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The glycoprotein G of rhabdoviruses

Brief Review

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Summary. Rhabdoviruses show an RNA-containing helically-wound nucleocapsid either enclosed by or enclosing a membrane M protein, surrounded by a lipid bilayer through which dynamic protein trimers made up of non-covalently associated monomers of glycoprotein G (G) project outside. Mature monomeric rhabdoviral G has more than 500 amino acids, 2–6 potential glycosylation sites, 12–16 highly conserved cysteine residues, 2–3 stretches of a–d hydrophobic heptad-repeats, a removed amino terminal hydrophobic signal peptide, a close to the carboxy terminal hydrophobic transmembrane sequence and a carboxy terminal short hydrophylic cytoplasmic domain. Association-dissociation between monomers-trimers and displacement of the trimers along the plane of the lipid membrane, are induced by changes in the external conditions (pH, temperature, detergents, etc.). Throughout conformational changes the G trimers are responsible for the virus attachment to cell receptors, for low-pH membrane fusion and for reacting with host neutralizing monoclonal antibodies (MAbs). Antigenic differences could exist between monomers and trimers, which may have implications for future vaccine developments. The family *Rhabdoviridae* is made up of the *Lyssavirus* (rabies), the *Vesiculovirus* (vesicular stomatitis virus, VSV) and many rhabdoviruses infecting fish, plants, and arthropod insects. All these reasons make the G of rhabdoviruses an ideal subject to study comparative virology and to investigate new vaccine technologies.

Rhabdoviruses

Molecular morphology models

The infectious virion is round at one end and flat at the other, measuring about 200 × 70 nm. Shorter virions, from 50 to 80 nm, interfere with the replication of infectious virions (defective interfering particles). The ratio of infectious virions to defective interfering particles varies from 1/100 to 1/1000. Defective

Table 1. Proteins of rhabdoviruses

Protein	M.W. (KDa)	Estimated number of molecules/virion VSV	Rabies	IHNV	VHSV
L	≈200	50	72	43	14
G	57	1205	1335	166	107
N	50	1258	1325	766	892
M1 (NS)	33	466	691	270	464
M2 (M)	23	1826	1148	1192	956

VSV Vesicular stomatitis virus [140]; Rabies [54]; *IHNV* infectious haematopoietic necrosis virus [72]; *VHSV* viral haemorrhagic septicaemia virus [8]. IHNV and VHSV have a sixth protein not present in the virions of about 12 KDa (NV) [83]

particles are abundant in persistent infections and in undiluted in vitro passages [71].

Rhabdoviruses consist of a helically wound ribonucleocapsid (3.5 μ long) surrounded by a lipid bilayer through which spikes project outside at 5 nm intervals. Trypsin treatment of the virion removes the spikes leaving behind a short peptide traversing the lipid bilayer. Removal of the lipid bilayer with phospholipases and fixation with formaldehyde yields the protein skeleton of the virus with the spikes attached to the ribonucleocapsid. Disruption of the lipid bilayer with detergents (Triton, CHAPS, etc.) releases the free spikes and the ribonucleocapsid, coiled in the absence of salts or uncoiled in the presence of high salt concentrations.

Rhabdoviruses have about 11000 bases of negative RNA to code for 3700 amino acid (aa) residues corresponding to an average of 5 6 proteins (Table 1). Rhabdoviruses possess 2 membrane proteins, the glycoprotein G(G) that forms the non-covalently-bound homotrimer spikes (65 KDa monomers) and the matrix protein M (M_2) (20 KDa). Rhabdoviruses have several internal proteins, the RNA polymerase L (200 KDa), a second matrix protein sometimes phosphorylated, M_1, NS or P (24 KDa) and the nucleoprotein N phosphorylated in most rhabdoviruses except in VSV (38 KDa). The internal proteins, L, N, and the viral RNA form the viral ribonucleocapsid. The approximate composition of rhabdoviruses is 74% of protein, 20% of lipid (from the membrane of the infected cell), 3% of carbohydrate (in the G) and 3% of RNA.

The M protein is resistant to protease digestion and lactoperoxidase iodination and at least in part, attaches both to the G on the internal side of the membrane and to the ribonucleocapsid (demonstrated by cross-linking) to the opposite side [28, 73, 155] (Fig. 1A). The M protein including that of fish rhabdoviruses [10] is basic [90], most probably to attach to the negatively charged residues of the inner surface of the membrane [155]. The cytoplasmic tail of the VSV G contains a sequence to interact with the M protein, to stabilize the G trimer [96] and to direct foreign chimeric proteins (pseudotypes) into VSV particles [106]. However, the results of negative staining and immuno-

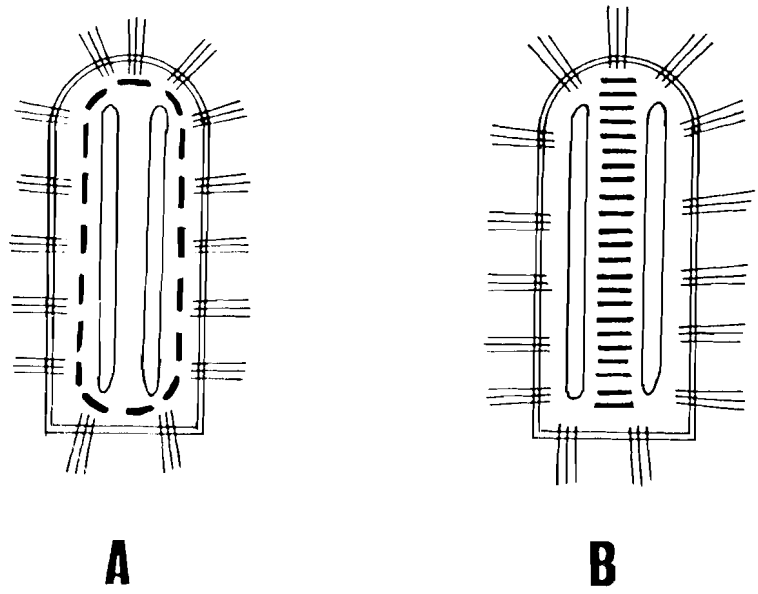

Fig. 1. The two proposed models for rhabdovirus molecular morphology. Rhabdoviruses contain an internal ribonucleocapsid (‖) surrounded by a lipid-bilayer (═══) through which G homotrimer spikes protrude (|||). The M protein (— — —) either covers all the internal side of the viral membrane (**A**) or is inside the ribonucleocapsid (**B**, drawn after Barge et al. [7])

electronmicroscopy, preliminarly confirmed by skeleton-liposome binding studies [7] have proposed a new model for the structure of rhabdoviruses in which the nucleocapsids are wound around a core of protein M (Fig. 1B). According to this model the protein M would be attached to the G and/or the lipid but only at the ends of the viral skeleton. The possibility also exists that both structures could be in a dynamic equilibrium depending on external conditions (for instance, pH) as suggested by some electron micrographs [15, 18].

Rhabdovirus types

The *Rhabdoviridae* family has been classified into the Lyssavirus (rabies) and the Vesiculovirus (vesicular stomatitis virus, VSV) genera [16] on the basis of seroneutralization and studies with monoclonal antibodies (Mabs) [144]. Other rhabdoviruses include those infecting fish, plants and arthropod insects. Closely-related strains can infect different species, for instance, VSV not only infects some vertebrates but also replicates in insect vectors as do some plant rhabdoviruses.

Lyssaviruses are rabies and rabies-related viruses, each one having several subtypes that affect mammals worldwide. All 5 viral gene products, the L, G, M_2, M_1 and N proteins, are present in the virion. The cDNA derived G sequences from fixed and street rabies strains have been recently compilated [9, 134] (Table 2) but new sequences continuously appear in the literature.

Table 2. Selected *Rhabdoviridae* viruses and the number of aa of G

Host	Virus description	# aa	Reference
Fish	VHSGER VHSV 07.71	507	[132]
	VHSDK VHSV DK	507	[94]
	IHNGP IHNV Cedar	507	[77]
Mammal	RABMOK rab Mokola	524	[134]
	RABPV rab PV	524	[135]
	RHRBGD rab ERA	524	[4]
	RABSAD rab SADB19	524	[33]
	RABMEP rab MEP	524	[101]
	RABLEP rab LEP	524	[133]
	RABCVS rab CVS	524	[111]
Cattle	VSVGPNO8 VSV N.J.-I	517	[103]
Horse	VSVGPNJA VSV N.J.-II	517	[103]
Swine	VSVGPN29 VSV N.J.-III	517	[103]
	RHGPORS VSV Orsay	511	[59]
	RHGM VSV San Juan	511	[118]
	RHVSVGR VSV Indiana	511	[137]
Insect	SIGMA Drosophila	526	[131]
	BEFV Bov. eph. fev.	623	[141]
Plant	SYNVG ATCC-PV263	628	[67]

VHS Viral haemorrhagic septicaemia; *IHN* infectious haematopoietic necrosis; *VPC* spring viremia carp; *RAB* rabies; *VSV* vesicular stomatitis virus; *BEFV* bovine ephemeral fever virus; *SYNV* sonchus yellow net virus

Vesiculoviruses are grouped in 2 main serotypes, New-Jersey (VSV-NJ) and Indiana (VSV-Ind), and in other minor strains [16, 103], each one having several subtypes. Vesiculoviruses affect cattle, horses and swine and are distributed throughout the Americas but are also present in Europe. All 5 viral gene products, the L, G. M, NS and N, are present in the virion. The cDNA derived G sequence has been studied in 34 VSV-NJ isolates [103] and 26 VSV Ind isolates [14]. Other studies have also reported cDNA derived VSV G sequences [59, 118, 137].

The main fish rhabdoviruses include those of cold-water teleost, like infectious hematopoietic necrosis virus (IHNV) or viral haemorrhagic septicaemia virus (VHSV) and those of warm-water teleost like spring viraemia of carps (SVCV) or others affecting eels and perch. Five mRNAs encoded the L, G, M_1, M_2, and N structural IHNV proteins, whereas the sixth encoded a nonviral (NV) protein of about 12 KDa [83]. The gene order by R-loop was 5′ L-NV-G-M_2-M_1-N. A similar gene structure has been discovered also in VHSV (B. Basurco, pers. comm. 1994). Only the cDNA derived G sequences from IHNV [77] and from VHSV [94, 132] have been published to date.

Only the cDNA derived G sequence of the plant rhabdovirus sonchus yellow net virus has been reported to date [67]. The cDNA derived G sequence of

bovine ephemeral fever [141] and of Sigma virus have also been reported [130, 131]. More than 100 other rhabdoviruses remain uncharacterized.

The glycoprotein G of rhabdoviruses

Molecular structure

The virion of rhabdoviruses possesses a unique G, an homotrimeric membrane protein forming a protruding 83 Å spike [61, 62]. The G is responsible for virus attachment to cell receptors [152], it contains the fusion properties of the virus detectable at pH $\leqslant 6$ in VSV [78], in rabies [61] or in VHSV [87] and it reacts with the host neutralizing Abs in VSV [75], VHSV [93], IHNV [48] or rabies [40].

The G of VSV-Ind has 511 amino acids including a signal peptide, two sites of potential glycosylation [118], a nonglycosylated hydrophobic protease-resistant fragment of MW $\approx$ 5000, two acylated sites, two regions of a–d hydrophobic heptad-repeats [29] and an hydrophilic cytoplasmic carboxy-terminal peptide tail [142]. In all rhabdoviruses studied this basic structure is highly conserved (Fig. 2). The VSV-NJ G contains 517 aa with a 50.9% sequence identity to the VSV-Ind. The rabies G contains 524 aa with more than 90% sequence identity among strains and with about a 20% sequence identity with the VSV G.

The VSV G exists in the virion as a homotrimer, as shown by chemical crosslinking and sedimentation velocity analysis of detergent-solubilized molecules [46]. In the presence of octylglucoside, fluorescein or rhodamine-labelled G monomers undergo reversible dissociation (in the nM range or at 60 ng/ml)-association (at 1000-fold higher concentration) [97]. Isolated G in the presence of octylglucoside at pH 7.5 appears as trimers in the VSV-Ind or as monomers in the VSV-NJ (the trimers of the VSV-NJ G are more stable at pH 5.7) [45]. Exchange among genetically different G monomers also occurs among the trimers in vivo [154].

The G extracted from the virion membrane by octylglucoside [108] or by lysolecithin [3] forms tail to tail rosettes as viewed in the electron microscope. A mixture of isolated G and sonicated phosphatidylcholine when dialyzed free of detergent is capable of forming vesicles with the G spikes protruding asymmetrically from the exterior of the lipid membrane as determined by proteolytic digestion.

Importance of glycosylation in folding and assembly

The carbohydrate of the G accounts for 10% of its total mass with a branched structure attached to a mannose-rich pentasaccharide core by N-linked glycosylation [112]. Only two of the three glycosylation sites in the ectodomain of G in most of the rabies strains are glycosylated, giving rise to two molecular forms separable by gel electrophoresis [151]. The degree of final sialylation (negative charges) produces host-dependent heterogeneity because the virion depends on the host cell enzymes for the addition of carbohydrate [126].

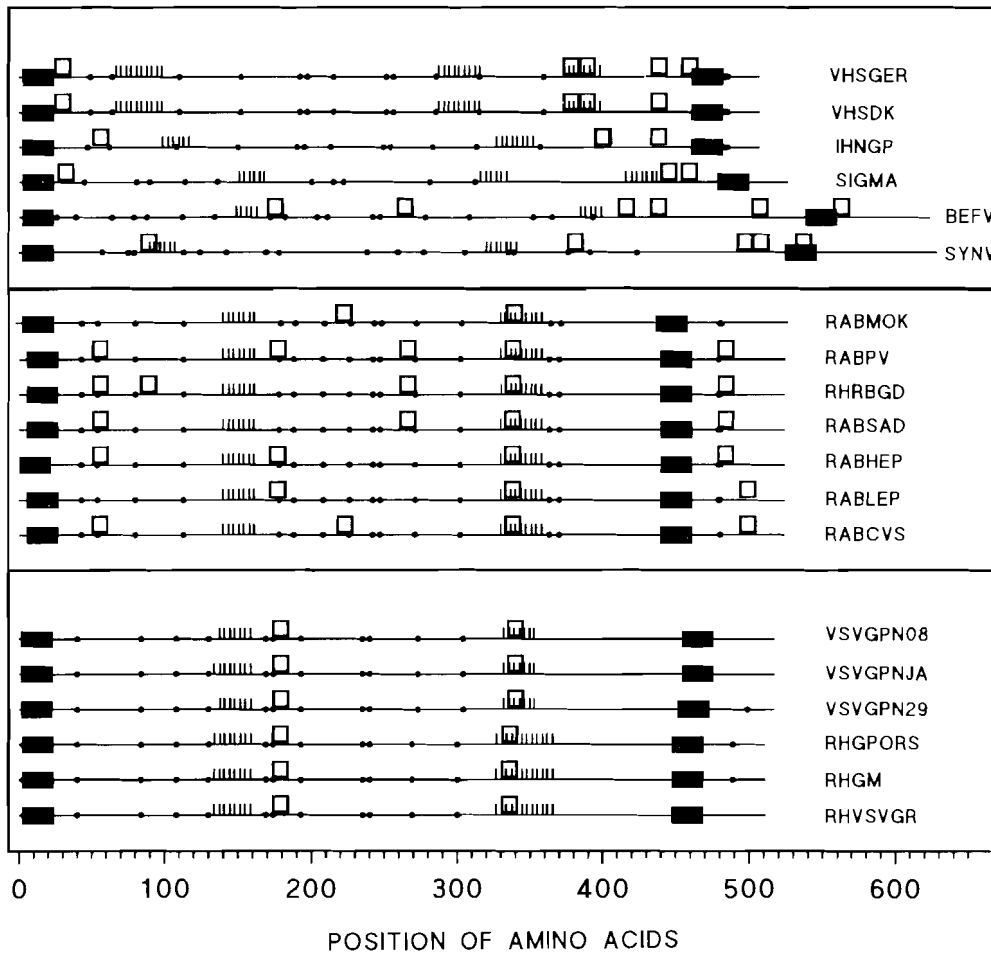

Fig. 2. Positions of structural elements of the G of rhabdoviruses. The names of the rhabdoviruses correspond to those of Table 2. ● Cysteins; □ putative glycosylation aa consensus sequences (in aa single letter code, NXS, T); ■ predicted transmembrane and signal peptides. ||||||, newly defined a–d hydrophobic heptad-repeats [29]

The current model of synthesis, folding, processing and membrane insertion of the VSV G, has been derived by using cell-free systems [64]. When the mRNA coded by the G gene is translated in a cell-free system, a 63 KDa nonglycosylated precursor is synthesized which after the addition of microsomal membranes, yields a 67 KDa glycosylated protein lacking the signal peptide [92]. Some glucose and mannoses are removed 10–20 min later at the Golgi apparatus where the peripheral sugars N-acetylglucosamine, galactose, sialic acid and fucose are added. All the processing is finished before the G is transported to the plasma membrane [124].

The addition of the carbohydrates is needed for the nascent protein chains to fold properly, probably to make hydrophobic folding intermediates more soluble and less likely to form irreversible aggregates. Elimination of some

cysteins [99] and/or inhibition of glycosylation either by site-directed mutagenesis in the consensus sequences or by inhibition of glycosylation by tunicamycin, leads to aggregated, misfolded, and/or disulfide crosslinked complexes in addition to high-temperature sensitive phenotypes [59]. For instance, in the presence of tunicamycin, cells infected with VSV released 10%–70% of virus particles at 30 °C but none at 37 °C [65]. At lower temperatures folding and subsequent transport can be normal while at high temperatures, the underglycosylated G misfolds. A similar phenomenon is seen in refolding experiments in vitro, which usually have to be performed at reduced temperatures to be efficient [44]. On the other hand, the antiviral effects of short hyperthermic treatments in rhabdovirus infected cells are most probably due to some alteration of glycosylation [36].

In many of the mutants, selected as spontaneous, obtained by mutagenesis (site directed or randomly induced) or a few of the MAb-resistant (MAR), the altered polypeptide structure affected its degree of glycosylation which in turn resulted in partial unfolding [116]. Temperature sensitive VSV G mutants had either the two sites underglycosylated [116] or only one site glycosylated [81]. G mutants containing only one glycosylated site are still transported to the plasma membrane and incorporated into viral infectious particles. However, at least in some strains of VSV, when neither of the two sites were glycosylated, the G was not transported [12]. Even a single aa substitution inhibits the transport of the VSV G at non permissive temperature, the G remains in an endoglycosidase H-sensitive site, and non infectious virions lacking spikes are produced [59].

Folding-assembly of homotrimers

Folding starts co-translationally and folding-assembly continues post-translationally as monitored in pulse-chase experiments by the acquisition of conformation-dependent epitopes, disulfide bond formation, differential effects of mutations and increased protease/glycosidase resistance. Besides glycosylation, folding is also critically dependent on the presence of both chaperones and folding enzymes to prevent irreversible aggregation (aggregation which occurs, for instance, when these molecules are expressed in *E. coli*) [44]. Only correctly and completely VSV folded molecules [45] are transported out of the endoplasmic reticulum to the Golgi apparatus were they might stay a total of 20 min [99, 109].

In the X-ray structures of the influenza hemagglutinin (homotrimer) and neuraminidase (homotetramer) there are two folding domains, a stem domain rich in α-helical structure and a globular top domain rich in β-sheet structure containing the active sites. The disulfide bonds and hydrophobic areas are inside the folded monomers or in the monomer-trimer interfaces. Similar models might explain the architecture of the G of rhabdoviruses. Since domains acquire their three-dimensional structures independently of each other, this probably could account for the successful expression of chimeric VSV G [35].

Assembly to trimers is a late event since it requires the complete and correct folding of monomers. Assembly takes place in about 7 min and needs ATP [44]. Coexpression of wild type and mutant or fluoresceinated monomers from different virus strains have demonstrated that monomers are randomly recruited from the newly synthesized pools to be assembled into trimers [96].

The mechanisms by which the transport into the secretory pathway of incompletely folded, misassembled, or unassembled G are selectively inhibited, called quality control [37, 44], are poorly understood and constitute an area for future research [68].

Fatty acid acylation

Palmitic acid attachment to the G was located in the cytoplasmic domain on the VSV-Ind G by thermolysin digestion and by site-directed mutagenesis [117]. Cysteines have been involved in the palmitate acylation because of its hydrolysis with hydroxylamine [100]. G lacking the palmitate was, however, glycosylated and transported normally to the cell surface, suggesting that fatty acid acylation might have only an effect on the long-term stability of the G in the membrane. Fatty acid acylation has been also demonstrated in rabies [63] but has not been found in VSV-NJ [60].

Soluble G

A soluble form of the G of VSV [26, 27], of rabies [43] and of VHSV (unpubl.), accumulates in the extracellular medium of cells infected in vitro. For VSV, one every 6 G molecules is cleaved in the endoplasmic reticulum shortly after its synthesis, transported to the cell membrane, and shed out to the extracellular medium. Soluble G purified from virion-depleted extracellular medium is 5 KDa smaller than the virus associated G [43, 57], due to the absence of the carboxy terminal region which contains the hydrophylic cytoplasmic and the membrane-anchoring hydrophobic domains [19].

Cell-rhabdovirus interaction

The cellular rhabdovirus receptor(s)

The interaction of a rhabdovirus with a cell depends on the presence of a receptor(s) molecule(s) on the cell and of a cell-binding molecule (G) on the virion. There is little evidence for variation in cell-surface receptors as determinants for rhabdoviral cellular susceptibility. For instance, VSV has a very wide host range, varying from insect, to fish and to mammalian cells. However, the cellular susceptibility to rhabdoviral infection depends not only on the existence of external receptors but also on the intracellular environment required for rhabdoviral replication.

Saturation of rhabdoviral binding at 4 °C was complete at 4000 VSV labeled virions per cell implying the existence of a specific cellular receptor [123]. Dose-response curves and Scatchard plots of VSV binding affinities [123] and

binding of Ab-neutralized VSV to the cells, suggested the existence of two cell binding sites [120]. However, since many stocks of rhabdoviruses contain an unknown amount of non infective defective interfering particles, the studies of rhabdoviral absorption/infection are particularly difficult to interpret. Thus, there are two cell receptors or two types of rhabdoviral particles?

VSV competes with rabies virus binding, suggesting the existence of a common receptor for all rhabdoviruses. This common receptor has a chloroform-methanol soluble component(s) that blocks virus binding and infectivity [32, 152]. Treatment of susceptible cells with phospholipases but not with trypsin inhibited the binding of VSV or of rabies to its host cells [128, 152]. Only phosphatidylserine was capable of inhibiting both the binding of VSV to the saturable cell surface receptor and 90% of VSV plaque formation [119]. Both the serine head group and the hydrophobic fatty acid portions of the phosphatidylserine were essential for its inhibiting activity. Binding of phosphatidylserine to the G has been also demonstrated in VHSV [30]. The predominant segregation of the phosphatidylserine among other membrane phospholipids to the inner part of the cellular membrane (80–90%) could explain the limited number of the virus attachment sites observed in the outer part of the cellular membrane. Despite the apparent role of phosphatidylserine in VSV binding and infection [78], it is also possible that it constitutes only a portion of a larger protein-lipid complex receptor [119, 120].

The rabies G sequence aa 170 to 255 is highly homologous to the regions of the snake venoms that bind to the acetylcholine receptor (AChR) [134]. Furthermore, rabies G binds to the AChR and behaves as an AChR antagonist [69]. Consequently, polyclonal antibodies (PAbs) against rabies virus-neutralizing MAbs bound to AChR. Even though more experiments with MAbs confirmed that the AChR could be one of the rabies receptors [69], all these experimens did not address the possible complexity of multiple virus receptors, one or more than one for the cells in culture, two or more for the nervous system, etc. The complete characterization of the rhabdovirus cellular receptor(s) is still an open question.

The rhabdoviral cell-binding molecule

Removal of G by proteases reduces several orders of magnitude both the infectivity and the agglutination of erythrocytes by rabies, VSV and other rhabdoviruses. Confirming these observations, isolated VSV G also hemagglutinates goose erythrocytes.

Whereas rhabdoviral binding can occur at 4 °C and pH 7.5, entry of the virus to the cell by endocytosis is an energy-dependent event which occurs at physiological temperature. Then fusion of the viral membrane with the host cell membrane occurs after the pH is lowered to ≈ 5, as suggested by fusion inhibition with lysosomotropic amines (chloroquine, ethylenediamine, tetracaine, ammonium chloride, etc.) in VSV [115, 123] and in rabies [127]. The rhabdoviruses can also bind (about 10 times more VSV or rabies bound to

cells at pH $\leqslant$ 6.5 than at higher pH) [152] and fuse directly to the plasma membrane but only under acidic medium conditions [78].

G-phospholipic vesicles fused with acidic phospholipid vesicles (phosphatidylserine or phosphatidic acid) only at pH 5 as monitored by electron microscopy or fluorescence energy transfer [47]. Hemolysis of human erythrocytes by VSV was also maximal at pH 5 [6] and exposure to pH 5 of infected or transfected cells expressing G causes cell to cell fusion in VSV [61] as well as in VHSV ([87] (results not shown)). The 25 aminoterminal aa of the VSV G had pH-dependent hemolytic activity [121] with the minimum requirement of only its 6 amino terminal aa [122]. However, since site-directed mutagenesis mutants obtained without hemolytic activity still showed pH-dependent fusion activity [150], other viral G regions must be implicated in the fusion process [82, 122].

Exposure of isolated VSV virions to pH 5 induces an accumulation of the G at the ends of the intact virion as shown by electron micrographs [18]. Exposure of isolated G to pH 5 induces a conformational change(s) which allows the protein to interact simultaneously with the viral and the host cell membranes. This interaction in VSV-Ind seems to occur via one or several stretch(es) of hydrophobic aa as mapped by fusion-defective mutants generated by aa insertion either after aa residues 123, 194, 410 and 415 [90] or in the aa 118–136 region [143]. Site-directed mutagenesis identified a VSV peptide involved in the low-pH induced membrane fusion between aa 123–137 [156].

There is a pH-dependent reversible equilibrium between the native fusion-active (83 Å) and the fusion-inactive (113 Å) rabies G trimers, in the absence of membrane lipids. Exposition of the G trimers to pH 5.9, resulted either in the inhibition of fusion in the absence of lipids or in the activation of fusion in the presence of lipids. Activation of the G trimers for fusion requires a conformational change that exposes hydrophobic regions at its surface as demonstrated by binding of MAbs to region II and by fluorescence assays [61].

Phospholipid binding to synthetic peptides from VHSV G allowed the mapping of one of the binding site(s) to be located in an a–d hydrophobic heptad-repeat region followed by 2 closely spaced arginines (aa 82–109) [31]. These newly defined heptad-repeats differ from the ones previously described to form coiled coils [95] because of the inclusion of the hydrophobic aa W, H and T in the a–d sites. Similar but not identical new a–d hydrophobic heptad-repeat regions do exist not only in VHSV (Table 2) but also in all other rhabdoviral G sequenced to date [29] (Fig. 2 and Table 4). Only the binding of phosphatidylserine to VHSV but not to the isolated peptide (aa 82–109) was dependent of low pH, suggesting that a pH-induced G conformational change is needed in the virion to make functional the phospholipid binding peptide.

A model which could have high similarities to the above has been proposed for influenza hemagglutinin in which a classical a–d hydrophobic heptad-repeat region with random conformation in the native trimer, only coils at low pH, relocating an amino terminal fusion peptide from the inside of the trimer toward

Table 3. Position of the phospholipid-binding peptides p2 and p106 in the G sequence of VHSV

```
...RPAQLRCPHEFEDINKGLVSVPTRIIHLPLSVTSVSAVASGHYLHRVTYRVTCSTS.1
                                                HYLHRVTYRVTCSTS 2
                                    IIHLPLSVTSVSAVASGHYLH       3
                                    IIHLPLSVTSVSAVASGHYLHRVTYRVT 4
|    |    |    |    |    |    |    |    |    |    |    |    |
55   60   65   70   75   80   85   90   95   100  105  110
```

1 Partial sequence of the G of VSHV. *2* p106 sequence of a 15-mer peptide that binds phospholipids. *3* Computer predicted α-helix. *4* p2, synthetic peptide containing the α-helix plus the Arg (R) containing sequence (RVTYRVT) that binds phospholipids with a 10-fold higher specific activity than p106. The hydrophobic aa of the a–d hydrophobic heptad-repeats are in bold. The positively charged aa (Arg) are underlined. Numbers are the amino terminal aa positions on the G [31]

Table 4. Complete sequences of the amino terminal heptads of the hydrophobic heptad-repeats (positions a b c d e f g) of the G of rhabdoviruses and its carboxy terminal parts containing basic residues

VHSGER	68	FEDINKG	LVSVPTR	IIHLPLS	VTSVSAV	ASGHYLH	RVTYR	107
VHSDK	68	FEDINKG	LVSVPTK	IIHLPLS	VTSVSAV	ASGHYLH	RVTYR	107
IHNGP	99	IHKV	LYRTICS	TGFFGGQ	TIE		KALVEMK	126
SIGMA	151	VKDHPVM	LDPYTNN	YVDAIFP			GGISSPG	178
BEFV	149	LIQHKPF	LNPYDNI	IYD			SRFLTPC	173
SYNVG	90	ITGARWN	YVGISIP	VFKIVTN			EVCYTSH	118
RABMOK	140	WLRT	VTTTKES	LLLISPS	IVEMDIY		GRTLHSP	171
RABPV	140	WLRT	VKTTKES	LVIISPS	IADMDPY		DRSLHSR	171
RHRBGD	140	WLRT	VKTTKES	LVIISPS	IADMDPY		DRSLHSR	171
RABSAD	140	WLRT	VKTTKES	LVIISPS	IADMDPY		DRSLHSR	171
RABHEP	140	WLRT	VKTTKES	LVIISPS	ITDMDPY		DRSLHSR	171
RABLEP	140	WLRT	VKTTKES	LVIISPS	ITDMDPY		DRSLHSR	171
RABCVS	140	WLRT	VRTTKES	LIIISPS	ITDMDOY		DRSLHSR	171
VSVGPN08	134	TVTD	AEAHIIT	VTPHSVK	VDEYTGE	WID	PHFIGGR	168
VSVGPNJA	134	TVTD	AEAHIVT	VTPHSVK	VDEYTGE	WID	PHFIGGR	168
VSVGPN29	138		AEAHIIT	VTPHSVK	VDEYTGE	WID	PHFLGGR	168
RHGPORS	134	TVTD	AEAHIVQ	VTPHHVL	VDEYTGE	WVD	SQFINGK	167
RHGM	134	TVTD	AEAHIVQ	VTPHHVL	VDEYTGE	WVD	SQFINGK	168
RHVSVGR	134	TVTD	AEAHIVQ	VTPHHVL	VDEYTGE	WVD	SQFINGK	168

The relative positions of all the heptads of hydrophobic repeats are given in Fig. 2. Hydrophobic residues in heptad positions a and d are in bold. The heptads are separated by a space to better appreciate its position. The numbers to the left and to the right of the sequences are the amino terminal positions of the first and last aa, respectively. The positively charged aa residues to the carboxy terminal position of the heptad sequences are underlined. The region 123–137 of the VSV has been identified by site directed mutagenesis as implicated in the fusion activity of these viruses. *VHS* Viral haemorrhagic septicaemia; *IHN* infectious haematopoietic necrosis; *RAB* rabies; *VSVGPN* vesicular stomatitis virus, New Jersey serotype; *RH* vesicular stomatitis virus, Indiana serotype. Only the hydrophobicity of a–d positions is conserved ($-\Delta G$ values ≥ 0.4 KCal/mol, aa W, F, Y, I, L, M, A, H, T). Whole heptad repeat sequences are unrelated among rhabdovirus genus and some of the basic residues terminating the heptad repeats or the space between residues are weakly conserved

the target cell membrane [22, 25]. Another model recently proposed for Sindbis virus entry shows that a change of conformation at low pH is followed by reduction of disulfide bridges and posterior new disulfide rearrangements before fusion occurs [1]. The existence of an internal aa 206–255 region partially bound to either the aa 20–63 or the aa 262–310 regions by disulfide bonds in the rabies G [42], suggests the existence of an equilibrium between two forms of G. Whether or nor rhabdoviral membrane fusion could also be triggered or followed by rearrangement of some of the disulfide bridges in the rhabdoviral G molecule it is not known. The relationships, if any, among the newly defined a–d hydrophobic heptad-repeats, the phospholipid binding regions, the conformational changes associated with fusion and the identified fusogenic peptides constitute one of the most exciting roads of research in these viruses.

Antigenicity of the rabies G

Protection against rabies seems to be dependent of a complex interdependence of host effector mechanisms: virus-neutralizing Abs, antigen-presenting cells, T-lymphocytes, interferon, etc. The relative importance of each of these during the course of a natural infection or after vaccination are poorly understood. Some studies showed that protection against rabies correlates well with the level of anti-G neutralizing Abs [146] but other studies found no such strong correlation [39, 40].

Most vaccines used for human protection after rabies exposure are derived from a rabies virus originally isolated and adapted to rabbit brain by Pasteur [107]. It was assumed that this strain had sufficient cross-reactivity with all strains of rabies virus in different host species or geographical areas to protect exposed individuals. Subsequently the use of PAbs and MAbs revealed considerable antigenic variability among those viruses both in the G [55, 56] and in the nucleoprotein N [39].

The majority of the anti-G MAbs obtained by immunizing with inactivated rabies virus and screened by infected cell membrane immunofluorescence, preferentially recognize conformation dependent epitopes and neutralize viral infectivity [56, 147]. Most of the 266 anti-G rabies neutralizing MAbs recognized several overlapping epitopes that make up site II (72.5%) and site III (24.8%), the rest being directed towards sites I and IV, as shown by sequencing of MAb neutralization-resistant (MAR mutants) [11, 85]. The relative importance of each of the antigenic sites did not depend on the strain of virus or on the particular animal examined. Rabbit anti-idiotypic Abs prepared against neutralizing MAbs to regions I, II and III, showed no cross-reactivity between the 3 regions [113]. Only 1 of the 266 MAbs reacted against SDS and mercaptoethanol treated G and thus it probably reacts with a linear epitope [11]. However, it seems that the number of reports of anti-G MAbs which are independent of the native conformation is continuously increasing [23, 79]. MAR mutants to site II have aa substitutions located

between aa 34–42 or aa 198–200. In contrast, the mutants resistant to MAbs to site III have aa substitutions between aa 330–338. If those aa substitutions are in the MAb binding regions, site II could be a discontinuous, conformational antigenic site and site III a continuous, conformational antigenic site.

Neutralization occurred with an average of 1–2 IgG/3 spikes or 1 IgM/9 spikes as shown by binding and electron microscopy studies [54]. Although aa substitutions in the antigenic sites altered the neutralization of the rabies mutants, only the position of aa 333 seems to affect their pathogenicity in vivo [125].

A neutralization epitope highly conserved on the G of all the rabies virus strains sequenced to date was defined by pepscan analysis as ^{260}LHDFRSDE. This peptide has been used either alone or in combination with T cell epitopes to produce anti-rabies Abs and to induce protection in mice [38]. However, the frequency of Abs to this epitope in serum Abs induced by rabies vaccination was very low [138].

Only a couple of classical biochemical studies have been made to map regions of the rabies G important for neutralization [42] and for lymphoproliferation [98] by cleaving the protein after methionine with cyanogen bromide (CNBr). CNBr-cleaved peptides of the G were resolved into 6 peptides under non-reducing conditions and into 8 peptides under reducing conditions. Each of the peptides induced binding Abs but only peptides aa 20–63, aa 123–198 and aa 312–342 induced neutralizing Abs. The only peptide immunoprecipitated by hyperimmune sera was the aa 123–198. On the other hand, all the molecules of peptide aa 208–255 were bound by cysteine bonds to either peptide aa 20–63 or aa 264–310, leaving a portion of the molecules of these last 2 peptides free of interpeptide cysteine bonds. These results suggested the existence of 2 different conformations on the native structure of G. The first conformation would contain more aa (aa 20–63 bound to aa 208–255) and the most antigenic peptide (aa 123–198) (large loop). The second conformation would contain less aa (aa 264–310 bound to aa 208–255) (small loop). The existence of such structure related to one of the neutralization sites of G, correlates with the evidence that most neutralizing MAbs bind to conformational epitopes, reduction of the G reduces 95% of its antigenicity [42] and 72.5% of the neutralizing MAbs defining the conformation-dependent site II map both at aa 34–42 and 198–200 (inside the large loop). Further work into this area would no doubt increase our understanding of the structure-neutralization relationships in the G of rhabdoviruses.

Antigenicity of VSV G

Only the G of the VSV is the antigen that gives rise to and react with neutralizing Abs [75, 110, 145]. The existence of four non-crossreactive neutralizing MAbs (defining 4 non-overlapping epitopes) in the G of the VSV-Ind [139] and in the VSV-NJ [89] has been confirmed by both MAb production, protection against protease digestion by neutralizing MAbs and mapping of MAR mutants

[89, 102, 137]. Reduction of the disulfide bonds of VSV G, inhibited the binding of most of the neutralizing MAbs.

Of 19 MAbs anti VSV-Ind G, only 4 were neutralizing. Two of these MAbs also exhibited strong binding to the VSV M protein [139] suggesting a G–M interaction. None of the neutralizing MAbs have been found to inhibit the binding of radiolabeled VSV although most of the neutralizing MAbs inhibited VSV mediated cell fusion. In another study, deletion constructs and chimeras of the G genes of VSV-Ind and NJ of known aa sequences helped to delineate its epitope maps, based on binding affinity and cross-reactivity of MAbs. Four MAb neutralizable epitopes in VSV-NJ mapped to aa 193–289 whereas 2 MAb neutralizable epitopes in VSV-Ind mapped to aa 80–183 and other 2 to aa 286–428. Earlier mapping attempts by proteolysis of the G and by sequencing MAR mutants were confirmed by this analysis [74].

The stabilization of the monomer-trimer equilibrium of VSV G by protein M interactions [96, 154] and the stability of the trimer structure during the low-pH-induced conformational changes needed for fusion [61, 62], suggest the importance of the trimer structure as a target for neutralizing Abs [11, 79]. Antigenic differences must exist between monomeric and trimeric G since differential diagnostic between VSV serotypes is made possible by the use of PAbs raised against each of its isolated trimers [2]. However trimer-specific MAbs have not yet been described for rhabdoviruses, probably because G isolated under a variety of conditions is often found to be monomeric [97].

Not only neutralizing but also nonneutralizing anti-VSV MAbs caused some in vivo protection against subsequent challenge [88]. Because only Fc containing MAbs could exert this effect, possible explanation for the protection caused by non-neutralizing MAbs could be complement lysis of the VSV bound by MAb.

Stimulation of cellular immunology

Viral infection induces both cytotoxic T lymphocytes (CTL) and Ab responses. Defective or inactivated viral particles although induce Ab responses are poor inducers of CTL responses [5]. The presence of defective-interfering particles in the rhabdoviral preparations reduced the levels of proliferating, interleukin-secreting CTL in mice [21].

Although the class I-restricted CTL response to VSV is mainly specific for the nucleoprotein, mice defective in the class I gene [58] generated a specific (positive for VSV-Ind but not for VSV-NJ) class II-restricted CTL response to G. G could enter the class II pathway either through synthesis from replication-competent VSV and from vaccinia-rhabdovirus recombinants containing the G gene [114] or through exogenous addition of inactivated, defective-interfering particles [20], micelles of G and soluble G [20]. Soluble G was the most efficient in terms of the number of molecules required to sensitize class II expressing target cells (10^4 molecules per cell or about 0.3–3 μg of G/ml). Extrapolation of these results to the in vivo rhabdoviral infections is possible because soluble G is synthesized during those and class II molecules are

inducible in many different cells. The lysis of uninfected cells exposed to soluble G by class II-restricted CTL could explain the haemorrhagic symptoms of some rhabdoviral infections [20].

To produce viral Ab responses, T-helper cells are most often needed and those are estimated by lymphoproliferation assays. Lymphoproliferation of virus-primed mice was shown by CNBr G peptides 20–63, 264–310, 312–342 and 406–471. Under non-reducing conditions, 2 additional peptides 123–198 and 77–121 stimulated lymphoproliferation, suggesting that disulfide-stabilized conformation was required for the integrity of a least some determinants. Different preparations of rabies viral G (purified native, soluble, rosettes, CNBr peptides, propionolactone-inactivated virus) showed no differences in stimulation of lymphoproliferation in contrast to its different stimulation of Ab responses [98].

Lymphoproliferation of rabies specific human T-helper cells required rabies antigens to be presented in association with class II molecules by antigen-presenting cells. Some of the T cell clones thus activated provided help for an Ab response (including neutralizing Abs) by rabies immune B cells [23]. The nucleoprotein N was the major rhabdoviral antigen stimulating lymphokine-secreting T-helper cells. Inoculation of mice with the identified synthetic peptides bearing the immunodominant T-helper cell epitopes resulted in an accelerated and enhanced neutralizing Ab response upon booster immunization with inactivated rabies virus [49]. Main T-helper epitopes of the VSV G in inbred mice have been characterized by obtaining T-cell hybridomas in aa 52–71, 316–335 and 415–433. These epitopes were able to provide functional help for the production of anti-VSV specific neutralizing Abs [24].

Humoral and cellular immunology of fish rhabdoviruses

Studies on the induction of in vivo protective immunity against fish rhabdoviruses have focused on the response to the G [13, 48], since neutralizing Ab to VHSV and IHNV shows exclusive specificity for this protein [93]. G from IHNV [48] and a region of G expressed in *E. coli* were effective in inducing protective immunity in the trout [66]. G from VHSV stimulated anamnestic lymphoproliferation in VHSV-immunized [50] and survivors of VHSV infection trout [51]. Accordingly, recombinant G fragments of VHSV expressed in *E. coli*, *Y. ruckeri* (a trout pathogen) and *S. cerevisiae* also stimulated in vitro anamnestic lymphoproliferation in survivors of VHSV infection but not in uninfected trout [51, 52]. Some preliminary evidence was obtained which shows correlation of the in vitro neutralizing MAb recognition with the in vivo protection capacity of the cloned fragments both in IHNV [153] and in VHSV [52, 94]. However, it is not yet clear whether or not there are conserved as well as strain variable neutralizing epitopes on the G of both IHNV and VHSV [105, 148]. Much remains to be done because of the scarcity of neutralizing MAbs anti fish rhabdoviruses.

Bacterially expressed nucleoprotein N augments the protective immunity induced by the G vaccine in fish in IHNV [104] but not in VHSV [52].

However, these are few and still preliminary reports and more work should be done in this area.

Vaccines

Currently, most of the rabies vaccines for use in humans consist of inactivated virus produced in human diploid cells while those for use in wild animals consist of vaccinia recombinants expressing the rabies G. Such vaccines have proved effective. However, efforts toward a new generation of rhabdoviral vaccines that are less expensive, safer and effective have been developed during the last years, including the possible use in the vaccine formulation of the rhabdoviral nucleoprotein N [41, 53, 84].

Characterization by immunoprecipitation with neutralizing MAbs, extent of glycosylation and protection studies, showed that a conformational correct rabies G could be expressed in recombinant yeast and vaccinia [76]. Injection into mice produced high titer neutralizing Abs. The G expressed in vaccinia generated a rabies specific CTL response in mice and the immunized mice survived rabies challenge. Finally, mice given oral vaccines of live vaccinia were also protected against rabies challenge [80, 86]. Some of the best field results reported were those obtained in a large-scale fox vaccine-bate vaccination in 2200 km^2 in Belgium, 81% of the foxes were vaccinated and the cases of rabies in that area dropped from 50–120 per year to none [17].

First attempts of using subunit vaccines were made by using recombinant bacteria, but cDNA cloned copies of rabies G mRNA when expressed in bacteria failed to yield a product capable of immunizing against rabies. The failure of inducing protection by the rhabdoviral G produced in bacteria compared to the successful production of fully immunogenic G in eucariotic vectors [76, 80, 86] is probably due to the bacterial inability to glycosylate and to correctly form the G disulfide bonds, therefore yielding an incorrectly folded G with intermolecular crosslinking which would be quite different from the native form of G which most probably is the membrane anchored trimer [61, 96]. However, there is not yet a direct evidence that G trimer structures are required for vaccination against rhabdoviruses.

The need for better human vaccines still exists. Live-attenuated viruses may be immunosuppressive or cause clinical disease if not attenuated sufficiently. Alternatively if they are too attenuated, their ability to generate immunity is limited. On the other hand, killed vaccines are often unable to generate protective levels of immunity for reasons of antigen load or loss of important epitopes during inactivation and repeated immunizations are often necessary. Live recombinant vaccine vectors like the vaccinia one [17] are effective, but their repeated use in the same host may be limited by vector immunity; they are also subject to reversion events and can cause disease or death in immunocompromised hosts. First described in 1990 [91, 149], direct gene injection and expression has since been demonstrated with many genes in several tissues and species as diverse as mice and fish [34, 70]. Its potential as a vaccine for influenza in a mouse model has been reported [136]. Immunization

with plasmid DNA encoding viral proteins may be advantageous because no infectious agent is involved, no assembly of virus particles is required, a correct folding of the G is allowed and an epitope selection for antigen presentation on the membrane of the infected cells by each individual is permitted [129]. More work is needed, however, to make this technology a real alternative to existing vaccines.

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References

1. Abell BA, Brown DT (1993) Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. J Virol 67: 5496–5501
2. Allende R, Sepúlveda L, Mendes da Silva A, Martins M, Söndahl MS, Alonso A (1992) An enzyme-linked immunosorbent assay for the detection of vesicular stomatitis virus antibodies. Prev Vet Med 14: 293–301
3. Altstiel LD, Landsberger FR (1981) Lipid-protein interactions between the surface glycoprotein of vesicular stomatitis virus and the lipid bilayer. Virology 115: 1–8
4. Anilionis A, Wunner WH, Curtis PJ (1981) Structure of the glycoprotein gene in rabies virus. Nature 294: 275–278
5. Bachmann MF, Künding TM, Kalberer CP, Hengartner H, Zinkernagel RM (1993) Formalin inactivation of vesicular stomatitis virus imparis T-cell-but not T-help-independent B-cell responses. J Virol 67: 3917–3922
6. Bailey C, Miller D, Lenard J (1981) Hemolysis of human erythrocytes by vesicular stomatitis virus. J Cell Biol 91: 111–116
7. Barge A, Gaudin Y, Coulon P, Ruigrok RWH (1993) Vesicular stomatitis virus M2 protein may be inside the ribonucleocapsid coil. J Virol 67: 7246–7253
8. Basurco B (1990) Estudio, identificación y caracterización del virus de la septicemia hemorrágica vírica en España. PhD Thesis, Universidad Complutense de Madrid.
9. Benmansour A, Brahimi M, Tuffereau C, Coulon P, Lafay F, Flamand A (1992) Rapid sequence evolution of streer rabies glycoprotein is related to the highly heterogeneous nature of the viral population. Virology 187: 33–45
10. Benmansour A, Paubert G, Bernard J, De Kinkelin P (1994). The polymerase-associated protein (M1) and the matrix protein (M2) from a virulent and avirulent strain of viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus. Virology 198: 602–612
11. Benmansour H, Leblois H, Coulon P, Tuffereau C, Gaudin Y, Flamand A, Lafay F (1991) Antigenicity of rabies virus glycoprotein. J Virol 65: 4198–4203
12. Bergmann JE, Tokuyasu KT, Singer SJ (1981) Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein, through the Golgi apparatus en route to the plasma membrane. Proc Natl Acad Sci USA 78: 1746–1750
13. Bernard J, LeBerre MB, DeKinkelin P (1983) Viral haemorrhagic septicemia of rainbow trout: relation between the G polypeptide and antibody production of fish after infection with the F25 attenuated variant. Infect Immun 39: 7–14
14. Bilsel PA, Nichol ST (1990) Polymerase errors accumulating during natural evolution of the glycoprotein gene of vesicular stomatitis virus indiana serotype isolates. J Virol 64: 4873–4883

15. Booy FP (1993) Cryoelectron microscopy. In: Bentz J (ed) Viral fusion mechanisms. CRC Press, London, pp 21–54
16. Bourhy H, Kissi B, Tordo N (1993) Molecular diversity of the lyssavirus genus. Virology 194:70–81
17. Brochier B, Kieny MP, Costy F, Coppens P, Bauduin B, Lecocq JP, Languet B, Chappuis G, Desmettre P, Afiademanyo K, Libois R, Pastoret PP (1991) Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. Nature 354: 520–522
18. Brown JC, Newcomb WW, Lawrence-Smith S (1988) pH dependent accumulation of the vesicular stomatitis virus glycoprotein at the ends of intact virions. Virology 167: 625–632
19. Browning M, Reiss CS, Huang AS (1990) The soluble viral glycoprotein of vesicular stomatitis virus efficiently sensitizes target cells for lysis by CD4 + T lymphocytes. J Virol 64: 3810–3816
20. Browning MJ, Huang AS, Reiss CS (1990) Cytolytic T lymphocytes from the Balb/c-H-2dm2 mouse recognize the vesicular stomatitis virus glycoprotein and are restricted by class II MHC antigens. J Immunol 145: 985–994
21. Browning MJ, Huneycutt BS, Huang AS, Reiss CS (1991) Replication-defective viruses modulate immune responses. J Immunol 147: 2685–2691
22. Bullough PA, Hughson FM, Skehel JJ, Wiley DC (1994) Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 371: 37–43
23. Bunschoten H, Gore M, Classen IJTM, Uytdehaag FGCM, Wunner W, Osterhaus ADME (1989) Characterization of a new virus-neutralizing epitope that denotes a sequential site on the rabies virus glycoprotein. J Gen Virol 70: 291–298
24. Burkhart C, Freer G, Castro R, Adorini L, Wiesmuller KH, Zinkernagel RM, Hengartner H (1994) Characterization of T-helper epitopes of the glycoprotein of vesicular stomatitis virus. J Virol 68: 1573–1580
25. Carr CM, Kim PS (1993) A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell 73: 823–832
26. Chatis PA, Morrison TG (1983) Characterization of the soluble glycoprotein released from vesicular stomatitis virus-infected cells. J Virol 45: 80–86
27. Cheng SSL, Huang AS (1986) Further characterization of the vesicular stomatitis virus temperature-sensitive 045 mutant: intracellular conversion of the glycolprotein to a soluble form. J Virol 59: 210–217
28. Chong LD, Rose JK (1993) Membrane association of functional vesicular stomatitis virus matrix protein in vivo. J Virol 67: 407–414
29. Coll JM (1995) Heptad-repeat sequences in the glycoprotein of rhabdoviruses. Virus Genes (in press)
30. Coll JM (1995) Phospholipid binding domain mapped in the glycoprotein G of VHSV, a salmonid rhabdovirus. J Virol (submitted)
31. Coll JM (1995) Low-pH induces the binding of haemorrhagic septicemia rhabdovirus to membrane phospholipids. J Fish Dis (submitted)
32. Conti C, Mastromarino P, Ciuffarella G, Orsi N (1988) Characterization of rat brain cellular membrane components acting as receptors for vesicular stomatitis virus. Arch Virol 99: 261–269
33. Conzelmann KK, Cox JH, Schneider LG, Thiel HJ (1990) Molecular cloning and complete nucleotide sequence of the attenuated rabies virus SAD B19. Virology 175: 485–489
34. Cox GJM, Zamb TJ, Babiuk LA (1993) Bovine herpesvirus 1: Immune responses in mice and cattle injected with plasmid DNA. J Virol 67: 5664–5667
35. Crise B, Ruusala A, Zagouras P, Shaw A, Rose JK (1989) Oligomerization of

glycolipid-anchored and soluble forms of the vesicular stomatitis virus glycoprotein. J Virol 63: 5328–5333
36. DeMarco A, Santoro MG (1993) Antiviral effect of short hyperthermic treatment at specific stages of vesicular stomatitis virus replication cycle. J Gen Virol 74: 1685–1690
37. DeSilva M, Balch WE, Helenius A (1990) Quality control in the endoplasmic reticulum: Folding and misfolding of vesicular stomatitis virus G protein in cells and in vitro. J Cell Biol 111: 857–866
38. Dietzschold B, Gore M, Marchadier D, Niu HS, Bunschoten HM, Otvos L, Wunner WH, Ertl HCJ, Osterhaus ADME, Koprowski H (1990) Structural and immunological characterization of a linear virus neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine. J Virol 64: 3804–3809
39. Dietzschold B, Rupprecht CE, Tollis M, Lafon M, Mattei J, Wiktor TJ, Koprowsky H (1988) Antigenic diversity of the glycoprotein and nucleocapsid of rabies and rabies related viruses: implications for epidemiology and control of rabies. Rev Infect Dis 10: 785–798
40. Dietzschold B, Tollis M, Rupprecht CE, Celis E, Koprowski H (1987) Antigenic variation in rabies and rabies-related viruse cross-protection independent of glycoprotein mediated virus-neutralizing antibody. J Infect Dis 156: 815–822
41. Dietzschold B, Wang H, Ruprecht HE, Celis E, Tollis M, Ert H, Heber-Katz E, Koprowski H (1987) Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein, (L,N,NS,RNA). J Immunol 9165–9170
42. Dietzschold B, Wiktor TJ, MacFarlan R, Varrichio A (1982) Antigenic structure of rabies virus glycoprotein: Ordering and immunological characterization of the large CNBr cleavage fragments. J Virol 44: 595–602
43. Dietzschold B, Wiktor J, Wunner WH, Varrichio A (1983) Chemical and immunological analysis of the rabies soluble glycoprotein. Virology 124: 330–337
44. Doms RW, Lamb RA, Rose JK, Helenius A (1993) Folding and assembly of viral membrane proteins. Virology 193: 545–562
45. Doms RW, Ruusala A, Nachaner C, Helenius J, Rose JK (1988) Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J Cell Virol 107: 89–99
46. Dubovi EJ, Wagner RR (1990) Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J Virol 22: 500–509
47. Eidelman O, Schlegel R, Tralka T, Blumenthal R (1984) pH- dependent fusion induced by vesicular stomatitis virus glycoprotein reconstituted into phospholipid vesicles. J Biol Chem 259: 4622–4628
48. Engelking H, Leong JC (1989) The glycoprotein of infectious hematopoitic necrosis virus eluciting antibody and protective responses. Virus Res 13: 213–230
49. Ertl HCJ, Dietzschlod B, Gore M, Otvos L, Larson JK, Wunner WH, Koprowski H (1989) Induction of rabies virus-specific T-Helper cells by synthetic peptides that carry dominant T-Helper cell epitopes of the viral ribonucleoprotein. J Virol 63: 2885–2892
50. Estepa A, Coll JM (1991) Infection of trout kidney cells with infectious pancreatic necrosis and viral haemorrhagic septicaemia viruses. Bull Eur Ass Fish Pathol 11: 101–104
51. Estepa A, Coll JM (1992) In vitro immunostimulants for optimal responses of kidney cells from healthy trout and from trout surviving viral haemorrhagic septicaemia virus disease. Fish Shellfish Immunol 2: 53–68
52. Estepa A, Thiry M, Coll JM (1994) Recombinant protein fragments from haemorrhagic

septicaemia rhabdovirus simulate trout leucocyte anamnestic in vitro responses. J Gen Virol 75: 1329–1338
53. Fekadu M, Sumner JW, Shaddock JH, Sanderlin DW, Baer GM (1992) Sickness and recovery of dogs challenged with a street rabies virus after vaccination with a vaccinia virus recombinant expressing rabies virus N protein. J Virol 66: 2601–2604
54. Flamand A, Raux H, Gaudin Y, Ruigrok RWH (1993) Mechanisms of rabies virus neutralization. Virology 194: 302–313
55. Flamand A, Wiktor TJ, Koprowsky H (1980) Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. I. The nucleocapsid protein. J Gen Virol 48: 97–104
56. Flamand A, Wiktor TJ, Koprowsky H (1980) Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. II. The glycoprotein. J Gen Virol 48: 105–109
57. Florkiewicz RZ, Smith A, Bergmann JE, Rose JK (1983) Isolation of stable nouse cell lines that express cell surface and secreted forms of the vesicular stomatitis virus glycoprotein. J Cell Biol 97: 1381–1388
58. Forger JM, Bronson RT, Huang AS, Reiss CS (1991) Murine infection by vesicular stomatitis virus: initial characterization of the H-2d system. J Virol 65: 4950–4958
59. Gallione CJ, Rose JK (1985) A single amino acid substitution in a hydrophobic domain causes temperature-sensitive cell-surface transport of a mutant viral glycoprotein. J Virol 54: 374–382
60. Gallione CJ, Rose JK (1983) Nucleotide sequence of a cDNA clone encoding the entive glycoprotein from the New Jersey serotype of vesicular stomatitis virus. J Virol 46: 162–169
61. Gaudin Y, Ruigrok RWH, Knossow M, Flamand A (1993) Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. J Virol 67: 1365–1372
62. Gaudin Y, Ruigrok RWH, Tuffereau C, Knossow M, Flamand A (1992) Rabies virus glycoprotein is a trimer. Virology 187: 627–632
63. Gaudin Y, Tuffereau C, Benmansour A, Flamand A (1991) Fatty acylation of rabies virus proteins. Virology 184: 441–444
64. Gething MJ, Sambrook J (1992) Protein folding in the cell. Nature 355: 33–45
65. Gibson R, Kornfeld S, Schlesinger S. (1981) The effect of oligosaccharide chains of different sizes on the maturation and physical properties of the G protein of vesicular stomatitis virus. J Biol Chem 256: 456–462
66. Gilmore, RDJ, Engelking HM, Manning DS, Leong JC (1988) Expression in Escherichia coil of an epitope of the glycoprotein of infectious haematopoietic necrosis virus protects against challenge. Biotechnology 6: 295–300
67. Goldberg BK, Modrell B, Hillman BI, Heaton AL, Choi T, Jackson OA (1991) Structure of the glycoprotein gene of Sonchus yellow net virus, a plant Rhabdovirus. Virology 185: 32–38
68. Hammond C, Helenius A (1994) Folding of VSV G protein: sequential interaction with BiP and calnexin. Science 266: 456–458
69. Hanham CA, Zhao F, Tignor GH (1993) Evidence from the anti-idiotypic network that the acetylcholine receptor is a rabies virus receptor. J Virol 67: 530–542
70. Hansen E, Fernandes K, Goldspink G, Buterworth P, Umeda PK, Chang K-C (1991) Strong expression of foreign genes following direct injection into fish muscle. FEBS Lett 290: 73–76
71. Horodyski FM, Nichol ST, Spindler KR, Holland JJ (1983) Properties of D1 particle resistant mutants of vesicular stomatitis virus isolated from persistent infections and from undiluted passages. Cell 33: 801–810

72. Hsu YL, Leong JV (1985) A comparison of detection methods for infectious hematopoietic necrosis virus. J Fish Dis 8: 1–12
73. Kaptur PE, Rhodes RB, Lyles DS (1991) Sequences of the vesicular stomatitis virus matrix protein involved in binding to nucleocapsids. J Virol 65: 1057–1065
74. Keil W, Wagner RR (1989) Epitope mapping by deletion mutants and chimeras of two vesicular stomatitis virus glycoprotein genes expressed by a vaccinia virus vector. Virology 170: 392–407
75. Kelley JM, Emersom SU, Wagner RR (1972) The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and react with neutralizing antibodies. J Virol 10: 1231–1236
76. Klepfer SR, Debouck C, Uffelman J, Jacobs A, Bollen A, Jones EV (1993) Characterization of rabies glycoprotein expressed in yeast. Arch Virol 128: 269–286
77. Koener JF, Passavant CW, Kurath G, Leong J (1987) Nucleotide sequence of a cDNA clone carrying the glycoprotein gene of infectious haematopoietic necrosis virus, a fish rhabdovirus. J Virol 61: 1342–1349
78. Konieczko EM, Whitaker-Dowling PA, Widnell CC (1994) Membrane fusion as a determinant of the infectibility of cells by vesicular stomatitis virus. Virology 199: 200–211
79. Kontsekova E, Macikova I, Novak M, Dedek L, Vrzal V, Kontsek P (1992) Conformation-dependent accessibility of the linear epitopes located on the rabies virus glycoprotein. Viral Immunol 5: 213–220
80. Koprowski H, Reagan KJ, Macfarlan RI, Dietzschold B, Wiktor TJ (1985) New generation of rabies vaccines: rabies glycoprotein gene recombinants, anti-idiotypic antibodies and synthetic peptides. In: Lerne RA, Chanock RM, Brown F (eds) Vaccines 85. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, pp 151–156
81. Kotwal GJ, Buller RML, Wunner WH, Pringle CR, Ghosh MP (1986) Role of glycosylation in transport of vesicular stomatitis virus envelope glycoprotein. A new class of mutant defection in glycosilation and transport of G protein. J Biol Chem 261: 8936–8943
82. Kotwal GJ, Capòne J, Irving RA, Rhee SH, Bilan P, Toneguzzo F, Hofmann T, Ghosh HP (1983) Viral membrane glycoproteins: comparison of the amino terminal amino acid sequences of the precursor and mature glycoproteins of three serotypes of vesicular stomatitis virus. Virology 129: 1–11
83. Kurath G, Leong JC (1985) Characterization of infectious hematopoietic necrosis virus mRNA species reveals a nonvirion rhabdovirus protein. J Virol 53: 462–468
84. Lafon M, Lafage M, Martinez-Arends A, Ramirez R, Vuillier F, Charron D, Lotteau V, Scott-Algara D (1992) Evidence for a viral superantigen in humans. Nature 358: 507–510
85. Lafon M, Wiktor TJ, Macfarlan RI (1983) Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. J Gen Virol 64: 843–851
86. Lathe R, Kieny MP, Lecocq JP, Drillien R, Wiktor TJ, Koprowski H (1985) Immunization against rabies using a vaccinia-rabies recombinant virus expressing the surface glycoprotein In: Lerner RA, Chanock RM, Brown F (eds) Vaccines 85. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, pp 157–162
87. Lecocq-Xhonneux F, Thiry M, Dheur I, Rossius M, Vanderheijden N, Martial J, DeKinkelin P (1994) A recombinant viral haemorrhagic septicaemia virus glycoprotein expressed in insect cells induces protective immunity in rainbow trout. J Gen Virol 75: 1579–1587
88. LeFrancois L (1984) Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: a distinct mechanisms action in vivo. J Virol 51: 208–214

89. LeFrancois L, Lyles DS (1982) The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. II. Monoclonal antibody to nonneutralizing and crossreactive epitopes of Indiana and New Jersey serotypes. Virology 121: 168–174
90. Li Y, Drone C, Sat E, Ghosh HP (1993) Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. J Virol 67: 4070–4077
91. Lin H, Parmacek MS, Morle G, Bolling S, Leiden JM (1990) Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 82: 2217–2221
92. Lodish HF, Zilverstein A, Porter M (1981) Synthesis and assembly of transmembrane viral and cellular glycoproteins. Methods Cell Biol 23: 5
93. Lorenzen N. Olesen NJ, Vestergaard-Jorgensen PE (1990) Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. J Gen Virol 71: 561–567
94. Lorenzen N, Olesen NJ, Vestergaard-Jorgensen PE, Etzerodt M, Holtet TL, Thorgersen MC (1993) Molecular cloning and expression in Escherichia coli of the glycoprotein gene of VHS virus and immunization of rainbow trout with the recombinant protein. J Gen Virol 74: 623–630
95. Lupas A, Van Dyke M, Stock J (1991) Predicting coiled coils from protein sequences. Science 252: 1162–1169
96. Lyles DS, McKenzie M, Parce JW (1992) Subunit interactions of vesicular stomatitis virus envelope glycoprotein stabilized by binding to viral matrix protein. J Virol 66: 349–358
97. Lyles DS, Varela VA, Parce JV (1990) Dynamic nature of the quaternary structure of the vesicular stomatitis virus envelope glycoprotein. Biochemistry 29: 2442–2449
98. MacFarland RI, Dietzschold B, Wiktor TJ, Kiel M, Houghten R, Lerner RA, Sutcliffe JG, Koprowski H (1984) T cells responses to cleaved rabies virus glycoprotein and to synthetic peptides. J Immunol 133: 2748–2752
99. Machamer CE, Rose JK (1988) Vesicular stomatitis virus G proteins with altered glycosylation sites display temperature-sensitive intracellular transport and are subject to aberrant intermolecular disulfide bonding. J Biol Chem 263: 5955–5960
100. Magee AI, Koyama AH, Malfer C, Wen D, Schlesinger MJ (1984) Release of fatty acids from virus glycoproteins by hydroxylamine. Biochim Biophys Acta 798: 156–166
101. Morimoto K, Ohkubo A, Kawai A (1989) Structure and transcription of the glycoprotein gene of attenuated HEP-Flury strain of rabies virus. Virology 173: 465–477
102. Nagata S, Okamoto Y, Inoue T, Ueno Y, Kurata, Chiba J (1992) Identification of epitopes associated with different biological activities on the glycoprotein of vesicular stomatitis virus by use of monoclonal antibodies. Arch Virol 127: 153–168
103. Nichol ST, Rowe JE, Fitch WM (1989) Glycoprotein evolution of vesicular stomatitis virus New Jersey. Virology 168: 281–291
104. Oberg LA, Wirkkula J, Mourich D, Leong JC (1991) Bacterially expressed nucleoprotein of infectious haematopoietic necrosis virus augments protective immunity induced by the glycoprotein vaccine in fish. J Virol 65: 4486–4489
105. Olesen NJ, Lorenzen N, Jorgensen PEV (1993) Serological differences among isolates of viral haemorrhagic septicemia virus detected by neutralizing monoclonal and polyclonal antibodies. Dis Aquatic Organ 16: 163–170
106. Owens RJ, Rose JK (1993) Cytoplasmic domain requirement for incorporation of a foreing envelope protein into vesicular stomatitis virus. J Virol 67: 360–365
107. Pasteur L (1885) Méthode pour prévenir la rage après morsure. CR Acad Sci Paris 101: 765–775

108. Petri WA, Wagner RR (1979) Reconstitution into liposomes of the glycoprotein of vesicular stomatitis virus by detergent dialysis. J Biol Chem 254: 4313–4316
109. Polakova K, Russ G (1992) Use of brefeldin A to localize block in intracellular transport of vesicular stomatitis virus G protein interferon-treated cells. Arch Virol 124: 171–179
110. Portner A, Webster RG, Bean W (1980) Similar frequency of antigenic variants in sendai vesicular stomatitis virus, and influenza A viruses. Virology 104: 235–238
111. Prehaud C, Takehara K, Flamand A, Bishop DHL (1989) Immunogenic and protective properties of rabies virus glycoprotein expressed by baculovirus vectors. Virology 173: 390–399
112. Reading CL, Penhoet EE, Ballou CE (1978) Carbohydrate structure of vesicular stomatitis virus glycoprotein. J Biol Chem 253: 5600–5612
113. Reagan KJ, Wunner WH, Wiktor TJ, Koprowski H (1983) Anti-idiotypic antibodies induce neutralizing antibodies to rabies virus glycoprotein. J Virol 48: 660–666
114. Reiss CS, Gapud CP, Keil W (1992) Newly synthesized class II MHC chains are required for VSV G presentation to CTL clones. Cell Immunol 139: 512–521
115. Rigaut KD, Birk DE, Lenard J (1991) Intracellular distribution of input vesicular stomatitis virus proteins after uncoating. J Virol 65: 2622–2628
116. Robertson JS, Etchison JR, Summers DF (1982) Comparison of the oligosaccharide structure of the glycoprotein of vesicular stomatitis virus and a thermolabile mutant tl17. J Gen Virol 58: 13–18
117. Rose JK, Adams GA, Gallione CJ (1984) The presence of cysteine in the cytoplasmic domain of the vesicular stomatitis virus glycoprotein is required for palmitate addition. Proc Natl Acad Sci USA 81: 2050–2054
118. Rose JK, Gallione CJ (1981) Stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J Virol 39: 519–528
119. Schlegel R, Sue TT, Willingham MC, Pastan I (1983) Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site? Cell 32: 639–646
120. Schlegel R, Wade M (1983) Neutralized vesicular stomatitis virus binds to host cells by a different receptor. Biochem Biophys Res Commun 114: 774–779
121. Schlegel R, Wade M (1984) A synthetic peptide corresponding to the NH2 terminus of vesicular stomatitis virus glycoprotein is a pH-dependent hemolysin. J Biol Chem 259: 4691–4694
122. Schlegel R, Wade M (1985) Biologically active peptides of the vesicular stomatitis virus glycoprotein. J Virol 53: 319–323
123. Schlegel R, Willigan MC, Pastan IH (1982) Saturable binding sites for vesicular stomatitis virus on the surface of Vero cells. J Virol 43: 871–875
124. Schlesinger S, Malfer C, Schlesinger MJ (1984) The formation of vesicular stomatitis virus (San Juan strain) becomes temperature-sensitive when glucose residues are retained on the oligosaccharides of the glycoprotein. J Biol Chem 259: 7597–7601
125. Seif G, Coulon P, Rollin PE, Flamand A (1985) Effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoproteins. J Virol 53: 926–934
126. Stanley P, Vivona G, Atkinson PH (1984) H NMR spectroscopy of carbohydrates from the G glycoprotein of vesicular stomatitis virus grown in parental and lec4 chinese hamster ovary cell. Arch Biochem Biophys 230: 363–371
127. Superti F, Derer M, Tsiang H (1984) Mechanism of rabies virus entry into CER cells. J Gen Virol 65: 781–789

128. Superti J, Seganti L, Tsiang H, Orsi N (1984) Role of phospholipids in rhabdovirus attachment to Cer cells. Arch Virol 81: 321–328
129. Tang DC, DeVit M, Johnston SA (1992) Genetic immunization is a simple method for eliciting an immune response. Nature 356: 152–154
130. Teninges D, Bras F, Dezelee S (1993) Genome organization of the sigma rhabdovirus: six genes and gene overlap. Virology 193: 1018–1023
131. Teninges D, Bras-Herreng F (1987) Rhabdovirus sigma, the hereditary CO2 sensitivity agent of drosophila: nucleotide sequence of a cDNA clone encoding the glycoprotein. J Gen Virol 68: 2625–2638
132. Thiry M, Lecoq-Xhonneux F, Dheur I, Renard A, Kinkelin D (1991) Sequence of a cDNA carrying the glycoprotein gene and part of the matrix protein M2 gene of viral haemorrhagic septicaemia virus, a fish rhabdovirus. Biochim Biophys Acta 1090: 345–347
133. Tordo N (1991) Contribution of molecular biology to vaccine development and molecular epidemiology of rabies disease. Mem Inst Butantan 53: 31–51
134. Tordo N, Bourhy H, Sather S, Ollo R (1993) Structure and expression in baculovirus of the Mokola virus glycoprotein: an efficient recombinant vaccine. Virology 194: 59–69
135. Tordo N, Poch O, Ermine A, Keith G, Rougeon F (1986) Walking along the rabies genome: is the large G-L intergenic region a remnant gene? Proc Natl Acad Sci USA 83: 3914–3918
136. Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A, Hawe LA, Leander KR, Martinez D, Perry HC, Shiver JW, Montgomery DL, Liut MA (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259: 1745–1749
137. Vandepol SB, Le Francois L, Holland JJ (1986) Sequences of the major antibody binding epitopes of the Indiana serotype of vesicular stomatitis virus. Virology 148: 312–325
138. VanderHeijden RWJ, Langedijk JPM, Groen J, UytdeHaag FGCM, Meloen RH, Osterhaus ADME (1993) Structural and functional studies on a unique linear neutralizing antigenic site (G5) of the rabies virus glycoprotein. J Gen Virol 74: 1539–1545
139. Volk WA, Snyder RM, Benjamin DC, Wagner R (1982) Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus: comparative neutralizing activity. J Virol 42: 220–227
140. Wagner RR, Prevec L, Brawn F, Summers DF, Sokol F, McLeod JM (1972) Classification of rhabdovirus proteins: a proposal. J Virol 10: 1228–1230
141. Walker, PJ, Byrne KA, Riding GA, Cowley JA, Wang Y, McWilliam S (1992) The genome of bovine ephemeral fever rhabdovirus contains two related glycoprotein genes. Virology 191: 49–61
142. Whitt MA, Chong L, Rose JK (1989) Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant. J Virol 63: 3569–3578
143. Whitt MA, Zagouras P, Crise B, Rose JK (1990) A fusion-defective mutant of the vesicular stomatitis virus glycoprotein. J Virol 64: 4907–4913
144. WHO (1984) Expert committee on rabies. WHO Techn Rep Ser 709: 9
145. Wiktor TJ, Clark HF (1973) Comparison of rabies virus strain by means of the plaque reduction test. Ann Microbiol Inst Pasteur 124: 283–288
146. Wiktor TJ, Gyorgy E, Schlumberger HD, Sokol F, Koprowski, H (1973) Antigenic properties of rabies virus components. J Immunol 110: 269–276

147. Wiktor TJ, Koprowsky H (1980) Antigenic variants of rabies virus. J Exp Med 12: 99–112
148. Winton JR, Arakawa CK, Lannan CN, Fryer JL (1988) Neutralizing monoclonal antibodies recognize antigenic variants among isolates of infectious haematopoietic necrosis virus. Dis Aquatic Organ 4: 199–204
149. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL (1990) Direct gene transfer into mouse muscle in vivo. Science 247: 1465–1468
150. Woodgett C, Rose JK (1986) Amino-terminal mutation of the vesicular stomatitis virus glycoprotein does not affect its fusion activity. J Virol 59: 486–489
151. Wunner WH, Dietzchold B, Smith CL, Lafon M (1985) Antigenic variants of CVS rabies virus with altered glycosylation sites. Virology 140: 1–12
152. Wunner WH, Reagan KJ, Koprowski H (1984) Characterization of saturable binding sites for rabies virus. J Virol 50: 691–697
153. Xu L, Mourich DV, Engelking, HM, Ristow S, Arnzen J, Leong JC (1991) Epitope mapping and characterization of the infectious hematopoietic necrosis virus glycoprotein, using fusion proteins synthesized in Escherichia coli. J Virol 65: 1611–1615
154. Zagouras P, Ruusala A, Rose JK (1991) Dissociation and reassociation of oligomeric viral glycoprotein subunits in the endoplasmic reticulum. J Virol 65: 1976–1984
155. Zakowsky JJ, Wagner RR (1980) Localization of the membrane-associated proteins in vesicular stomatitis virus by use of hydrophobic membrane probes and cross-linking reagents. J Virol 36: 93–97
156. Zhang L, Ghosh HP (1994) Characterization of the putative fusogenic domain in vesicular stomatitis virus glycoprotein G. J Virol 68: 2186–2193

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