Structure-Function Relationships and Mode of Replication of Animal Rhabdoviruses

(functions of rhabdoviruses/proteins of rhabdoviruses/replication in enucleated cells)

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ABSTRACT Recently accumulated knowledge allows more precise comparison of the structural (and possibly evolutionary) relationships of several different animal rhabdoviruses: vesicular stomatitis virus, rabies virus, Kern Canyon virus, and spring viremia of carp virus. Each virus is composed primarily of a glycoprotein, an RNA-associated nucleoprotein, and one or two membrane proteins. Vesicular stomatitis virus group viruses contain lesser amounts of two additional distinct polypeptides, NS and L. The separate viruses undergo structural polypeptide phosphorylation in vitro according to characteristic patterns. In vesicular stomatitis virus the NS protein is selectively phosphorylated. In rabies group viruses and in spring viremia of carp virus, the nucleoprotein is the predominant phosphoprotein; in these viruses only the phosphorylated moiety is selectively cleaved off by trypsin. In Kern Canyon virus, only membrane protein and glycoprotein are weakly phosphorylated. Each virus possesses a virion-bound protein kinase. Vesicular stomatitis virus group viruses, Kern Canyon virus, and spring viremia of carp virus only contain virion-bound transcriptases of respectively decreasing levels of activity demonstrable in vitro. Vesicular stomatitis and Kern Canyon viruses replicate efficiently in enucleated cells; rabies virus does not. Based upon these observations, it is suggested that vesicular stomatitis virus may represent the most highly evolved of these rhabdoviruses, whereas spring viremia of carp and Kern Canyon viruses may represent "evolutionary links" between the vesicular stomatitis and rabies virus groups.

In general, a newly discovered virus is classified as a rhabdovirus on the basis of its morphology and ultrastructural features (bullet-shape; helical nucleocapsid surrounded by an envelope with surface projections). Until now, however, division of rhabdoviruses into subgroups has been based principally on their antigenic characteristics. The vesicular stomatitis virus (VSV) subgroup comprises several antigenically related members (VSV Indiana, VSV Argentina, VSV Cocal, VSV Brazil, VSV New Jersey, and possibly Chandipura and Piry viruses), and the subgroup of rabies virus consists of five members (rabies, Mokola, Lagos bat, Kotonkan, and Obodhiang viruses) sharing common antigens. These cross-relationships were established mainly as the result of the study of serological crossreactivities between the core proteins of various members of these subgroups (1-4), since the envelope antigens were found more distantly related than internal antigens even among members of the same subgroup. Further studies indicated that two other animal rhabdoviruses, the Kern Canyon virus (KCV) and the spring viremia of carp virus (SVCV), are immunologically unrelated to either members of VSV or rabies virus subgroups (4, 5).

The study of the homology of the nucleotide sequences between genomes of various members of the rhabdovirus subgroup indicated that although the nucleotide sequences of the fixed strains of rabies virus were nearly identical, a very low, or barely significant, degree of homology existed between various members of VSV serological subgroups, and as expected, there was no homology between the genomes of rabies virus and SVCV on one hand, and VSV on the other (6, 7) (D. H. L. Bishop, personal communication).

Recently, advances in the knowledge of the structure of protein composition and other biochemical properties of rhabdoviruses and of their mode of replication in the host cell have indicated that different interrelationships between members of this group of viruses may have to be contemplated. Data presented in this article suggest that these characteristics have to be considered in any meaningful classification of rhabdoviruses.

Analysis of composition of proteins of animal rhabdoviruses revealed the presence in all of them of three major proteins. (i) The G protein (envelope glycoprotein) constitutes the basic unit of surface projections of the virus particles emerging from the lipid-coated membrane protein(s) and is the antigen capable of binding virus neutralizing antibodies and of eliciting their formation (8-10). This surface antigen is also responsible for agglutination of erythrocytes by certain rhabdoviruses (11) and for the adsorption of the virus particles onto the surface of the host cells. (ii) The M protein(s) form(s) the envelope membrane surrounding the viral nucleocapsid. (iii) The N protein is associated with the viral RNA to form the helical nucleoprotein (4, 12, 13). In addition, all members of the VSV subgroup contain two minor (L and NS) proteins (Fig. 1a). The relative proportions of the protein constituents and the molecular size of the structural proteins differ slightly between viruses of the VSV subgroup (14-16). In contrast, rabies virus contains in addition to G and N proteins, two membrane proteins (M1 and M2) and no clearly defined minor polypeptides (Fig. 1b). Mokola and Lagos bat viruses, belonging to the rabies subgroup serologically, contain the three major (G, N, and M) and two minor polypeptide components (Fig. 1c). The polypeptide composition of SVCV is similar to, but not identical with, that of VSV (Fig. 1d). KCV contains the three major proteins (G, N,

Abbreviations: VSV, vesicular stomatitis virus; KCV, Kern Canyon virus; SVCV, spring viremia of carp virus; G protein, glycoprotein; M protein, membrane protein; N protein, nucleoprotein.

In addition to the glycosylation of the envelope spike protein, core proteins of rhabdoviruses can be modified by phosphorylation of serine or threonine residues (17) (Table 1). In viruses of VSV subgroup the core-associated minor NS protein is the only phosphoprotein (4, 13, 18, 19). With rabies and rabies-related viruses, the N protein becomes phosphorylated in the infected cells even before the envelopment of the viral nucleocapsid. The phosphorylation of the N protein of these viruses is confined to a terminal segment of the polypeptide, which can be cleaved off by treatment of the viral nucleocapsid with trypsin (4, 13, 20). For comparison, the N protein of VSV is not phosphorylated and cannot be cleaved by exposure of the nucleocapsid to trypsin. Although the N protein is the only structural phosphoprotein of rabies virus, the rabies-related Mokola and Lagos bat viruses contain two additional phosphoprotein components. SVCV exhibits a pattern of intracellular protein phosphorylation that resembles that of rabies subgroup viruses: the N protein and one additional protein are phosphorylated in vivo. The phosphorylated segment of the SVCV N polypeptide can also be removed by treatment of the nucleocapsid with trypsin (4).

In KCV, the G and N proteins are phosphorylated but to a far lesser extent than the phosphoproteins of other rhabdoviruses. We believe that this marginal phosphorylation occurs during or after virus assembly. Correspondingly, the N protein is not phosphorylated in the intracellular free nucleocapsids of KCV and cannot be cleaved by treatment of the nucleocapsid with trypsin (13).

All animal rhabdoviruses mentioned here contain a virus-bound protein kinase that, in a cell-free system, can catalyze the transfer of the γ-phosphate group of ATP or dATP to viral proteins. The viral phosphoproteins are usually the best phosphate acceptors, in vitro, although among the viral proteins that are not phosphorylated in vivo there are some that can accept phosphate in a cell-free system (13, 17, 20, 21). The biological function(s) of the virus-bound protein kinase and of rhabdovirus protein phosphorylation is not known.

In addition to protein kinase, the members of the VSV subgroup contain a particle-bound RNA-dependent RNA polymerase, transcriptase (22), which transcribes, both in vivo and in vitro, the viral RNA (the minus-RNA) into a complementary RNA (plus-RNA) (23–26). The transcriptase activity seems to be connected with the minor L protein of VSV (26). KCV contains a similar transcriptase; its specific activity is, however, much lower than that of VSV (27).

The transcriptase activity bound to SVCV has been found to be even lower than that bound to KCV. The infectivity and transcriptase activity of subviral fractions of members of the VSV subgroup can be restored by combining the template (nucleoprotein) of one virus with the solubilized fraction (containing also the L protein) of another closely related virus. This type of reconstitution does not seem to be operative with distant members of the subgroup (D. H. L. pro-
Bishop, personal communication). No RNA transcriptase activity has yet been detected in rabies, Mokola, and Lagos bat viruses tested in quantities (45–50 µg of protein) adequate to demonstrate marked activity in VSV, KCV, and SVCV.

In cells exposed to viruses of the VSV subgroup, the events involved in the replication of the virus particles (uncoating of parental virus, transcription of the viral genome, transcription of the virus-specific messenger RNA, viral RNA replication, and virus assembly) are confined to the cytoplasm of the host. The replication of these viruses is not affected by inhibitors of DNA synthesis or of DNA transcription, and assembly of infectious VSV proceeds unhindered in the enucleated host cells (28, 29). Parental rabies virus particles are also uncoated in the cytoplasm of the host cells (K. Hummeler and F. Sokol, unpublished observation), where

**Table 1. Properties of structural proteins, glycoproteins, and phosphoproteins of animal rhabdoviruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Designation (mol. wt. × 10⁻¹) and localization of Phosphoproteins</th>
<th>Changes in the N protein after treatment of the nucleocapsid with trypsin</th>
<th>Proteins capable of accepting phosphate in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSV (Indiana)</td>
<td>NS (53), core (not bound to RNA)</td>
<td>L (190), core (not bound to RNA) G (69), envelope surface N (50), core (bound to RNA) M (29), envelope membrane</td>
<td>No change</td>
</tr>
<tr>
<td>KCV*</td>
<td>N† (54), core (bound to RNA) G (85), envelope surface</td>
<td>M (25), envelope membrane</td>
<td>No change</td>
</tr>
<tr>
<td>SVCV</td>
<td>N (52), core (bound to RNA) P (43), core (not bound to RNA)</td>
<td>L (150), unknown localization G (88), envelope surface Minor protein (35), unknown localization M (23), envelope membrane</td>
<td>Phosphorylated segment cleaved off</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>N (62), core (bound to RNA)</td>
<td>G (80), envelope surface M1 (40), envelope membrane M2 (25), envelope membrane</td>
<td>Phosphorylated segment cleaved off</td>
</tr>
<tr>
<td>Lagos bat virus</td>
<td>N (61), core (bound to RNA) P (45), core (not bound to RNA)</td>
<td>G (65), envelope surface M (25), envelope membrane</td>
<td>Phosphorylated segment cleaved off</td>
</tr>
<tr>
<td>Mokola virus</td>
<td>N (64), core (bound to RNA) P (46), core (not bound to RNA)</td>
<td>G (73), envelope surface M (25), envelope membrane</td>
<td>Phosphorylated segment cleaved off</td>
</tr>
</tbody>
</table>

* Both N and G protein are phosphorylated in vivo to a very low degree; probably only a small proportion of the molecules possesses phosphate group(s).
† The phosphorylated N molecules migrate more slowly in sodium dodecyl sulfate-polyacrylamide gel than the nonphosphorylated ones.
P = Protein of unknown function.

**Table 2. Evolutionary trends in rhabdoviruses**

<table>
<thead>
<tr>
<th>Subgroup of virus</th>
<th>Site of assembly of nucleocapsids and of virus particles*</th>
<th>Nuclear function required for virus production</th>
<th>RNA transcriptase activity (relative to activity in VSV Indiana)†</th>
<th>Cleavage of the phosphorylated core protein (N) by trypsin treatment of the nucleocapsid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato yellow dwarf virus</td>
<td>Nucleus</td>
<td>Yes</td>
<td>Not done</td>
<td>Not known</td>
</tr>
<tr>
<td>Rabies</td>
<td>Cytoplasm</td>
<td>Yes</td>
<td>Not detectable (&lt;0.005)</td>
<td>Yes</td>
</tr>
<tr>
<td>SVCV</td>
<td>Cytoplasm</td>
<td>Not known</td>
<td>0.01</td>
<td>Yes</td>
</tr>
<tr>
<td>KCV</td>
<td>Cytoplasm</td>
<td>No</td>
<td>0.04</td>
<td>No</td>
</tr>
<tr>
<td>VSV</td>
<td>Cytoplasm</td>
<td>No</td>
<td>1.00†</td>
<td>No</td>
</tr>
</tbody>
</table>

* Based on ultrastructural observations.
† The transcriptase activity was assayed at 30° in the presence of actinomycin D (4 µg/ml) in a described cell-free system (27) (P. Madore, H. F. Clark, and F. Sokol, to be published). Activities were compared on the basis of pmol of UTP incorporated per mg of viral protein.
† B (infectious) particles recovered from cells concomitantly infected with defective interfering T particles show extremely low levels of RNA transcriptase activity (32).
the transcription (in the presence or absence of cycloheximide) of the viral genome (D. H. L. Bishop, personal communication) and translation of the virus-specific messenger RNA also takes place. Electron microscopic observation indicates that maturation of rabies virus takes place either at the plasma membrane or in association with intracytoplasmic matrices (30). Infectious KCV is also produced by enucleated cells.

In contrast to the results obtained with VSV or KCV, infectious rabies virus cannot be produced by enucleated cells, but synthesis of rabies antigen (as detected by immunofluorescence) and virus-directed plus-RNA takes place in these cells (29). The synthesized virus-specific messenger RNA may be defective so that it cannot be translated into all viral proteins needed for the assembly of the infectious virus. Alternatively, the progeny viral minus-RNA may replicate exclusively in the nucleus. Even if this is not the case and viral RNA can replicate in the cytoplasm, the block for virus assembly may exist at a post-translational step and functional nucleus must be present for virus maturation to occur. Whether the fact that replication of rabies virus is somewhat inhibited by the DNA inhibitor, cytosine arabinoside (31), is related to the inability of the virus to replicate in enucleated cells is difficult to say at present. Nuclei of the host cells are known to be involved in the replication of several plant rhabdoviruses, the vicinity of the inner nuclear membrane being the site of nucleocapsid and virion assembly (1).

With these advances in our knowledge concerning rhabdoviruses, it is clear that the classification of animal rhabdoviruses into meaningful subgroups must be on the basis of the comparative structure of their components and of the virus-host interrelationship during replication. If rhabdoviruses evolved from an agent highly dependent on functions of the host cell, specifically on those that were required for the transcription and the replication of the viral genome (1), then viruses that do replicate and mature in the nucleus, such as potato yellow dwarf virus, would represent an ancestor prototype of the rhabdovirus group. Conversely, viruses of the VSV subgroup, which acquired a relatively high degree of independence from host cell functions, could represent the most recent evolutionary stage in this group (Table 2). It is interesting to note that under certain conditions of propagation (see the legend of Table 2) even VSV can become more dependent on host functions through a marked decrease in its transcriptase activity (32). Such functional modification brings VSV closer to the members of the rabies subgroup. The characteristics of VSV and KCV seem much more similar than their antigenic differences would indicate. Conversely, the SCV appears much more closely related to the rabies virus group even though its polypeptide composition more closely resembles that of the VSV group. SCV is also antigenically unrelated to members of rabies subgroup and shows no genome homology with members of this group. It seems, therefore, that SCV and KCV may represent evolutionary links between the members of VSV group on one hand and rabies group on the other.

Thus, further studies along these lines may not only unravel meaningful relationships within the group of rhabdoviruses, but may also throw some light on the evolution of these agents in nature.

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