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Reversible Conformational Changes and Fusion Activity of Rabies Virus Glycoprotein

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In an attempt to understand the implication of the rabies virus glycoprotein (G) in the first steps of the viral cycle, we studied the pH dependence of virus-induced fusion and hemagglutination, as well as modifications of the structure and properties of the viral glycoprotein following pH acidification. Our results suggest that the G protein adopts at least three distinct configurations, each associated with different properties. At neutral pH, G did not fuse membranes or hemagglutinate erythrocytes. It was insensitive to digestion with bromelain and trypsin. At pH 6.4, the glycoprotein became sensitive to proteases. Hemagglutination was at its maximum and then sharply decreased with the pH. No fusion was detected. Aggregation of virus was also observed. The third configuration, at below pH 6.1, was associated with the appearance of fusion. Some neutralizing monoclonal antibodies were able to differentiate these three configurations. Preincubation of the virus at below pH 6 inhibited fusion, but this inhibition, like the structural modifications of the glycoprotein, was reversible when G was reincubated at neutral pH.

Rabies virus causes a fatal disease associated with intense viral replication in the central nervous system. Its negative-strand RNA genome encodes successively N, M1, M2, G, and L proteins. The nucleoprotein N, the phosphoprotein M1, and the RNA polymerase L are associated with the RNA molecule and compose the transcriptionally active nucleocapsid. This nucleocapsid is surrounded by a lipid bilayer associated with M2 and the transmembrane glycoprotein G (for a review, see reference 3).

The glycoprotein initiates the interaction between the virus and the target cell: it is responsible for virion attachment to specific receptors on the cell membrane (45). The nature of this receptor is still not known: rabies virus is a strict neuropathogen in vivo and yet it has a wide host range in vitro, infecting nearly all mammalian and avian cell types tested (45). In vivo, nicotinic acetylcholine receptor has been suggested to be rabies virus receptor (18). Other studies (17) show that the problem is probably more complex, suggesting the existence of at least two receptors at the surface of nerve cells. In vitro, gangliosides via their sialic acid or phospholipids play the role of receptors (38, 39, 45).

Rabies virus has also been found to cause fusion of cells and hemolysis of erythrocytes under acidic conditions (19). In addition, chloroquine and ammonium chloride have been shown to prevent infection (37). It has been proposed that rabies virus enters the cell via the endocytic pathway and subsequently fuses with the membrane of the endosome after its acidification. However, little is known about the behavior of the rabies virus glycoprotein during virus internalization. We studied membrane fusion induced by the CVS strain of rabies virus using different systems such as hemolysis, fusion from within (FFWI) of infected cells, and fusion of artificial liposomes studied by fluorescence resonance energy transfer assay.

Because of its suggested role in the process of fusion, we also investigated structural modifications of the rabies virus glycoprotein at acidic pHs. Our results strongly suggest that this protein undergoes two conformational changes. The first, at around pH 6.4, is responsible for the hemagglutination properties of the virus and allows tight binding of the virus to membranes. The second, at below pH 6, corresponds to the appearance of the fusion.

As in the case of influenza virus (29, 32, 34, 42), preincubation of the virus at below pH 6 inhibited fusion. However, like the conformational changes, this inhibition was reversible by reincreasing the pH.

MATERIALS AND METHODS

Chemicals. *N*-(Lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (RHO-PE) and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) were purchased from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Cholesterol, phosphatidylcholine, phosphatidylethanolamine (PE), gangliosides (type III from bovine brain), and trypsin (type XIII from bovine pancreas) were supplied by Sigma Chemical Co. Bromelain was bought from Boehringer.

Cells. BSR, a clone of BHK 21 (baby hamster kidney) cells, was grown in Eagle minimal essential medium supplemented with 10% calf serum.

Viruses. The CVS strain of rabies virus and the antigenic mutant P3, mutated at amino acid 198 of the viral glycoprotein (Lys to Met), were cultivated and purified as previously described (21, 27).

MAbs. Six neutralizing antiglycoprotein monoclonal antibodies (MAbs) with different specificities were used. They were produced in BALB/c mice immunized with UV-inactivated strain CVS virus and characterized in our laboratory (4, 21, 27).

FFWI. For FFWI, a subconfluent monolayer of BSR cells in sterile chambers (Lab-Tek; Miles Laboratories) was inoculated with CVS at a multiplicity of infection of 2, kept at room temperature for 1 h, and then incubated with Eagle minimal essential medium plus 2% calf serum at 37°C. At 24 h postinfection, the cells were washed with phosphate-

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buffered saline and exposed to prewarmed (37°C) Eagle minimal essential medium with 10 mM morpholinoethanesulfonic acid (MES) adjusted to the appropriate pH with NaOH. Cells were incubated at 37°C for 1 h, fixed, and stained with cresyl violet.

Hemagglutination. For hemagglutination determination, fifty microliters of 2% human erythrocytes (type B+) in phosphate-citrate McIlvaine buffer at the required pH was diluted with an equal volume of CVS in the same buffer in a 96-well plate (Falcon). The plate was kept at 4°C for 1 h before reading.

Hemolysis. For hemolysis determination, ten micrograms of CVS was mixed with 500 μl of 2% human erythrocytes (type B+) in phosphate-citrate McIlvaine buffer at the required pH and incubated in an ice bath for 5 min. Test tubes were further incubated for 20 min at 37°C. After centrifugation, the A_{540} of the supernatant was measured with a spectrophotometer.

Preparation of liposomes. Two hundred fifty micrograms of phosphatidylcholine, 500 μg of PE, and 125 μg of gangliosides dissolved in organic solvents were mixed with 10 μg of RHO-PE and 10 μg of NBD-PE and dried in vacuo. After addition of 1 ml of 0.15 M NaCl, the mixture was sonicated for six 3-min periods. The liposome suspension was clarified at 3,500 × *g* for 5 min, and the supernatant was used in the subsequent assay.

Assay for fusion. The resonance energy transfer method of Struck et al. (35), which involves the nonexchangeable probes NBD-PE as a fluorescent donor and RHO-PE as a fluorescent acceptor, was used to assay for fusion. Fusion of fluorescent liposomes with an unlabelled viral bilayer results in dilution of the fluorescent probes in the membrane. A consequent decrease in resonance energy transfer was detected by an increase in NBD-PE fluorescence and a decrease in RHO-PE fluorescence. Fluorescence was measured with a Jobin Yvon JY3 spectrofluorimeter with excitation and emission slit widths set at 10 nm. Excitation was at 455 nm. Ten microliters of fluorescent liposomes was mixed with 970 μl of phosphate-citrate buffer at the required pH. Twenty microliters of virus was then added. The fluorescence spectra were registered before and after the addition of the virus. The ratio of NBD (535 nm) to rhodamine (592 nm) fluorescence 10 min after the addition of the virus divided by the same ratio measured at the initial time was used as the measure of fusion. In some experiments, the increase of NBD fluorescence resulting from probe dilution was monitored continuously at excitation and emission wavelengths of 455 and 535 nm, respectively.

Digestion of G with bromelain. Bromelain was diluted in phosphate-citrate McIlvaine buffer at various pHs at a concentration of 4 μg/ml. One volume of virus (0.5 mg/ml) was added to one volume of proteases, and the mixture was incubated for various durations at 37°C. Digestion products were analyzed by 10% polyacrylamide gel electrophoresis (PAGE).

Binding of neutralizing MAbs to purified virus at different pHs. Ten microliters of virus (1 mg/ml) was incubated for 30 min with 10 μl of phosphate-citrate McIlvaine buffer at various pHs and then incubated with 80 μl of diluted MAbs at the same pH for 1 h at 37°C. Viruses and antibodies were then overlaid on a 25% glycerol cushion in McIlvaine buffer at the same pH and centrifuged for 1 h at 4°C and 40,000 rpm in an SW50 Beckman rotor. The pellet was directly dissolved in Laemmli buffer and analyzed by PAGE.

Immunosorbent assay. Enzyme-linked immunosorbent assays were performed as previously described (21).

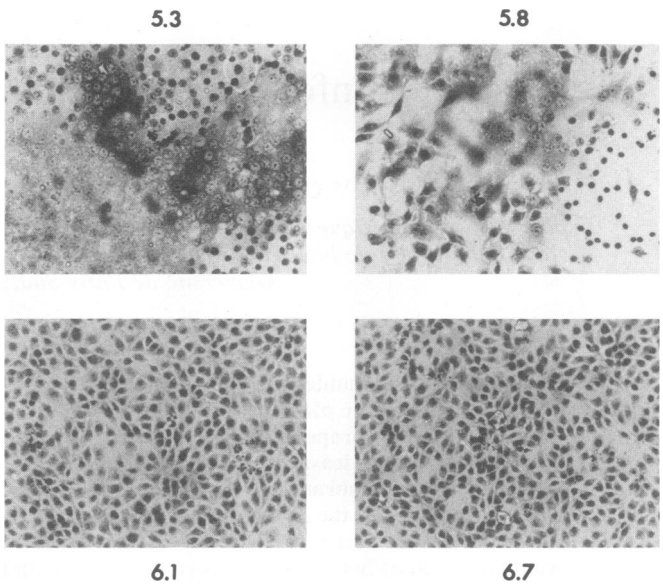

FIG. 1. FFWI of BSR cells at different pHs. Final magnification, ×75.

Electron microscopy. The virus negatively stained with 2% phosphotungstic acid was directly observed in a Philips 300 electron microscope at 60 kV.

RESULTS

Cell fusion. Because cell-to-cell fusion has been extensively used for the demonstration of fusion activity in several virus families (14, 43), we studied FFWI of BSR cells infected with the CVS strain of rabies virus. After 24 h of incubation at 37°C, the glycoprotein was highly expressed at the plasma membranes, as demonstrated in an indirect fluorescence assay performed on intact cells (data not shown). The cells were then exposed to Eagle minimal essential medium adjusted to the required pH (see Materials and Methods). Fusion of infected cells appeared when the pH fell below 6.1 and was maximal when the pH was below 5.9 (Fig. 1). In some experiments, lysis of infected cells also occurred at acidic pHs.

Hemagglutination and hemolysis. Hemagglutination of goose erythrocytes by rabies virus has been well documented (13, 15). Hemagglutination of human erythrocytes showed the same pH dependence as described in references 13 and 15: it was not detectable at neutral pH, was maximal at pH 6.4, and then decreased sharply (Fig. 2).

Rabies virus also induced a pH-dependent hemolysis of erythrocytes (19). At 37°C, hemolysis was detectable only at below pH 6. It was maximal at pH 5.5 and decreased at lower pHs (Fig. 3).

Fusion of liposomes. Because hemolysis requires not only the fusion but also the loss of integrity of cellular membranes, it might not be the best system for studying membrane fusion. A resonance energy transfer assay was used to study fusion of artificial liposomes with viral membranes. This assay, involving the nonexchangeable probes NBD-PE as a fluorescent donor and RHO-PE as a fluorescent acceptor, has already been used in the case of influenza virus (29, 31, 32, 34).

We first tried to optimize the composition of the liposomes. In view of the results obtained by other groups (6, 7,

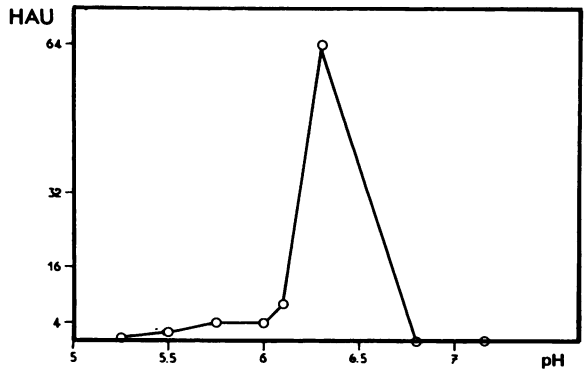

FIG. 2. Hemagglutination of human erythrocytes by the CVS strain of rabies virus. Hemagglutination was performed as described in Materials and Methods. HAU, hemagglutinating units.

32, 33), negatively charged phospholipids (such as cardiolipin or phosphatidylserine) were not used in this study: they do not specifically respond to viral fusion. It appeared that increasing the amount of PE in liposomes was favorable for fusion, as was the presence of gangliosides; cholesterol did not seem to be necessary (data not shown). Consequently, liposomes containing phosphatidylcholine, PE, and gangliosides in a weight ratio of 1:2:0.5 were used in subsequent studies. We observed that fusion occurred predominantly within 10 min, after a short lag period. At 20°C, lag time was around 40 s at pH 5.85 (see Fig. 5, curve 1); it increased with the pH (data not shown) and varied between virus preparations. Using this system, we also studied the pH dependence of fusion. We compared the extent of fusion after 10 min at different pHs. Fusion was detectable only at below pH 6.2 and was maximum at pH 5.8. It then decreased with the pH (Fig. 4).

Published results have reported that preincubation of influenza A virus at acidic pHs leads to a total, irreversible

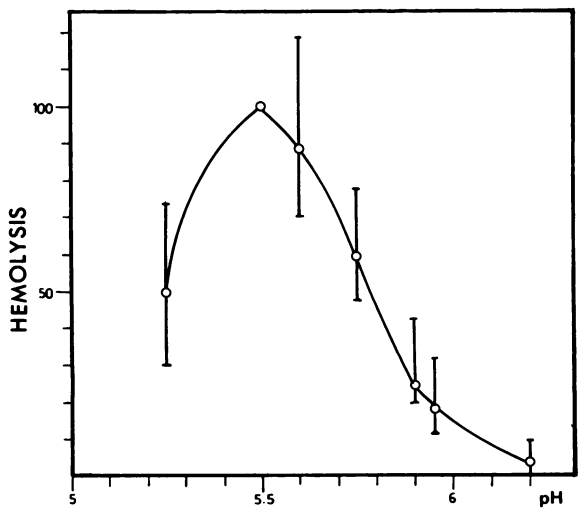

FIG. 3. Hemolysis of human erythrocytes by the CVS strain of rabies virus. Experiments were performed as described in Materials and Methods. We arbitrarily gave the value 100 to hemolysis at pH 5.5. Each point is the average of six experimental points. The bars on the curve show the range of results.

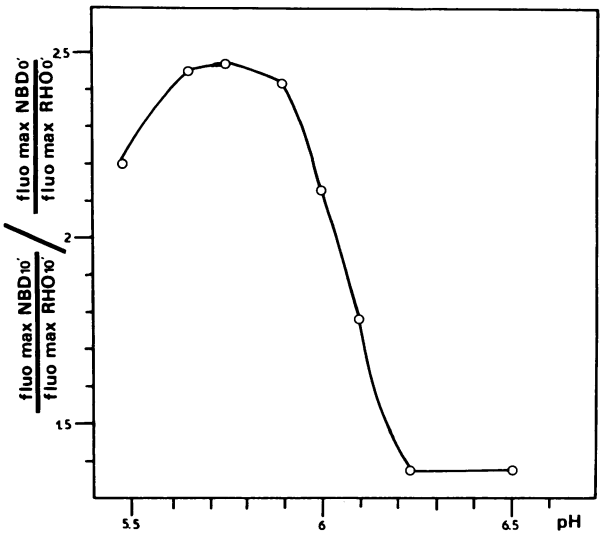

FIG. 4. Fusion of liposomes with CVS: dependence on pH. Liposomes contained phosphatidylcholine, PE, and gangliosides in a weight ratio of 1:2:0.5. Fusion was monitored as described in Materials and Methods. fluo, fluorescence.

inhibition of its ability to fuse liposomes (29, 32, 34, 42), while preincubation of vesicular stomatitis virus (VSV) at acidic pHs enhances its fusion activity (22). In view of these results, we also studied the influence of rabies virus preincubation at acidic pHs. Virus was preincubated at 20°C for various durations at pH 5.85 or 6.4. Liposomes in a buffer at pH 5.85 were then added, and fusion was monitored by registering the increase of fluorescence at 535 nm. Preincubation of the virus at pH 6.4 for periods as long as 20 min did not seem to have an influence on the initial rate and the final level of fusion (data not shown). On the contrary, preincubation of the virus at pH 5.85 led to an inactivation of its fusogenic properties which was total after 20 min (Fig. 5).

G protein is the main outer component of rhabdoviruses. In the case of VSV, it has been demonstrated that this protein can mediate a low-pH-induced fusion of cells (11, 23). Thus, G was a logical candidate as the entity responsible

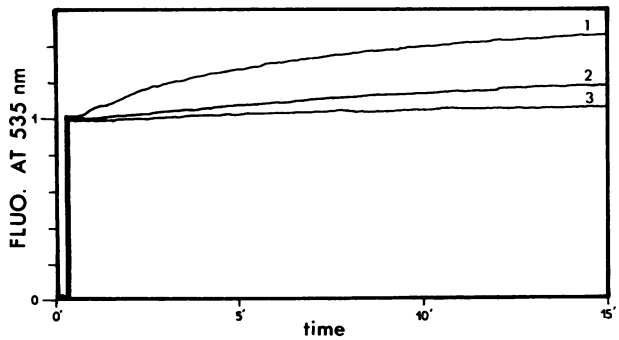

FIG. 5. Kinetics of fusion at pH 5.9 and effect of preincubation at low pH. Curve 1, fusion of liposomes at pH 5.9; curve 2, fusion of liposomes at pH 5.9 after 5 min of pretreatment of CVS at pH 5.9; curve 3, fusion of liposomes at pH 5.9 after 20 min of pretreatment of CVS at pH 5.9. FLUO, fluorescence.

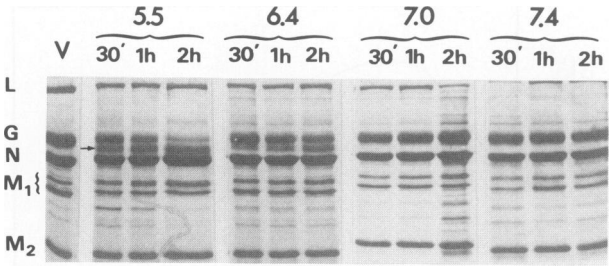

FIG. 6. Time course of bromelain susceptibility of antigenic mutant P3 at different pHs. Virus (final concentration, 0.25 mg/ml) was added to bromelain (final concentration, 2 μg/ml) at the indicated pHs and incubated at 37°C. Aliquots (10 μg of virus) were taken at different times of incubation and immediately boiled in Laemmli buffer and analyzed by 10% PAGE. Lane V, untreated virus. The arrow indicates the position of Gb (see text).

for rabies virus-triggered fusion, and we investigated eventual structural changes of the glycoprotein at acidic pHs.

pH-dependent sensitivity of G to proteases. A mutant called P3 (21), which is mutated at amino acid 198 of the glycoprotein, 6 amino acids before the first glycosylation site, was used. One effect of this mutation is the systematic glycosylation of the site, which is not the case with the parental strain. The G protein of P3 thus appears as a single band in polyacrylamide gels, and the presence of a cleaved form of G, which migrates slightly faster, is easier to follow. The fusogenic properties of P3 were indistinguishable from those of CVS (data not shown). Bromelain had no effect on the virus at pHs 7 and 7.4, the optimal pH of the enzyme. At a lower pH (6.4 or 5.5), G became sensitive to the protease and the digestion led to a stable product of 60 kDa called Gb (Fig. 6). Cleavage was more efficient at pH 5.5 than at pH 6.4. The other viral proteins were not sensitive to bromelain treatment.

Given the molecular weight of Gb, cleavage must have occurred near the transmembrane domain or near the N terminus of G. When the virus was pelleted after cleavage at pH 6.4 (Fig. 7), most of Gb remained in the supernatant, indicating that cleavage occurred at the beginning of the ectodomain, close to the transmembrane domain. Gb appeared to have a sedimentation coefficient of 4S in a sucrose

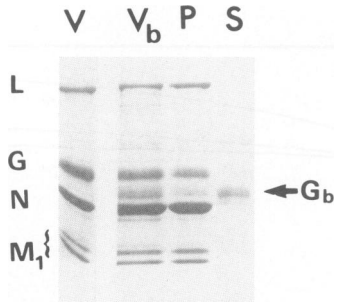

FIG. 7. Location of the bromelain cleavage site. Virus (lane V) was incubated with bromelain for 2 h at 37°C and pH 6.4. Next, an aliquot was taken and immediately boiled in Laemmli buffer for further analysis (lane V_b). The rest was diluted in cold NaCl (150 mM) and centrifuged in an SW50 rotor with an adaptor for small volumes at 25,000 rpm and 4°C for 2 h. The pellet (lane P) was directly solubilized in Laemmli buffer. The supernatant was precipitated with trichloroacetic acid and analyzed in the same gel (lane S).

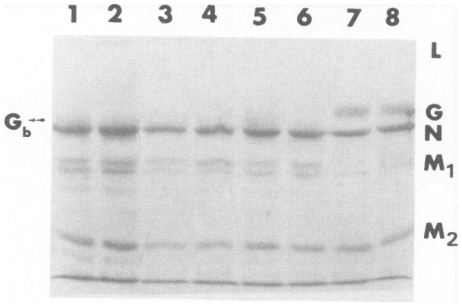

FIG. 8. Reversibility of the sensitivity to bromelain. Virus (mutant P3) was incubated for 1 h at 37°C and pH 5.9. Then an aliquot (10 μg of virus) was taken and digested with bromelain (2 μg/ml) for 1 h (lane 1) or 3.5 h (lane 2). The rest was pelleted through a 25% glycerol cushion in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–50 mM NaCl. The pellet was then dissolved in 150 mM NaCl. Aliquots (10 μg of virus) were then digested with bromelain (1 μg/ml) at pH 5.9 (lanes 3 and 4), pH 6.4 (lanes 5 and 6), or pH 7.5 (lanes 7 and 8) for 1 h (lanes 3, 5, and 7) or 3.5 h (lanes 4, 6, and 8). They were boiled immediately after the digestion in Laemmli buffer and analyzed by 14% PAGE.

gradient at pH 7.4, indicating that it sedimented as a monomer (data not shown).

We cannot exclude the possibility that the action of bromelain at acidic pH is due to a modification of protease specificity at below pH 6.4. However, similar results were obtained with trypsin, a protease which cleaves at the same sites whatever the pH: at pH 6.4, the glycoprotein became sensitive to trypsin. In this case, the digestion did not lead to a stable product (data not shown). These experiments suggested that viral G undergoes a conformational change at acidic pHs. However, when the virus was incubated for 1 h at 37°C at pH 6.4 or 5.9, pelleted, and then resuspended in a buffer at neutral pH, G was no longer sensitive to bromelain (Fig. 8). This suggested that the conformational change was reversible.

pH-dependent recognition of G by MAbs. We also analyzed the effect of pH on the antigenicity of G by reacting the molecule with MAbs of different specificities. The virus was incubated with MAbs at various pHs (see Materials and Methods) and then centrifuged through a 25% glycerol cushion at the same pH. The pellet, dissolved in Laemmli buffer (16), was analyzed by PAGE to detect the presence of virion-associated MAbs (Fig. 9). The results obtained for six MAbs are shown in Table 1. We clearly detected the presence of three categories of antibodies. The first category was composed of MAbs which recognized the viral glycoprotein with the same efficiency at neutral and acidic pHs. The second category was composed of MAbs which recognized the glycoprotein at pH 7.4 and 6.4 but not at pH 5.9. The third category was composed of MAbs which recognized the glycoprotein at pH 7.4 but not at acidic pHs. The loss of recognition at acidic pHs might be due to modifications of the active site of the MAbs. This possibility could be ruled out for MAb 29EC3, which recognized the glycoprotein at pHs 7.4 and 5.9 with the same efficiency when the virus was attached to a solid support (data not shown), probably because G could not undergo normal conformational changes when the pH was lowered. Similarly, 29CA4 recognized coated virus at pH 6.4. In this latter case, 29CA4 still differentiated the mutant escaping neutralization from the parental strain, indicating that this MAb bound the same domain of the protein when the virus was attached on a solid

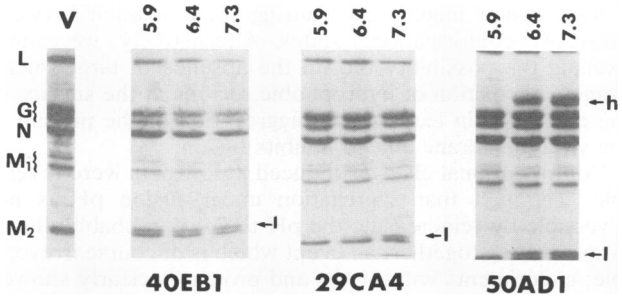

FIG. 9. Recognition of G by MAbs 40EB1, 29CA4, and 50AD1 at different pHs. Experiments were performed as described in Materials and Methods. Heavy and light chains of MAbs associated with the viruses are indicated (h and l, respectively). In the case of 40EB1 and 29CA4, which are immunoglobulin G (while 50AD1 is immunoglobulin M), heavy chains were not detectable (they comigrated with N).

support. As shown with proteases, this conformational change was reversible: viral G incubated at acidic pHs for 30 min and then diluted in a buffer at neutral pH was recognized by MAbs 29EC3 and 30AA5 as efficiently as the native form.

Electron microscopy. We used electron microscopy to detect eventual morphological changes of the virus at acidic pHs. The virus was incubated for 30 min at various pHs before negative staining. Virions which were well dispersed at pH 7.4 formed large aggregates at acidic pHs (Fig. 10). The presence of a dissociating agent (EDTA, 10 mM) had no influence on aggregation. In addition, at pH 5.9, the virions seemed to lose their regular shape. Virus aggregation at pH 6.4 was at least partially reversible after 1 h of incubation at pH 7.4. This was not the case for aggregation at pH 5.9 (data not shown).

Reversibility of fusion inhibition. Since conformational changes of G seemed to be reversible, we decided to investigate whether the loss of fusogenic properties of G observed after preincubation at an acidic pH (5.85) was also reversible. Virus which had been exposed to pH 5.85 for 20 min and was no longer able to fuse liposomes was reincubated at pH 7.15 for various durations and then mixed with liposomes at a final pH of 5.94. Seventy percent of the initial fusion ability was restored after 40 min at pH 7.15, indicating that the loss of fusogenic properties of G was also reversible (Fig. 11).

DISCUSSION

In an attempt to understand the involvement of rabies virus glycoprotein in the first steps of the viral cycle, we have investigated hemagglutination and membrane fusion induced by the CVS strain of rabies virus. We have been able to show that, as in the case of numerous other viruses (for a review, see reference 30), the viral glycoprotein undergoes conformational changes at acidic pHs. Half-maximal fusion is at pH 6 (FFWI or fusion of artificial liposomes), a value similar to that obtained with VSV (5, 11, 23, 43). Hemolysis occurs at a lower pH (0.3 pH unit). This phenomenon has already been described in the case of influenza A (42) and influenza C (12) viruses. Fusion kinetics reveals the existence of a lag which becomes longer at higher pHs. This phenomenon has been reported in the case of influenza C (12) and influenza A (32, 34) viruses. As in the case of influenza A virus (29, 32, 34, 42), incubation of the virus at a low pH prior to its interaction with a target membrane inactivated the fusion activity of the virus. However, this inactivation is reversible when the virus is reincubated at pH 7.15.

Biochemical and immunological studies strongly suggest the occurrence of conformational changes of G at acidic pHs. A similar effect has been extensively studied in the case of the hemagglutinin of influenza A virus (9, 24, 25, 28, 41, 44). In the case of hemagglutinin (HA1 + HA2), the conformational change is irreversible and allows the exposition of the hydrophobic, highly conserved N terminus of HA2 which can interact with the target membrane (for a review, see reference 40). In the case of VSV G glycoprotein, such a conformational change has also been reported to lead to the

TABLE 1. Recognition of the CVS strain by different MAbs at different pHs

MAb	Mutation (amino acid) conferring resistance to MAb	Antigenic site	Recognition at pH:			Reversibility[a]
			7.4	6.4	5.9	
40EB1	343	Minor a	+	+	+	ND
20A2	342	Minor a	+	+	+	ND
29EC3	336	III	+	+	−	Yes
50AD1	333	III	+	+	−	ND
30AA5	36	II	+	+	−	Yes
29CA4	198	II	+	−	−	ND

[a] ND, not determined.

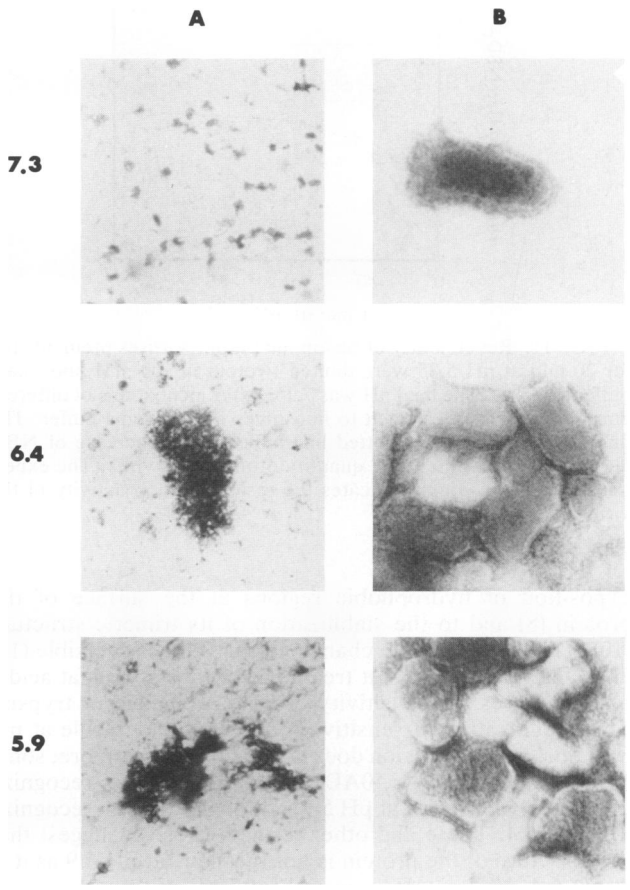

FIG. 10. Electron micrographs of CVS strain of rabies virus at different pHs. Magnification, ×8,650 (A) and ×79,500 (B).

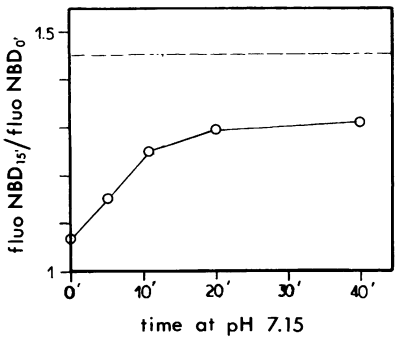

FIG. 11. Reversibility of fusion inhibition. Viruses preincubated for 20 min at pH 5.85 were diluted twofold in 100 mM phosphate buffer (pH 7.4). The final pH was 7.15. After incubations of different durations, they were added to liposomes in McIlvaine buffer. The final pH was 5.95. The dotted line indicates the increase of NBD fluorescence with the same quantity of untreated virus. The experimental point at 0 min indicates the residual fusion activity of the virus after inhibition.

exposition of hydrophobic regions at the surface of the protein (8) and to the stabilization of its trimeric structure (10). This conformation change appears to be reversible (10, 22). Here, we report that treatment of rabies virus at acidic pHs increases the sensitivity of G to bromelain or trypsin. This modification of sensitivity is already detectable at pH 6.4, a pH at which fusion does not occur. Furthermore, some MAbs (e.g., 29EC3 or 50AD1; Table 1) no longer recognize the viral glycoprotein at pH 5.9, although they still recognize it at pH 6.4. These and other results (Table 2) suggest that the structure of the protein is not the same at pH 5.9 as it is at pH 6.4.

Structural modifications observed at pH 6.4 correspond to the maximal hemagglutination and the appearance of viral aggregates, as detected in electron microscopy. This first conformational change allows a tight binding of the virus to membranes and is likely to occur upon acidification of the endocytic vesicle. It could be a prerequisite for a fusion event. The nature of the binding is unknown, but it does not seem to involve only hydrophobic interactions, because (i) hemagglutination is only poorly inhibited by PE or neutral lipids and (ii) it is efficiently inhibited by gangliosides or negatively charged phospholipids such as phosphatidylinositol or phosphatidylserine (reference 13 and our unpublished results).

The second structural modification, observed around pH 6, corresponds to the appearance of viral fusogenic properties. As for numerous other viruses, these properties probably involve exposition of hydrophobic regions at the protein surface. However, as no fusion is detected at pH 6.4 and preincubation of virions at pH 5.9 inhibits their fusion ability, fusion might occur during the transition between these two conformational states. Alternatively, we cannot exclude the possibility that in the absence of target membranes, exposition of hydrophobic regions at the surface of the glycoprotein leads to self-aggregation of the protein in the viral membrane and so inhibits fusion.

TABLE 2. Properties of CVS virions at different pHs

pH	Fusion	Hemagglutination	Aspect[a]	Sensitivity to bromelain	Recognition by MAb(s)[b]:		
					A	B	C
7.4	−	−	Isolated	−	+	+	+
6.4	−	+	Aggregated	+	+	+	−
5.9	+	−	Aggregated	+	+	−	−

[a] Aspect of the virus in electron microscopy.
[b] A, 40EB1 and 20A2; B, 29EC3, 50AD1, and 30AA5; C, 29CA4.

Conformational changes induced at low pHs were reversible. The fact that aggregation under fusion pH is not reversible by reincreasing the pH to 7.4 is probably due to viruses fusing together, an event which is of course irreversible; experiments with MAbs and proteases clearly showed that the structural modifications are reversible. These results are similar to those observed with VSV (10, 22). Is this reversibility biologically significant? As with other transmembrane proteins, G is synthesized in the rough endoplasmic reticulum and subsequently transported to the Golgi apparatus. Whether virion mutation occurs in the Golgi apparatus or at the plasma membrane is not quite clear in the case of rabies virus. Whatever the case, G is transported to the periphery in acidic vesicles (1, 2, 26). The pH of these compartments is at least sufficient to induce the first conformational change in G and might be lower if the infection process decreases the intracellular pH, as reported for Sindbis virus (20). Reversibility could then be necessary to obtain an active glycoprotein with its neutral conformation at the surface of the virion. In the case of influenza virus, reversibility would not be necessary, as influenza virus M2 protein counteracts the acidity of vesicular compartments of the exocytic pathway in infected cells and then protects the structural integrity of acid-sensitive hemagglutinin (36).

To investigate which regions of the glycoprotein are modified at acidic pHs, MAbs may be useful tools. We found that among the six MAbs used in this study, one (29CA4) no longer recognized G at pH 6.4 and four no longer recognized G at pH 5.9. Those four MAbs are directed against the two major antigenic sites of the protein (sites II and III), while the two MAbs (40EB1 and 20A2) directed against minor site a (close to site III [see reference 4]) continued to bind the protein with the same efficiency at pHs 6.4 and 5.9. It is possible that the conformation of the minor site was modified less at a low pH than the configuration of the major antigenic sites. It would be interesting to confirm this finding with additional MAbs.

As G does not possess any obvious hydrophobic peptide, we cannot clearly delineate a region of the protein implicated in fusion. Additional data are required for a better understanding of the membrane fusion triggered by G; the results presented here are a first step in this direction.

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