypical class I fusion protein influenza HA results in a more favorable energy state, an event which is coupled to the energetically unfavorable event of membrane fusion.[76] Within the constraints of this model it would be expected that a fusion protein could not return to its original energy state after reorganization has occurred—a hypothesis that has been experimentally verified.[76] As discussed, Rhabdovirus glycoprotein G also mediates membrane fusion through a

dramatic pH-dependent structural reorganization, however the mechanism appears to be quite distinct from that of HA and other class I viral fusion proteins.

The glycoprotein G of Rhabdoviruses is proposed to exist in three forms: the native state (N), which is observed above pH 7.0 and that which is displayed on the viral envelope after budding; the activated state (A), which is optimal near pH 6.0, involves dramatic conformation reorganization and is capable of facilitating membrane fusion. Below the optimal pH of fusion is the inactivated state (I), which appears structurally similar to the activated state but in which no fusion activity is detectable.[53] Whereas the prototypical class I fusion protein HA remains permanently inactive after the undergoing similar low-pH induced structural reorganizations,[76] the conformational states of glycoprotein G are fully reversible and occur in a pH-dependent manner.[53]

Considering that there is believed to be only one viral fusion event required for entry, the evolution of a reversible fusion protein must be required for a separate event. It is possible that glycoprotein G undergoes reversible folding in order to prevent irreversible preactivation as it travels through the acidic environment of the Golgi, a process which is facilitated by the activation prerequisite of proteolytic cleavage in other class I fusion proteins such as influenza HA.[76] Although not normally considered to be a syncytial virus, cell-cell fusion can be facilitated by VSV at apparently neutral pH in certain polarized cells, which may more closely represent in vivo conditions.[77] Interestingly a number of other low-pH dependent viruses, including coronaviruses and flaviviruses,[78-80] also do not follow the influenza model of low pH inactivation, suggesting a possible common strategy for fusion activation. For Rhabdoviruses, cell-cell fusion may turn out to be a relevant form of viral spread and an important strategy for evading the host immune response.

CONCLUSION

Rhabdovirus entry is a complex process—far more so than initially described -and warrants further study, especially considering the increasing effort to use Rhabdovirus and glycoprotein G pseudotyped vectors for use in human gene therapy.[81] Proteinaceous receptors await identification, which will allow an understanding of their relationship to the viral entry process, especially regarding the role of multivesicular bodies and late endosomes. The process of glycoprotein G-mediated fusion itself remains controversial and fundamentally unclear. Ultimately, the acquisition of a definitive model for Rhabdovirus fusion and virus entry will be dependent on the solving of an X-ray crystal structure for glycoprotein G.

NOTE ADDED IN PROOF

Since the completion of this chapter, the crystal structure of the VSV (Indiana) G protein has been solved.[82] An ectodomain was produced by limited proteolysis of virions at pH 6.25 using thermolysin, yielding G_{th} (residues 1-410 of VSV G), which was then crystallized at pH 7. The structure was solved at 2.4Å resolution, and showed a novel structure for a viral fusion protein. The overall structure of G_{th} is an inverted cone and is apparently in a post-fusion form, consisting of four domains that have features of both class I and class II viral fusion proteins. Domains I and III are predominantly b-sheet,

with domain III having a pleckstrin homology (PH) domain implicated in lipid binding and signaling events. Domain II is termed the trimerization domain and comprises a six-helix bundle reminiscent of the core of the post-fusion form of a class I fusion protein. Domain IV (the so-called fusion domain) has an extended b-sheet structure that has extensive similarity to class II fusion proteins, with four hydrophobic residues on the tip of the domain forming a bipartite "fusion patch" comprising residues W^{72}, Y^{73}, Y^{116} and A^{117}. The p2 domain previously implicated in PS binding and fusion, lies within domain IV—although the contribution of histidine residues, previously proposed to be central to fusion activation, is currently unclear. Also, G_{th} is missing the membrane-proximal region previously reported to be essential for fusion. To account for the reversibility of the conformational changes, it is proposed that a large number of acidic amino acids are brought together in the six-helix bundle, with their deprotonation at higher pH inducing strong repulsive forces that destabilize the core trimer and allow reversion to a pre-fusion state. Despite the relative lack of sequence similarity of the G protein, the basic overall topology of the G protein is likely to be conserved across the *Rhabdoviridae*. The VSV G protein clearly represents a novel structural class of viral membrane fusion protein, and perhaps the most remarkable feature is the finding that it shares the same overall domain structure as glycoprotein B of herpes simplex virus (HSV), indicating that these very different viruses may have common evolutionary origins.

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