Mutations in the glycoprotein of viral haemorrhagic septicaemia virus that affect virulence for fish and the pH threshold for membrane fusion

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To study the molecular basis of virulence of viral haemorrhagic septicaemia virus (VHSV), we used a cross-reactive neutralizing MAb to select MAb-resistant (MAR) mutants with reduced pathogenicity for fish. From sequence determination of the G gene of MAR mutants, attenuated laboratory variant and avirulent field strains, we identified two distant regions of the glycoprotein associated with virulence: region I (aa 135–161), homologous to the putative fusion peptide of both rabies virus (RV) and vesicular stomatitis virus (VSV), and region II (surrounding aa 431–433), homologous to RV and VSV domains controlling the conformational changes necessary for the fusion process to take place. Simultaneous mutations in both regions resulted in the most attenuated phenotype and we obtained genetic evidence that regions I and II may be structurally linked. As the MAR mutants had mutations in or near domains involved in fusion, the fusion properties of VHSV and its variants were analysed. This work allowed us to postulate that the fusion domain of VHSV is probably constituted of two distinct regions of the protein connected through a disulfide bridge between cysteines 110 and 152. Finally, we obtained evidence suggesting that the pH threshold for fusion is a determinant for virulence: restriction of fusion to a more acidic pH was associated with attenuation for the variant tr25 which had a shift of the threshold for maximal fusion from pH 6.30 (for the parental strain) to pH 6.00; conversely, two field strains which had maximal fusion at pH 6.60 were the most virulent.

Introduction

Viral haemorrhagic septicaemia virus (VHSV), also known as Egtvved virus, is a fish rhabdovirus that was initially isolated from rainbow trout (Oncorhynchus mykiss). It is the agent that causes the most economically serious viral disease for the fish farming industry in continental Europe (de Kinkelin et al., 1979).

VHSV belongs to the family Rhabdoviridae. It has a single-stranded RNA genome that encodes five structural proteins: the nucleocapsid protein N (Bernard et al., 1990), the polymerase-associated protein P, the matrix protein M (Benmansour et al., 1994), the transmembrane glycoprotein G (Thiry et al., 1991) and the polymerase L. In addition, a sixth functional gene, which encodes a non-structural protein (NV), was recently characterized (Basurco & Benmansour, 1995).

Like other rhabdoviruses (Coll, 1995a), VHSV has a transmembrane G protein that is responsible for attachment to the cell membrane and entry into the cell by a process of receptor-mediated endocytosis (Lecoq-Xhonneux et al., 1994). Fusion of the viral membrane with the endosomal membrane, triggered by the low pH of the endosomal compartment, allows delivery of the viral nucleocapsid into the cytoplasm. As with other viruses (Gaudin et al., 1995), fusion involves the insertion of a region of the glycoprotein in the target membrane. The location of this so-called fusion peptide has been recently identified for both rabies virus (RV) (aa 121–198) and vesicular stomatitis virus (VSV) (aa 117–136) (Li et al., 1993; Zhang & Ghosh, 1994; Durrer et al., 1995; Fredericksen & Whitt, 1995, 1996). In both RV and VSV the fusion peptide contains a stretch of prolines and aromatic residues which are also found in the VHSV glycoprotein between aa 144–154.
Therefore, this region of VHSV G is thought to contain the fusion peptide.

Neutralizing antibodies are directed exclusively against the G protein and are the most important component of the protective immune response against VHSV (Lorenzen et al., 1990). They exert their neutralizing activity, either at early stages of virus attachment to the cell membrane by blocking binding to the cellular receptor, or later in the cycle by inhibiting the fusion process. Consequently, as for other rhabdoviruses (Seif et al., 1985; Préhaud et al., 1988), resistance to the neutralizing effect of MAbs may proceed through selection of mutations within regions of the glycoprotein involved in virulence. Such mutations may affect the structural stability of the glycoprotein, its ability to recognize its receptor or its membrane fusion properties.

In a previous study, we described MAb-resistant (MAR) mutants and mapped the mutations responsible for the resistant phenotypes (Béarzotti et al., 1995). Of the MAbs used in this previous study, one (MAb C10), a cross-type neutralizing MAb, allowed the selection of mutants which had a reduced virulence for fish. In the present study, we further extended the characterization of the mutations involved in the attenuated phenotype and identified two distant regions of G associated with virulence. One of these regions is located in or near the putative fusion peptide of VHSV described above; the other is in a region which has been proposed to be involved in regulation of low-pH-induced conformational changes for both RV and VSV (Gaudin et al., 1996; Shokralla et al., 1998). Therefore, the fusion properties of VHSV (and of its variants) were also studied. Analysis of an attenuated laboratory variant indicated that this variant presented a shift of the pH threshold for membrane fusion towards a more acidic pH. Conversely, hypervirulent field strains presented a shift of the pH threshold for membrane fusion towards neutral pH. These latter results suggest that the pH threshold for fusion is a determinant for virulence. Finally, this work allows us to predict the position of the fusion domain of VHSV, which seems to be constituted of two distant segments of the primary sequence ([110–118] and [144–154]) maintained together by a disulfide bridge between cysteines 110 and 152.

Methods

**Cells and viruses.** VHSV strains 07-71 and tr25 and their antigenic mutants were grown as previously described (de Kinkel and Le Berre, 1977) on EPC cells (of carp origin) or RTG2 cells (of trout origin). Virions were purified from clarified supernatants by centrifugation through a 25% glycerol cushion.

**Selection of antigenic mutants and revertants.** This was done as described previously (Seif et al., 1985). Briefly, 10^6 p.f.u. from several different cloned viral stocks was incubated for 1 h at 14 °C with ascitic fluid and plated onto monolayers of EPC cells. Well-separated plaques were selected and small stocks of each putative mutant were prepared on EPC cells. The stocks were confronted with the selecting MAbs, and only those showing at least 90% resistance were used for this study. Revertants were selected, using essentially the same plating procedure, from well-characterized stocks of attenuated escape mutants passaged on the RTG2 trout cell line instead of the cyprinid cells (EPC).

**RNA isolation.** The viral RNA, for use in the RT–PCR amplification, was extracted from purified viral particles by the SDS–proteinase K procedure described by Benmansour et al. (1992). RNA from tissue or infected cells was extracted with the guanidinium–HCl procedure (Chomczynski & Sacchi, 1987) or an Rneasy kit (Qiagen).

**RT–PCR amplification and sequence determination.** RT–PCR was performed on viral RNA as previously described (Benmansour et al., 1992), except that the concentration of the four deoxynucleotides was reduced to 20 mM. For direct consensus sequencing, RT–PCR products representing the whole G gene were purified with a Qiaquick PCR kit (Qiagen) and aliquots were used directly in an automated cycle sequencing reaction with specific internal primers (ABI dye-terminator protocol).

**Sequence analysis.** The sequences were assembled and analysed with the Genetics Computer Group package (Devereux et al., 1984) run on a Sun/Unix minicomputer station.

**Virulence test.** Pathogenicity tests were performed on virus-free rainbow trout juveniles from the Spring strain (INRA, France) maintained in flow-through aquaria at 10 °C. For injection trials, groups of 40 trout weighing 2–3 g each were injected intramuscularly (i.m.) with 50 p.f.u. of wild-type (wt) viruses or MAR mutants in 20 µl of cell culture medium. For trials on infection by the natural route, groups of 40 trout of the same origin were waterborne-infected by immersion for 3 h in a suspension of wt virus or MAR mutants at a concentration of 5 × 10^5 p.f.u./µl. Meckleinfected control groups were included in each series and for each route of infection. The course of infection was monitored on the basis of daily records of mortality and clinical signs.

**Preparation of liposomes and assays for fusion.** Phosphatidylcholine (700 µg) plus phosphatidylethanolamine (300 µg) dissolved in organic solvents were mixed with 10 µg N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine and 10 µg N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine and dried in vacuo. After addition of 1 ml 150 mM NaCl, the mixture was sonicated for six 3 min periods in a bath sonicator. The liposome suspension was clarified at 3500 g for 5 min, and the supernatant was used in the subsequent assay. Fusion was assayed as previously described (Gaudin et al., 1996). Briefly, 10 µg fluorescent liposomes was mixed with about 50 µg virus in a buffer containing 150 mM NaCl and 5 mM Tris—HCl pH 7.5 (final volume 600 µl) in the cuvette of a thermostatically controlled Perkin Elmer LS50B spectrophotometer. After 5 min incubation at 14 °C, 400 µl phosphate—citrate buffer at the required pH (prepared from 100 mM citric acid and 200 mM dibasic sodium phosphate solution) was added and the increase of NBD fluorescence was monitored continuously. Excitation was at 455 nm (slit width 4 nm) and emission at 535 nm (slit width 10 nm). The mixture was stirred continuously during the experiment.

Results

**MAR mutants to a cross-reactive neutralizing MAb**

Although MAb c10 was derived from a mouse immunized with strain 07-71 (serotype 1), it was able to neutralize VHSV strains from the four known serotypes (Benmansour et al., 1997). Therefore, we postulated that the epitope recognized by MAb c10 should be conserved and may be involved in an
The VHSV glycoprotein, virulence and fusion

Fig. 1. Regions of the glycoprotein involved in determination of virulence. Only amino acid sequence differences from the reference sequence of strain 07-71 are noted. The sequence of escape mutants and revertants is shaded. Strains He and Makah are two field isolates with low virulence for rainbow trout.

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important function of the G protein. We used MAb c10 to select antigenic mutants from different cloned stocks of strain 07-71. We obtained four independent MAR mutants, m07-1, m07-2a, m07-2b and m07-2c, which were expanded and used to prepare purified mutant viruses. Viral RNA from each mutant was extracted, reverse transcribed and the cDNA used in a PCR reaction to amplify the G gene. The purified PCR products were subjected to automated sequencing with a set of G specific primers. The entire G gene sequence of the four independent MAR mutants was determined unambiguously from both strands of the amplified DNA.

As summarized in Fig. 1, MAR mutants to MAb c10 had either one point mutation at position 139, or two or three mutations including position 140. MAR mutants m07-1 and m07-2b had a different mutation at the same codon, which resulted in the amino acid changes S139–N and S139–I respectively; m07-2c had two point mutations, K140–R and I433–T; m07-2a had the same mutations as m07-2c and a third additional point mutation, K161–R. As the simultaneous occurrence of two or three mutations is normally a rare event, we repeated the sequencing on RT–PCR products from different m07-2c and m07-2a stocks and confirmed the presence of the two and three concomitant mutations respectively. Thus, their occurrence seemed to be co-selected under the neutralizing pressure of MAb c10. This feature is probably a consequence of the quasispecies nature of the virus population (Benmansour et al., 1997).

Virulence and pathogenicity of MAR mutants in fish

To test the effect of the observed mutations on virulence, we performed a series of infection trials. Juvenile trout were injected i.m. with 50 p.f.u. wt or mutant viruses. Under these conditions, strain 07-71 provoked symptoms characteristic of viral haemorrhagic septicaemia, and a 90% cumulative mortality. In this experiment, MAR mutants m07-1 or m07-2b (a single mutation at position 139) behaved apparently as the parental strain with a total cumulative mortality of 80% (Fig.
Fig. 2. Influence of glycoprotein mutations on virulence for fish. Groups of 40 fish, 1000–1500 degree-days old (1–3 g weight), were injected with 50 p.f.u. of wild-type or mutant virus and kept in flow-through aquaria with the water temperature maintained at 10 °C. Fish mortality was recorded for 20, 50 or 90 days depending on the virulence of the strains. (a) Virulence of MAR mutants selected from strain 07-71; (b) virulence of revertants selected from attenuated MAR mutants m07-2a and m07-2c; (c) virulence of the temperature-resistant variant tr25 and MAR mutants selected from it; (d) comparison of different virulence profiles, from hypervirulent to attenuated.

2a). However, when the natural route of infection was used (virus dispensed in the water) we recorded 40% cumulative mortality for m07-1 versus 70% for wt virus, which represents a significant attenuation of MAR m07-1(data not shown). By contrast, the attenuation of m07-2c and m07-2a was well marked in the i.m. injection experiment, with respectively 30% and 17% cumulative mortality versus 90% for wt virus (Fig. 2a). Therefore, virulence was affected by a single mutation at position 139 (and probably 140), but was further notably decreased by an additional mutation at position 433. Since mutation I433–T is present in field strain VB without affecting its virulence, then the attenuated phenotype observed in m07-2c could be either due to the effect of mutation 140 alone, or to a synergistic effect of the association of mutations 140 and 433. Interestingly, addition of a single mutation at position 140 in the context of the tr25 variant sequence did not increase attenuation (see below). This observation could indicate that the second hypothesis is more likely. A third additional mutation at position 161 resulted in the most attenuated phenotype.

Selection of revertant viruses

To substantiate the exact contribution effect of positions 140, 161 or 433 on the attenuated status, we tried to isolate each mutation and eliminate the associated mutations by selection of revertants while maintaining the selective pressure on position 140. For this purpose, both m07-2a and m07-2c were passaged at a high m.o.i. in the presence of MAb c10 on an homologous cell line (trout cell line RTG2) instead of the heterologous EPC cell line used for the initial selection. Then, the progeny viruses were subjected to plaque selection in the presence of MAb c10. Virus isolates from selected plaques were expanded and assayed for virulence on juvenile trout by i.m. injection. Surprisingly, viruses selected from both MAR mutants regained virulence for trout by the i.m. route (Fig. 2b), and were considered revertants. Sequence determination of revertants r07-2a and r07-2c confirmed that both had indeed reverted to the wt genotype at positions 140/161/433 or 140/433 respectively. Not surprisingly, both had acquired a new single mutation at position 139, which now accounted for their resistance to MAb c10 (Fig. 1). As we were not able to select mutation 140 without mutation 433, these positions are probably structurally linked to generate the resistant phenotype associated with a marked attenuated status.

G gene sequence of a laboratory attenuated variant and a non-virulent field isolate

To further investigate the contribution of mutations of the G protein to the attenuated status, we determined the G gene sequence of tr25, an attenuated laboratory variant of strain 07-71, which was obtained through serial several passages on an heterologous cell line (EPC cells), combined with a stepwise increase of the incubation temperature from 14 to 25 °C (unpublished results). Compared to the 07-71 parent strain subjected to the same number of passages at 14 °C, the tr25 variant had seven nucleotide substitutions, of which four
resulted in an amino acid change: Q118–R, T135–I, S339–R, and L431–P. Two of these substitutions were within close proximity of the substitutions found in the attenuated MAR mutants (Fig. 1). Moreover, sequence determination of the G gene of a non-virulent VHSV strain isolated from a healthy salmon in the United States (strain Makah) demonstrated three interesting differences, D136–N, S139–G and L431–P, among a total of 39 amino acid differences from strain 07-71 (Fig. 1). Although these observations were not a direct confirmation, they strongly suggested that two discrete regions of the glycoprotein represented respectively by positions 135/139/140/161 and 431/433 were involved in the attenuation of virulence of VHSV.

**Further attenuation of variant tr25**

Despite the presence of two amino acid substitutions within the vicinity of the c10 epitope, tr25 was fully neutralized by MAb c10. Therefore, we used the same experimental procedure as above to select independent MAR mutants from different stocks of tr25: mtr25-1, mtr25-2, mtr25-4, and mtr25-5. Compared to tr25, mtr25-1 and mtr25-2 had an identical single mutation, S139–N, while mtr25-4 and mtr25-5 had a single mutation at K140 which was changed to N or E respectively (Fig. 1). These results were another confirmation of the critical importance of positions 139 and 140 for the integrity of the c10 epitope. When injected into fish, mtr25-1 and mtr25-2 were significantly less virulent than tr25, with a cumulative mortality of 7.5% versus 20% for tr25. However, the virulence of mtr25-4 and mtr25-5 was no different from that of the parent virus tr25 (Fig. 2c). Thus, in the context of the tr25 sequence an additional mutation at position 139 increased the attenuation, while additional mutations at position 140 were without effect. Nonetheless, these results confirmed the importance of this region in the determination of virulence.

**Effect of mutations on the pH threshold for membrane fusion and relation to virulence**

Like mammalian rhabdoviruses, VHSV was shown to have a low-pH-induced fusogenic activity mediated by the viral G protein (Lecocq-Xhonneux et al., 1994; Estepa & Coll, 1996). As some of our attenuated mutants had mutations near domains involved in fusion (mutations at positions 139 and 140) or in control of low-pH-induced conformational change (mutations at positions 431 and 433), we decided to investigate this critical step in virus entry. For this purpose, we used an in vitro procedure which permitted real-time monitoring of the fusion process between purified VHSV virions and fluorescent liposomes (Gaudin et al., 1993). We first observed that, similar to virus growth, VHSV-mediated fusion was strictly dependent on a low temperature and was optimal at 14 °C (data not shown).

Fig. 3 (a) showed typical fusion kinetics obtained for the wt virus (strain 07-71) at different pH values. Fusion was maximal at pH 6.00 to 6.30, decreased to about 60% at pH 6.45 and 20% at pH 6.90. Similar experiments were performed for MAR mutants. To allow direct comparison between different viruses, variation in fusion was expressed as a percentage of the maximal fusion for each virus and plotted against the pH.
Fig. 4. Comparison of the fusogenic activity of different field strains and MAR mutants. To allow comparison between different viruses the fusion activity at a given pH value is expressed as a percentage of the maximal fusion recorded for each virus. (a) strain 07-71 and its MAR mutants; (b) variant tr25 and its MAR mutants; (c) strains with different virulence profiles.

Mutation S139–N, S139–I, K140–R (in association with I433–T) and K161–R had no detectable effect on the pH threshold for maximal fusion, since a similar fusion profile was obtained with mutants m07-1, m07-2a, m07-2b and m07-2c (Fig. 4a).

Compared to the parent strain 07-71, variant tr25 had a marked change in the pH threshold for maximal fusion (Fig. 3b). Fusion was maximal at pH 6·00, decreased to 40% at pH 6·30, and was only 10% at pH 6·45 (Fig. 4b). Similar to results with 07-71 MAR mutants, additional mutations S139–N, K140–N/E had no effect on the fusion profile of tr25 (Fig. 4b). From these results, we deduced that changes at position 139 or 140 did not affect the fusion profile.

Since attenuated variant tr25 had differed from 07-71 at four amino acid positions it was not possible to assign unambiguously the difference in the fusion profile to a specific position. In an effort to narrow the analysis, we examined the fusion profile of field strains with similar mutations to tr25. We found that change L431–P was present in field strains Makah and He without affecting their fusion profile, which was similar to strain 07-71 (Fig. 4c). However, two different field strains, 23-75 and 02-84, which appeared to be extremely virulent (Fig. 2d), had a shift to 6·60 of the pH threshold for maximal fusion (Fig. 4c) and still retained 15–25% of activity at pH 6·90 (Fig. 4c). Both strains had in common Q118–K. Then, it was tempting to connect the change in the pH threshold for maximal fusion of tr25 to mutation Q118–R, although we have no argument to explain how a change of K (in attenuated variant tr25) to R (in virulent field strains 23-75 and 02-84) could promote a shift of 0·60 pH units, except that arginine and lysine give different hydrogen-bonding patterns. Nevertheless, these latter results suggest that the pH threshold of fusion is indeed a determinant of virulence.

**Discussion**

In this paper, we have demonstrated that some VHSV mutants that are resistant to neutralization by a cross-neutralizing MAb have an attenuated phenotype. This result is another illustration of the concept that neutralizing epitopes may overlap with regions of the glycoprotein important for virulence. We also showed that different selective conditions which generated attenuated virus (escape to neutralization, adaptation to heterologous cell line, rise in growth temperature, natural selection) typically resulted in selection of mutations within the same regions of the glycoprotein. This could mean that amino acids of the glycoprotein which control virulence are limited to a few discrete regions of the protein.

From sequence determination of MAR mutants, laboratory variants and field strains of VHSV, we deduced that maximal attenuation of virulence was invariably associated with the simultaneous occurrence of mutations in two distant regions of the glycoprotein: region I (aa 135–161) and region II (centred on aa 431–433). A comparable situation was described for IHNV, another salmonid fish rhabdovirus, where attenuation was connected with two sets of associated mutations, either aa 78 and 218 or 276 and 419 (Kim et al., 1994). For RV, a mammalian rhabdovirus, a single mutation at aa 352 (333 for the mature protein) was shown to completely abolish neuro-
Virulence for adult mice (Coulon et al., 1983; Dietzschold et al., 1983; Seif et al., 1985; Tuffereau et al., 1989). However, attenuated phenotypes were also observed with mutations in the composite antigenic site II: aa 53–61 and 217–222 (Préhaud et al., 1988).

Most interestingly, region I is homologous to the region of the putative fusogenic peptide of both VSV and RV (Fig. 5a) and region II has evident sequence similarities with regions 395–425 of VSV or 392–418 of RV (Fig. 5b). Mutations in this latter region have been shown to affect the fusogenic properties of RV and VSV G proteins (Gaudin et al., 1996; Shokralla et al., 1998), possibly by influencing the low-pH-induced conformational changes necessary for fusion activity. Our finding that (i) mutations at position 140 were always associated with a concomitant change at position 431 or 433 and that (ii) we could not select a revertant with a single mutation at this position, provided the first concrete argument that regions I and II are structurally linked in the native G protein. Whether in one of the G conformations, these two interacting domains form a trimeric six-helical bundle with an interior triple coiled-coil structure similar to the one recently determined for the TM fusion proteins of retroviruses (Chan et al., 1997; Lu et al., 1995; Weissenhorn et al., 1997; Blacklow et al., 1995; Fass et al., 1996) remains to be determined. It is noteworthy that there is no observed coiled-coil predicted for rhabdoviruses glycoproteins although some sequences reminiscent of heptad repeats have been detected (Coll, 1995b; Estepa & Coll, 1996).

Since the MAR mutants have mutations in two regions involved in the complex process of fusion, we analysed the fusogenic characteristics of MAR mutants of VHSV. We have unambiguously shown that the attenuated MAR mutants have the same pH-dependent fusion profiles as the parent strains. Thus, for these mutants, attenuation of virulence is not due to a change in the pH threshold for membrane fusion. Attenuation was neither due to a radical change in tissue tropism, although we observed an increase in the frequency of the delayed nervous form of the disease with our most attenuated mutant (data not shown). However, we could not unambiguously link this phenomenon to the specific amino acid changes since the wt virus gave similar late nervous forms in a few fishes with no consistent amino acid change in the G sequence retrieved from the nervous tissue. Therefore, for the MAR mutants, the exact reason for attenuation remains to be determined.

Determination of the fusion profile of different field strains (Makah, He, 23-75 and 02-84) and laboratory variants of VHSV (tr25) was also undertaken. On the basis of the results, VHSV strains were divided into three groups according to their fusogenic pH profile. Group 1, which comprised variant tr25 and its MAR mutants, had a pH threshold for maximal fusion at pH 6.00; all had an attenuated phenotype. In group 2 the threshold was observed at pH 6.30. This group comprised the 07-71wt virus and its derived MAR mutants. Viruses in this group displayed a wide range of virulence characteristics, from high to low. Group 3 had a threshold for maximal fusion at pH 6.60 and contains the hypervirulent field strains 23-75 and 02-84. This classification suggests that there is a correlation between the pH at which fusion occurs and the magnitude of virulence: the lower the pH threshold for fusion, the more attenuated the virus. An explanation for such a relationship could be that the site of uncoating (which depends on the pH of fusion) is important for further steps of infection, such as transcription. This explanation is consistent with results obtained on VSV which have shown that a shift toward lower values of the pH threshold for fusion could lead to a loss of infectivity for cell culture (Fredericksen & Whitt, 1996, 1998). Moreover, in animals, rapid uncoating after endocytosis could prevent viral antigens from being efficiently processed for presentation by MHC class II molecules, thus preventing the immune system from mounting a specific response. Indeed, the proteases involved in antigen processing for MHC class II presentation reside mostly within highly acidic compartments deep within the cell (for a review see Fineschi & Miller, 1997).
Finally, this study allows a more precise characterization of the fusion domain of VHSV. Our results indicate that single mutations in positions 139, 140 and 161 do not affect the fusion properties of the virus. Furthermore different strains (07-71, Makah and He), despite having different amino acids in positions 136, 138, 139 (Fig. 1), have a similar fusion profile. This indicates that these amino acids are probably not directly involved in the fusion process. An analysis of VHSV G primary sequence suggests that the putative fusion peptide could begin at tyrosine-144, because peptide 130–143 contains eight charged and three polar amino acids (Fig. 1) and is thus very hydrophilic and unlikely able to interact with a target membrane. This latter peptide may constitute a loop which forms the epitope for MAb C10. More interestingly variant tr25, which has a shift in the pH threshold for membrane fusion, has four amino acid differences from its parental strain, 07-71 (Q118–R, T135–I, S339–R and L431–P). As change L431–P is present in field strains Makah and He without affecting their fusion profile, which is similar to that of strain 07-71, this rules out a role of this mutation in the fusion phenotype of tr25. Because field strains 23–75 and 02-84 also have a shift of their fusion pH and both have a difference at position 118 (R in place of Q) when compared to strain 07-71, it is tempting to connect the fusion phenotype to the mutation at position 118. Interestingly, sequence analysis of rhabdovirus glycoproteins reveals that VHSV and vesiculoviruses have two extra cysteines when compared to lyssaviruses. These cysteines (positions 110 and 152 for VHSV, 84 and 130 for vesiculoviruses) are connected by a disulfide bridge in VHSV (Einer-Jensen et al., 1998). Cysteine-152 is located in the putative fusion peptide described above (i.e. the peptide beginning at tyrosine-144) whereas cysteine-110 is separated from glutamine-118 by the heptapeptide STFFGG. As phenylalanine and glycine are often found in fusion peptides, this peptide may also interact with one membrane during the fusion process. Therefore, we suggest that the complete fusion domain of VHSV is constituted by these two distant regions of the primary sequence put together in the tertiary structure of the glycoprotein by disulfide bridge between cysteines 110 and 152. This might be also the case for VSV and other vesiculoviruses.

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