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Review

Molecular and Cellular Aspects of Rhabdovirus Entry

Aurélie A. V. Albertini, Eduard Baquero, Anna Ferlin and Yves Gaudin *

Laboratoire de Virologie Moléculaire et Structurale, Centre de Recherche de Gif, CNRS (UPR 3296), Avenue de la Terrasse, 91198, Gif sur Yvette Cedex, France;
E-Mails: alberti@vms.cnrs-gif.fr (A.A.V.A.); baquero@vms.cnrs-gif.fr (E.B.);
ferlin@vms.cnrs-gif.fr (A.F.)

* Author to whom correspondence should be addressed; E-Mail: gaudin@vms.cnrs-gif.fr;
Tel.: +33-1-6982-3836; Fax: +33-1-6982-4308.

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Abstract: Rhabdoviruses enter the cell via the endocytic pathway and subsequently fuse with a cellular membrane within the acidic environment of the endosome. Both receptor recognition and membrane fusion are mediated by a single transmembrane viral glycoprotein (G). Fusion is triggered via a low-pH induced structural rearrangement. G is an atypical fusion protein as there is a pH-dependent equilibrium between its pre- and post-fusion conformations. The elucidation of the atomic structures of these two conformations for the vesicular stomatitis virus (VSV) G has revealed that it is different from the previously characterized class I and class II fusion proteins. In this review, the pre- and post-fusion VSV G structures are presented in detail demonstrating that G combines the features of the class I and class II fusion proteins. In addition to these similarities, these G structures also reveal some particularities that expand our understanding of the working of fusion machineries. Combined with data from recent studies that revealed the cellular aspects of the initial stages of rhabdovirus infection, all these data give an integrated view of the entry pathway of rhabdoviruses into their host cell.

Keywords: rhabdovirus; rabies virus; vesicular stomatitis virus; endocytosis; membrane fusion; glycoprotein

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1. Introduction

The Rhabdoviridae family is grouped in the order Mononegavirales together with the Filoviridae (e.g., Ebola Virus), the Paramyxoviridae (e.g., measles and respiratory syncytial viruses) and the Bornaviridae (e.g., Borna disease virus). All of these viruses are enveloped and have a non-segmented genome made of a single stranded negative-sense RNA molecule.

Among Mononegavirales, Rhabdoviruses have the most diverse hosts. They are widespread among a great diversity of organisms such as plants, insects, fishes, mammals, reptiles and crustaceans [1]. This family has a long history and it has been recently shown that the genomes of several arthropods contain numerous integrated elements from Rhabdoviruses with some integration events that are at least 11 million years old [2].

Based on their structural properties, antigenicity and phylogenetic analyses, Rhabdoviruses have been grouped into six genera. Lyssavirus (prototype: rabies virus—RABV) and Vesiculovirus (prototype: vesicular stomatitis virus—VSV) are the two best studied genera. Other genera include the Ephemerovirus genus (prototype: bovine ephemeral fever virus), the Novirhabdovirus genus (which contains many fish viruses such as the infectious hematopoietic necrosis virus) and two genera that are arthropod-borne and infect plants: Cytorhabdoviruses (prototype: Lettuce necrotic yellows virus) and Nucleorhabdoviruses (prototype: Potato yellow dwarf virus). In addition, numerous identified rhabdoviruses are still unclassified.

All rhabdoviruses have a rigid bullet shape with a flat base and a round tip. The genome of rhabdoviruses comprises up to ten genes among which only five are common to all members of the family. These genes encode the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the viral polymerase (L). The genome is associated with N, L and P to form the nucleocapsid, which is condensed by the matrix protein into a tightly coiled helical structure. The condensed nucleocapsid is surrounded by a lipid bilayer containing the viral glycoprotein G that constitutes the spikes that protrude from the viral surface.

G plays a critical role during the initial steps of the infectious cycle. First, it recognizes receptors at the viral surface and after virion endocytosis, it mediates the fusion between the viral and the endosomal membranes.

2. Basic Biochemical Properties of the Rhabdovirus Glycoprotein

G is a type I membrane glycoprotein. After cleavage of the amino-terminal signal peptide, the complete mature glycoprotein is approximately 500 amino acids long (495 for VSV and 505 for RABV). The bulk of the mass of G is located outside the viral membrane and constitutes the amino-terminal ectodomain (452 for VSV and 440 for RABV). G is anchored in the membrane by a single α-helical transmembrane (TM) segment. The small intraviral domain is likely involved in interactions with internal proteins and there is evidence for RABV G interaction with M protein [3].

For both VSV and RABV, the glycoproteins form trimers [4–8]. This oligomeric organization is not stable at high pH and is sensitive to detergent solubilization [5]. In the case of VSV, there exists a dynamic equilibrium between monomers and trimers of G, both *in vitro* after detergent solubilization [9,10] and *in vivo* in the endoplasmic reticulum [11].

The rhabdovirus glycoprotein is N-glycosylated. The number of glycosylation sites may vary from one virus to another. For both VSV G and RABV G, it has been shown that the oligosaccharide chains are required for proper folding of the protein at two different levels: first, they increase the solubility of the folding intermediates and second, they allow the interaction of these folding intermediates with calnexin and calreticulin, which are chaperones with lectin properties [12–14].

The G ectodomain is the target for neutralizing antibodies [15–19]. The major antigenic sites of both VSV G and RABV G have been characterized. In the case of RABV, several hundred monoclonal antibodies (Mabs) have been used to characterize the antigenic structure of G. RABV G has two major antigenic sites: antigenic site II is located between amino-acids 34 and 42 and amino-acids 198 and 200 [17], and antigenic site III extends from amino acid 330 to 338 [18]. This latter site is associated with virulence and the replacement of arginine 333 by any other amino acid (except lysine) leads to an attenuated phenotype [18,20,21]. In addition to these major antigenic sites, one minor antigenic site and a few isolated epitopes have been described [22–26].

3. Rhabdovirus Receptors

VSV has a wide host spectrum: It infects both vertebrates and insects cells. Therefore, its receptor is a rather ubiquitous molecule. Phosphatidylserine (PS) has long been considered to be a viral receptor [27] despite the fact that it is only present at the surface of apoptotic cells. Recent results indicate that PS is not a receptor for VSV [28]. Other studies have suggested that gangliosides might play the role of the receptors in CER (chicken embryo related) cells [29]. Moreover, recent work has demonstrated that the endoplasmic reticulum chaperone gp96 is essential for infection with VSV [30]. Cells without gp96 or with catalytically inactive gp96 do not bind VSV-G. From these data, it has been proposed that gp96 is essential for the occurrence of functional VSV-G receptors at the cell surface, most likely because it is required for correct folding of either a proteinaceous receptor or an enzyme required for the synthesis of a glycolipid receptor [30].

In the case of RABV, apart from the very beginning and end of the infectious process, non-adapted isolates exclusively multiply and propagate in neurons, and *in vitro*, they can only infect established cell lines of neuronal origin. Several passages are required to select a fixed strain that is adapted to the multiplication in non-neuronal cell lines (such as BHK21 and Vero cells) [31–33]. Most of the fixed RABV laboratory strains have resulted from such an adaptation process. Although they have kept their neurotropism and propagate in the nervous system like street viruses, they have also acquired the ability to use ubiquitous receptors that are present at the surface of non-neuronal cell types [34]. As a consequence, among the many molecules that have been proposed to be RABV receptors [35], it is not clear which are actually used by natural isolates during animal infection.

Host cell treatment with different phospholipases has been shown to reduce the binding of fixed RABV strains suggesting that some phospholipids can play the role of viral receptors [36]. Similarly, cells pretreated with neuraminidase were shown to be non-susceptible to viral infection [37]. After incorporation of exogenous gangliosides such as GT1b and GQ1b, the cells recovered their susceptibility to RABV infection [38]. These results indicate that highly sialylated gangliosides are part of the cellular membrane receptor structure for the attachment of fixed RABV strains.

In addition to phospholipids and gangliosides, three proteins have been proposed to play the role of viral receptors. Some evidence indicates that the nicotinic acetylcholine receptor (nAChR) acts as a RABV receptor [39]. First, a segment of RABV G has a sequence similarity to the snake venom curaremimetic neurotoxins which are potent ligands of nAChR [40]. Second, an interaction between RABV and purified Torpedo nAChR was demonstrated [41]. Finally, purified RABV was shown to bind the α subunit of nAChR in an overlay assay [42]. However, there is no direct evidence in animal models that this molecule is a RABV receptor. Furthermore, RABV can infect neurons that do not express nAChR [43], and nAChR is located mainly on muscle cells. Thus, although nAChR could account for the ability of street RABV to multiply locally in myotubes at the site of inoculation [44], which would facilitate the subsequent penetration into neurons, other molecules are needed to mediate viral entry into neurons.

The second protein that has been proposed to play the role of a RABV receptor is the neural cell adhesion molecule (NCAM) [45]. Preincubation of RABV with soluble NCAM inhibited its ability to infect susceptible cells. Moreover, the transfection of resistant L fibroblasts with the NCAM-encoding gene induces RABV susceptibility. Additionally, the infection of NCAM-deficient mice by RABV resulted in slightly delayed mortality and restricted brain invasion. This suggests that NCAM is a *bona fide* receptor *in vivo*.

The low-affinity nerve-growth factor receptor, p75NTR was identified as a ligand of a soluble form of RABV G [46]. The ability of RABV G to bind p75NTR was dependent on the presence of a lysine and an arginine in positions 330 and 333, respectively, which were known to control virus penetration into the motor and sensory neurons of adult mice [18,20,21]. Replacement of amino acids 318 and 352 were also shown to abolish interaction between p75NTR and RABV G [47]. Furthermore, p75NTR-expressing BSR cells were permissive for a non-adapted fox RABV isolate (street virus). The glycoprotein from another genotype of lyssavirus (GT 6, European bat lyssavirus type 2) was also shown to bind p75NTR [48]. Nevertheless, mice lacking all the extracellular receptor domains were still susceptible to infection (of which the rate and specificity were unchanged), indicating that the RABVG-p75(NTR) interaction is not necessary for RABV infection of primary neurons [49].

Very little is known concerning the receptor of other rhabdoviruses, the only exception is the viral hemorrhagic septicemia virus (VHSV), a salmonid rhabdovirus, for which it has been shown that monoclonal antibodies (MAbs) directed against a fibronectin containing complex protect cells from the infection. Because the purified rainbow trout fibronectin was able to bind specifically to VHSV, fibronectin was proposed to be a receptor for VHSV and other fish rhabdoviruses [50].

4. Fusion Properties of Rhabdoviruses

After receptor binding, both RABV and VSV enter the cell by the endocytic pathway. The acidic environment of the endosomal compartment triggers a series of conformational changes of the viral glycoproteins that catalyze fusion between the viral and the endosomal membranes [51–53].

The pH dependence of fusion has been characterized for several rhabdoviruses [54–56]. Fusion is optimal around pH 6 and the threshold for fusion activity is approximately pH 6.5.

As for other viruses that fuse at low pH, the exposure of the virion to low pH in the absence of a target membrane leads to the inactivation of the fusion properties of G [57–59]. Remarkably, unlike

other viruses for which the low-pH induced fusion inactivation is irreversible [57], reincubation of the virus above pH 7 leads to the recovery of the initial fusion activity of rhabdoviruses [58,59].

Along with the fusion properties, it has been demonstrated that G can assume at least three different conformational states that have different biochemical properties [54,58]. The native pre-fusion state is observed at the viral surface above pH 7. In this conformation, G is thought to bind the viral receptors. Upon acidification, the virions are initially more hydrophobic [58], a feature that, in the absence of a target membrane, results in viral aggregation [59]. Using hydrophobic photolabeling, it has been demonstrated for both RABV and VSV, that G is then in an activated state that is able to interact with the target membrane as the first step of the fusion reaction [60]. After a longer incubation at low pH, the G post-fusion conformation is reached. In this conformation, the structure of G is antigenically distinct from both the pre-fusion and the activated conformation and, in electron microscopy, its ectodomain appears much more elongated than in the pre-fusion state (11 nm *versus* 8 nm) [58].

The structural transition is reversible and the pre-fusion state can be recovered from the post-fusion state by readjusting the pH above 7 [58]. In fact, there is a pH-dependent thermodynamic equilibrium between different states of G that is shifted toward the post-fusion state at low pH [61]. The transition toward the post-fusion state is highly cooperative upon proton binding: approximately 2.8 protons bind simultaneously to trimeric G to induce the conformational change [61].

This equilibrium explains why the low-pH induced inactivation is reversible. The reversibility of the conformational change is required to allow G to be transported through the acidic compartments of the Golgi apparatus and to recover its native pre-fusion state when incorporated in newly synthesized virions [62]. Other viruses, for which the fusogenic conformational change is irreversible (such as influenza virus, tick borne encephalitis virus or Semliki forest virus), have evolved different mechanisms to protect their fusion proteins from undergoing irreversible conformational changes in the Golgi apparatus [63]. For these viruses, it has been proposed that the high amount of energy released during the conformational change from the metastable pre-fusion state to the final stable post-fusion state is used to form the high energy lipidic intermediates during the fusion reaction [64]. In the case of rhabdoviruses, the existence of an equilibrium between the different states implies that the energy released during the structural transition of a single trimer is small compared with the energetic barrier of the fusion reaction (the activation energy of the fusion process has been estimated to be in the range of 40 kcal/mol [54,65,66]). This indicates that a concerted action of several trimers is required. Indeed, for RABV, the minimal number of spikes involved in a fusion complex has been estimated to be approximately 15 [61].

The fusion pathway of rhabdoviruses with liposomes has been studied in detail. Neither RABV nor VSV require a specific lipid for fusion [59,67,68]. However, recent studies have indicated that the target membrane composition has an influence on the efficiency of the process. Particularly, it has been demonstrated that lipid bis(monoacylglycero)phosphate (BMP, also called lysobisphosphatidic acid), present in the internal vesicles of the endocytic pathway, favors VSV fusion when it is present in the target membrane [69].

An investigation of the effects of lipids with various dynamic molecular shapes on RABV-induced fusion has suggested that, similar to other enveloped viruses [70,71], RABV-induced fusion proceeds via the formation of an intermediate stalk that is a local lipidic connection between the outer leaflets of the fusing membranes [57]. A radial expansion of the stalk would induce the formation of a transient

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