Membrane fusion activity of vesicular stomatitis virus glycoprotein G is induced by low pH but not by heat or denaturant. Virology

ARTICLE *in* VIROLOGY · JULY 2003

Impact Factor: 3.32 · DOI: 10.1016/S0042-6822(03)00146-6 · Source: PubMed

ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 310 (2003) 319–332

www.elsevier.com/locate/yviro

Membrane fusion activity of vesicular stomatitis virus glycoprotein G is induced by low pH but not by heat or denaturant

Yi Yao, Kakoli Ghosh, Raquel F. Epand, Richard M. Epand, and Hara P. Ghosh*

Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada

Received 18 November 2002; returned to author for revision 7 January 2003; accepted 4 February 2003

Abstract

The fusogenic envelope glycoprotein G of the rhabdovirus vesicular stomatitis virus (VSV) induces membrane fusion at acidic pH. At acidic pH the G protein undergoes a major structural reorganization leading to the fusogenic conformation. However, unlike other viral fusion proteins, the low-pH-induced conformational change of VSV G is completely reversible. As well, the presence of an α-helical coiled-coil motif required for fusion by a number of viral and cellular fusion proteins was not predicted in VSV G protein by using a number of algorithms. Results of pH dependence of the thermal stability of G protein as determined by intrinsic Trp fluorescence and circular dichroism (CD) spectroscopy show that the G protein is equally stable at neutral or acidic pH. Destabilization of G structure at neutral pH with either heat or urea did not induce membrane fusion or conformational change(s) leading to membrane fusion. Taken together, these data suggest that the mechanism of VSV G-induced fusion is distinct from the fusion mechanism of fusion proteins that involve a coiled-coil motif.

Introduction

Enveloped animal viruses enter the host cell by fusion of the viral membrane with the cellular membranes. The fusion of the two membranes is mediated by the viral envelope glycoprotein. A number of viruses such as paramyxoviruses and retroviruses, including the human immunodeficiency virus, fuse directly at the cell surface at neutral pH. In contrast, viruses, such as orthomyxo-, alpha-, and rhabdoviruses are initially internalized by receptor-mediated endocytosis and then fuse with the endosomal membranes via a low-pH-mediated fusion reaction (Hernandez et al., 1996; White, 1992). Fusion of the cellular and viral membrane mediated by the viral envelope glycoprotein requires conformational changes involving major structural reorganization. The conformational change(s) necessary for converting the nonfusogenic protein into the fusogenic conformation is triggered either by interaction with cellular receptor(s) at the cell surface or by the acidic pH of the endosomes (Eckert and Kim, 2001; Gaudin, 2000; Gaudin et al., 1995; Hughson, 1995, 1997; Skehel and Wiley, 1998, 2000; Skehel et al., 1995; Weissenhorn et al., 1999).

The spike glycoprotein hemagglutinin (HA) of influenza virus, a member of the orthomyxovirus family, has been most extensively studied to understand the mechanism involved in membrane fusion. The crystal structure of HA was determined both in the neutral (prefusogenic) and in acid pH (fusogenic) forms (Bullough et al., 1994; Wilson et al., 1981). A crystal structure was also determined for uncleaved HA precursor (Chen et al., 1998). A dramatic irreversible structural reorganization of the molecule is triggered during fusion activation at low pH. Fusion activation at acid pH results in the release of the fusion peptide that is buried inside the trimer interface at neutral pH and positioning it at the N-terminal tip of a newly formed coiled coil which is extended by 100 Å to reach the target membrane. In addition, an α-helical region at the carboxy terminus of the coiled-coil segment is refolded into a 180° turn such that the ectodomain of HA containing the membrane anchor sequence is folded back to form a rod-shaped molecule containing both the fusion peptide and the membrane anchor at the same end (Bullough et al., 1994; Carr and Kim, 1993; Hughson, 1995; Skehel and Wiley, 1998). At neutral

* Corresponding author. Department of Biochemistry, McMaster University, 1200 Main St. W., Hamilton, ON, L8N 3Z5 Canada. Fax: +1-905-522-9033.

E-mail address: ghosh@mcmaster.ca (H.P. Ghosh).

0042-6822/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0042-6822(03)00146-6

pH HA is nonfusogenic. However, the native form of HA can be converted to a fusogenic form at neutral pH by perturbants such as heat or urea, suggesting that at neutral pH HA is in a metastable state (Carr et al., 1997; Ruigrok et al., 1986). Similar activation of fusion at elevated temperature was also observed for paramyxoviruses (Paterson et al., 2000; Wharton et al., 2000). Structural studies of the fusion proteins of enveloped viruses belonging to retroviruses including human immunodeficiency virus 1 (HIV 1) (Lu et al., 1995; Weissenhorn et al., 1997), simian immunodeficiency virus (SIV) (Blacklow et al., 1995), Moloney murine leukemia virus (MLV) (Fass et al., 1996); paramyxovirus, SV5 (Baker et al., 1999), and human respiratory syncytial virus (Zhao et al., 2000); and filovirus, Ebola (Weissenhorn et al., 1998), suggest that similar to the HA protein of influenza virus a number of diverse viral fusion proteins also use coiled-coil structures for membrane fusion (Hughson, 1997; Skehel and Wiley, 1998; Weissenhorn, 1999). Viral fusion proteins using this type of mechanism are classified as class I fusion proteins (Lescar et al., 2001).

Studies with flavivirus and alphavirus fusion proteins identified a separate class of viral fusion proteins (class II) (Gibbons et al., 2000; Heinz and Allison, 2001; Lescar et al., 2001; Rey et al., 1995; Stiasny et al., 2001). Class II viral-fusion proteins are activated irreversibly by low pH and are not proteolytically cleaved from a precursor but need to be associated with a second spike protein whose cleavage is required for fusion. They contain an internal fusion peptide and are not predicted to form coiled-coils. X-ray structural analyses of two class II proteins, the E protein of the flavivirus tick-borne encephalitis (TBE) virus (Rey et al., 1995) and the E1 protein of the alphavirus Semliki Forest virus (SFV) (Lescar et al., 2001) have shown that both of these have very similar structures, which are different from the structure of class I fusion proteins, exemplified by influenza virus HA protein (Bullough et al., 1994; Wilson et al., 1981).

Vesicular stomatitis virus (VSV) belongs to the vesiculovirinae genera of the rhabdoviridae family. The single envelope glycoprotein G of VSV plays an essential role in virus infection. Initial interaction between VSV and the target cell occurs by binding of the G protein to the cellular receptor phosphatidylserine present at the cell surface (Wagner and Rose, 1996; Schlegel et al., 1983). The attached virus is then endocytosed and delivered to the endosome. The acid pH of the endosomal compartment triggers fusion of the viral membrane with the endosomal membrane mediated by the G protein to release the VSV nucleocapsid. Cells infected with VSV express G protein at the cell surface and exposure of the cells expressing G protein to low pH results in cell-cell fusion leading to polykaryon formation (White et al., 1981). Expression of G protein in cells transfected with a cloned G gene further showed that the G protein alone was sufficient for acid-pH-induced cell fusion (Florkiewicz and Rose, 1984; Reidel et al., 1984). VSV G protein is a transmembrane glycoprotein containing a cleaved N-terminal signal peptide and a single membrane anchor at the carboxy terminus (Rose and Gallione, 1981; Coll, 1995a). Like other viral envelope proteins it forms a trimer which is, however, stable only at low pH (Kreis and Lodish, 1986; Doms et al., 1987). Studies using the soluble ectodomain of VSV G showed that exposure to acid pH triggers a conformational change involving increased exposure of a hydrophobic region. However, the observed conformational change was reversible (Crimmins et al., 1983). Recent biophysical studies with isolated G protein confirmed that the low-pH-induced conformational changes involve major structural reorganization (Carneiro et al., 2001). Kinetics of VSV-mediated cell fusion at low pH further showed that VSV G can exist in different conformational forms and that the conformational change from the nonfusogenic to the fusogenic form was reversible (Clague et al., 1990; Puri et al., 1988, 1992; Gaudin, 2000). Studies with the G glycoprotein of rabies virus (RV), a member of the lyssavirinae genera of the rhabdoviradae family, also showed low-pH-triggered conformational changes that were reversible (Gaudin, 2000; Gaudin et al., 1991, 1993, 1999). Studies from our laboratory and others have shown that the fusion domain of VSV G is located internally (residues 116–137). However, unlike the fusion peptides present in other viral fusion proteins it is not hydrophobic (Durrer et al., 1995; Fredericksen and Whitt, 1995; Zhang and Ghosh, 1994). It was further shown that the low-pH-mediated insertion of the fusion domain of VSV G in the target membrane is reversible (Pak et al., 1997). Studies from our laboratory showed that a region located between residues 390 to 418 of VSV G protein played a role in the control of the low-pH-induced conformational change (Shokralla et al., 1998,1999). A similar region in the RV G protein controlling the low-pH-induced conformational change was also identified (Gaudin et al., 1996). It appears, therefore, that the fusion process involving VSV G protein is similar to rabies virus G but is distinct from the mechanism used by the class I and class II viral fusion proteins and new insights may emerge from studying G protein-mediated membrane fusion. The objective of the present study is to examine the possibility that the native (prefusogenic) conformation can be induced to change to the fusogenic conformation not only by low pH but also by destabilizing agents, such as heat or urea. The results show that the thermal stabilities of the native nonfusogenic and fusogenic G proteins, as monitored by intrinsic tryptophan fluorescence and CD analyses, did not differ markedly. It was further shown that at neutral pH neither heat nor urea treatment could trigger membrane fusion or induce conformational change(s) required for fusion.

Results

Recent structural studies of a number of virus membrane fusion proteins as well as cellular fusion proteins involved in neurotransmitter release and intracellular vesicle transport suggest a common mechanism of protein-mediated membrane fusion. The presence of a coiled-coil motif and

the formation of a rod shaped α-helical bundle of the ectodomain of the fusion protein containing the membrane-inserted sequences at one end of the rod is believed to be involved in fusion of two membranes (Skehel and Wiley, 1998; Weissenhorn et al., 1999). Coiled-coil sequences generally contain hydrophobic and hydrophillic amino acids in a repeating heptad pattern (the 4–3 hydrophobic repeat) (Lupas et al., 1991). Examination of the primary sequence of a number of viral fusion proteins suggested the presence of heptad repeat sequence adjacent to putative fusion domains (Chambers et al., 1990). Earlier, we reported that analysis of VSV G protein sequence by the algorithm of Lupas et al. (1991) failed to identify the presence of any predicted coiled-coil motif (Zhang and Ghosh, 1994). The availability of a number of newer algorithms predicting the probability of coiled-coil formation, such as COILS (Lupas, 1996), PairCoil (Berger et al., 1995), MultiCoil (Wolf et al., 1997), and LearnCoil-VMF (Singh et al., 1999), prompted us to reexamine the VSV G sequence to identify probable coiled-coil motifs. None of the algorithms predicted any coiled-coil regions in G protein. In the case of the COILS program, the G protein was analyzed by changing three separate variables: the scoring matrix (MTIDK vs MTK); the window size of 14, 21, or 28 residues; and the weighting of the a and d amino acids residues (1.0 vs 2.5). The validity of prediction of all of the programs used was checked by analyzing viral fusion proteins, such as HA of influenza virus (Concannon et al., 1984), gp160 of HIV-1 (Douglas et al., 1997), F protein of New Castle disease virus (Sakaguchi et al., 1989), and GP64 of baculovirus (Ayres et al., 1994), all of which showed the presence of coiled-coil regions. Taken together, the computational data confirm our previous suggestion that VSV G does not contain a predicted coiled-coil sequence. Previously, Coll (1995b) predicted two regions containing heptad repeat units to be present in the ectodomain of G glycoproteins of a number of rhabdoviruses. However, our analyses failed to identify any of these predicted regions. A possible reason or this discrepancy could be the algorithm used by Coll (1995b).

The fact that fusion activation of VSV G protein is not dependent on the proteolytic cleavage of a precursor further suggested that the mechanism of conversion of the G protein to the fusion-active state may be different from those of the fusion proteins belonging to the influenza HA prototype. It was earlier shown that the nonfusogenic form of influenza HA2 is in a metastable condition such that the low-pH-induced conversion to the fusogenic state can be substituted by perturbants such as heat or urea (Carr et al., 1997). It was also suggested that fusion proteins that use the coiled-coil-based fusion mechanism are metastable under nonfusogenic conditions (Carr et al., 1997). Induction of fusion at elevated temperature was also reported for paramyxoviruses, Sendaivirus, and SV5 (Paterson et al., 2000; Wharton et al., 2000). We, therefore, decided to examine if the G protein shows altered thermostability under fusogenic conditions or if destablizing agents such as heat or urea could induce

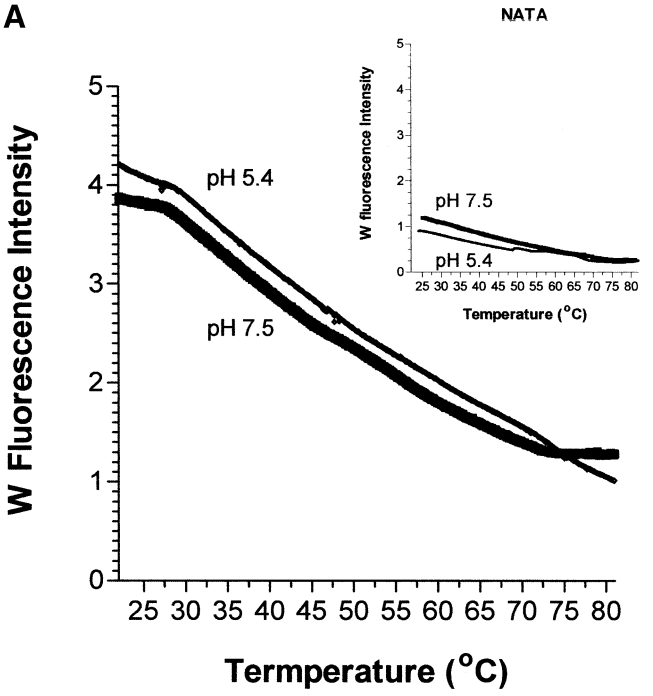

Fig. 1. Thermal stability of VSV G protein at neutral and acid pH. G protein was isolated from VSV Indiana by extraction of the virions with octylglucoside as described earlier (Kotwal et al., 1983). (A) Thermal stabilities of the G protein at pH 7.4 and 5.4 were monitored by measuring intrinsic Trp fluorescence. Samples of G protein were adjusted to pH 7.4 or 5.4 and incubated at room temperature for 15 min before starting the fluorescence measurements. At each temperature the fluorescence intensity relative to the signal at 25°C is shown. The thermal stability profiles of NATA at pH 7.4 and 5.4 are shown as insets. (B) Thermal denaturation and renaturation curves of the G protein at pH 7.4 and 5.4. Samples of G protein at pH 7.4 or 5.4 were heated gradually from 25 to 95°C. Renaturation of the G protein was monitored by cooling the samples from 95 to 25°C at a programmed rate of 0.5°C/min. The curves were obtained by monitoring the CD signal at 222 nm. The curves are representative of two independent experiments. Representative spectra at 25°C of G protein at pH 7.4 or pH 5.6 are shown as insets.

conformational change(s) in VSV G protein, leading to virus-induced membrane fusion at neutral pH.

Thermostability of G protein at neutral and acid pH

To examine the thermostability of the G protein under nonfusogenic (neutral pH) and fusogenic (acid pH) conditions, we measured the thermally induced changes in (a) intrinsic Trp fluorescence as a function of pH as well as (b) changes in secondary structures of the G protein at neutral or acid pH by monitoring the changes in the mean residue ellipticity at 222 nm.

In the nonfusogenic state at pH 7.4, the fluorescence spectra represents the hydrophobic environment of the 10 Trp residues, all present in the ectodomain of the G protein. Acidification of the G protein results in the exposure of these Trp residues that can be observed as a decrease in the intrinsic Trp fluorescence. Temperature-induced changes in the intrinsic Trp fluorescence of G protein at pH 7.4 and 5.4

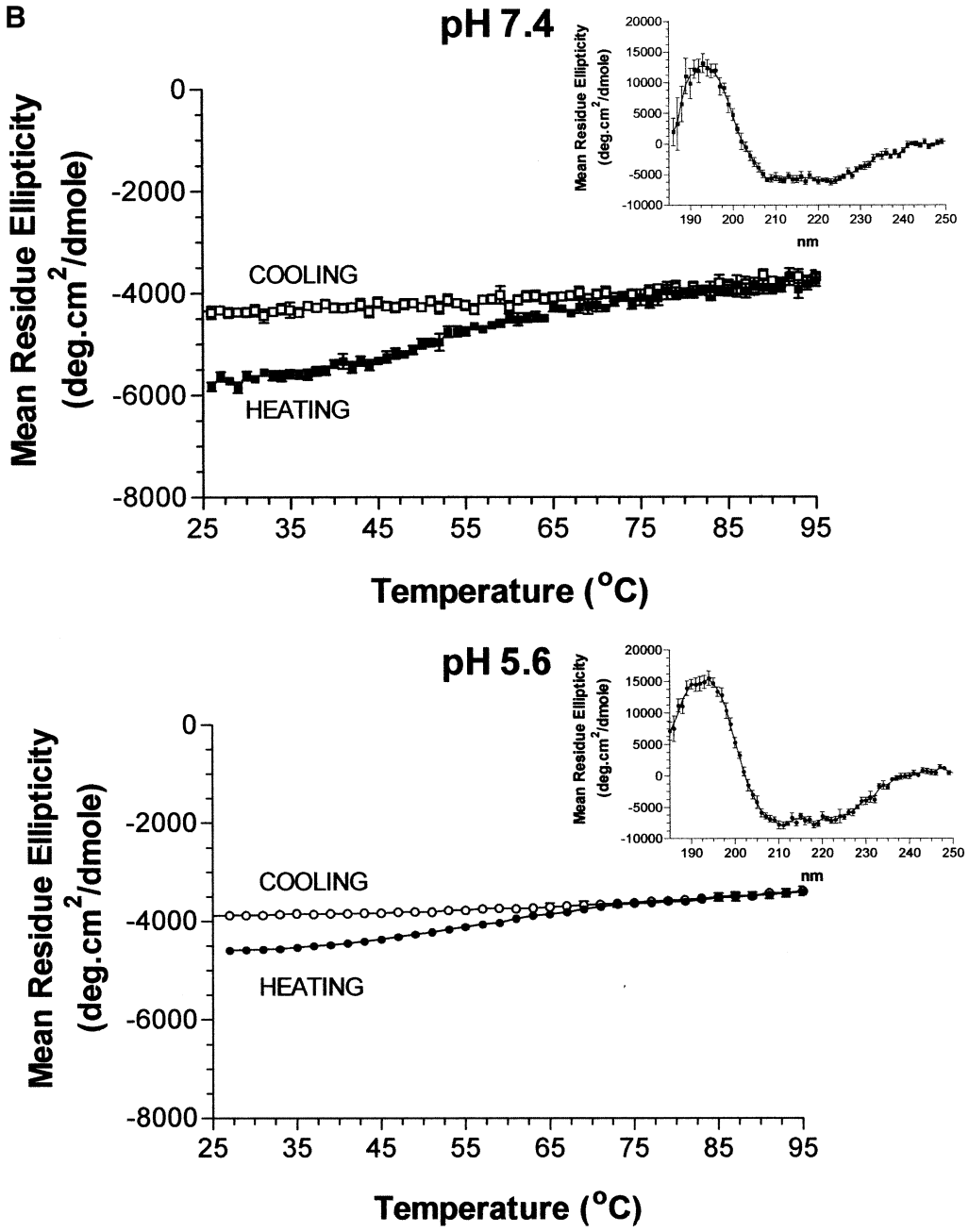

Fig. 1 (*continued*)

are shown in Fig. 1A. The melting profiles of G protein at pH 7.4 and 5.4 appear to be identical, and the increase in the temperature from 25 to 80°C resulted in a continuous decrease in Trp fluorescence (Fig. 1A). The melting profiles were similar to the temperature-dependent fluorescence decrease exhibited by *N*-acetyl-L-tryptophanamide (NATA) at the corresponding pH. No changes were observed in the emission maximum of 330 nm at both pH.

Perturbations in the secondary structure of G protein was also measured for both the nonfusogenic (pH 7.4) and the fusogenic (pH 5.4) conformations of G protein by determining the mean residue molar ellipticity at 222 nm. Results presented in Fig. 1B show that at both pH 7.4 and pH 5.4 G proteins show similar thermal stability with gradual changes in the ellipticity with increasing temperature. No changes in the molar ellipticity were, however, observed when samples of G protein heated to 95°C at acid or neutral pH were gradually cooled to 25°C. Thus, the conformational changes induced by heating G protein at neutral or acid pH were irreversible. The far-UV CD spectra were analyzed for secondary structures by a method based on neural network theory (Bohm et al., 1992). The results showed changes in secondary structure induced by lowering the pH to 5.6 at 25°C. Changes in secondary structures were also observed

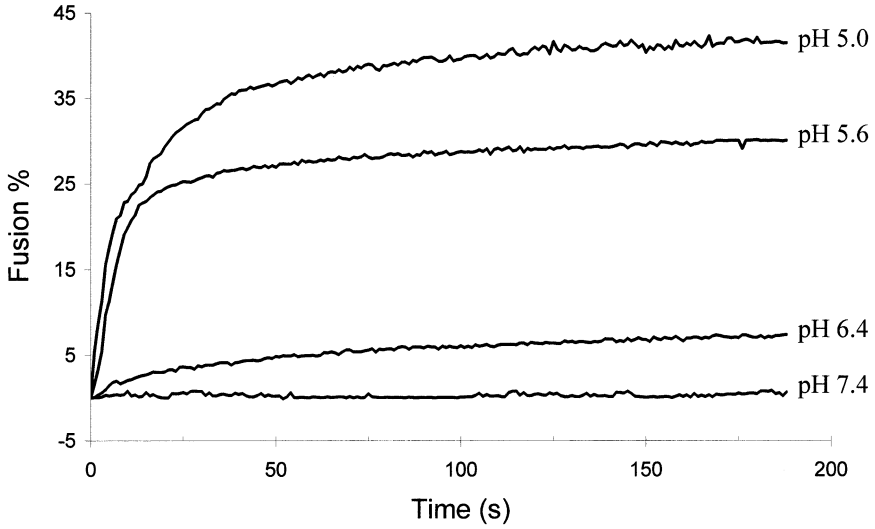

Fig. 2. pH dependence of VSV-mediated fusion of liposomes. SUVs (150 μM lipid) containing PC:PE:PS:cholesterol in molar ratios of 1:0.5:0.5:1 and 2 mol% of NBD-PE and RHO-PE were mixed with purified VSV (50μg protein) at 37°C. Acid was added at time zero to change the pH from 7.4 to 6.4, 5.6, or 5.0 and the fluorescence was monitored. After 4 min Triton X-100 was added to a final concentration of 0.1% to induce complete loss of FRET (100% fusion). The data are representative of three experiments.

after heating the G protein at either neutral or acid pH. The percentage of β-structure was significantly increased after heating to 95°C at pH 7.4 or 5.6. Taken together, the results of intrinsic Trp fluorescence and secondary structure analyses of G protein at neutral and acid pH indicate no significant difference in thermal stability.

Effect of heat or urea treatment on VSV-induced membrane fusion

Previously VSV was shown to induce fusion of liposomes containing the phospholipid PS (Yamada and Ohnishi, 1986; Pal et al., 1988; Puri et al., 1992; Carneiro et al., 2001). We used a fluorescent lipid-mixing assay that measures the change in resonance energy transfer in the liposomes as a result of virus-induced membrane fusion (Struck et al., 1981). This fluorescent lipid-mixing assay has been extensively used to monitor fusion of viruses with membranes (Carr et al., 1997; Gaudin et al., 1991, 1993). We used SUVs containing PC, PE, PS, and cholesterol in a molar ratio of 1:0.5:0.5:1 and the fluorescent lipids NBD-PE and RHO-PE. In agreement with previous reports, the fusion of liposomes by VSV required low pH, with a threshold of fusion at pH 6.4 (Fig. 2). Fusion of liposomes at pH 5.6 or 5.0 was very fast and was almost complete within 60 s. No fusion of liposomes at pH 7.4 was observed even after 30 min.

To investigate whether the denaturant urea could induce fusion of VSV with membranes at neutral pH, fusion of the liposomes at pH 7.4 in the absence or in the presence of urea was monitored. The presence of 3, 4, or 5 M urea did not trigger a significant level of fusion at pH 7.4 (Fig. 3). No further increase in the extent of fusion at pH 7.4 was observed when the experiment was continued for 30 min. Control experiments showed a similar small increase in fluorescence at pH 7.4 in the absence of any virus but in the presence of urea, indicating that the low level of fusion at pH 7.4 observed in the presence of urea could be due to nonspecific effects of urea (data not presented). The presence of urea, however, did not affect fusion at pH 5.0 except that the extent of fusion in the presence of urea was reduced, possibly due to general disruption of structure of G protein (Fig. 3). Treatment of VSV at pH 5.0 with 3 M urea for 1 h led to complete loss of fusion activity (data not shown).

To test triggering of fusion at the neutral pH by heat, liposomes were mixed with VSV and incubated at 37, 55, 58, or 60°C and the changes in the fluorescence were measured. As shown in Fig. 4; a very small increase in fusion was observed at pH 7.4. In contrast, significant fusion of liposomes at pH 5.0 was observed at 37, 55, 58, or 60°C (Fig. 4). The extent of fusion at elevated temperatures were reduced as compared to that at 37°C, possibly due to general disruption of the structure of the G protein. No further increase in the extent of fusion at pH 7.4 was observed when the experiment was continued for 30 min. As in the case of urea, control experiments in the absence of any VSV showed a similar small increase in fusion at pH 7.4 at 55, 58, or 60°C, suggesting that the low level of fusion observed after heat treatment may be due to a nonspecific increase in fluorescence (data not shown). Assays of virus infectivity showed that preincubation of VSV at 55°C for 10 min resulted into 99% loss in the plaque-forming activity. However, complete loss of liposome fusion activity of VSV at pH 5.0 was observed after preincubation at 55°C for 60 min (data not shown).

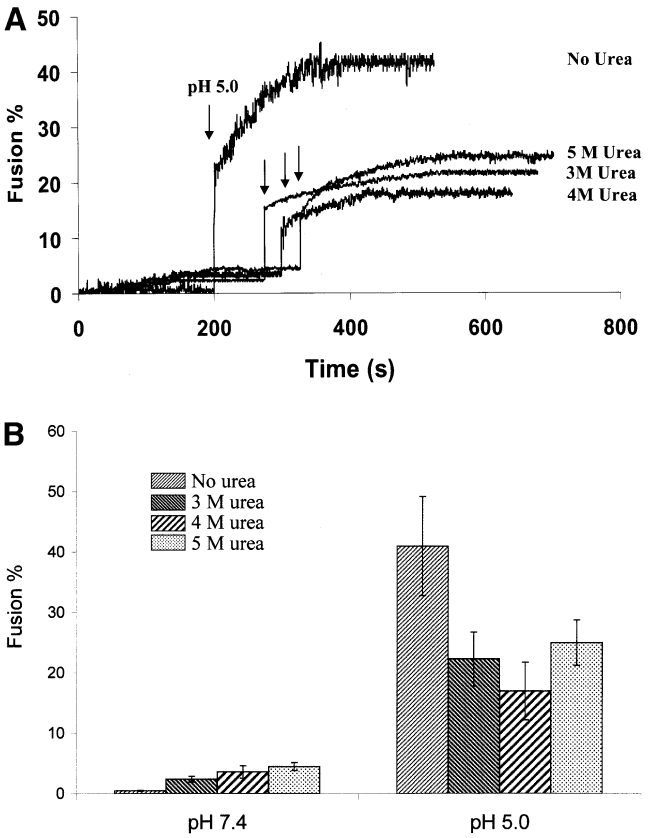

Fig. 3. Effect of urea on VSV-induced membrane fusion. Liposomes were added to buffer (pH 7.4) containing 3, 4, or 5 M urea at 37°C. VSV was then added and the fluorescence was recorded at pH 7.4. After 5 min complete loss of FRET was attained by adding Triton X-100. (A) Kinetics of liposome fusion in the presence of urea at pH 7.4. The pH of the liposome-virus mixture was lowered to pH 5.0 at the time points indicated on the curve by arrows and the fluorescence was monitored. (B) Virus-liposome mixtures at pH 7.5 or 5.0 were incubated at 37°C for 5 min in the presence of urea and the extent of fusion was calculated. Average data and standard deviation of three experiments are shown.

Effect of urea or heat on trypsin sensitivity of VSV G protein

A biochemical proteolysis assay has been used to monitor the low-pH-induced conformational changes in VSV G protein (Fredericksen and Whitt, 1996; Shokralla et al., 1998), HA of influenza virus (Carr et al., 1997; Skehel et al., 1982, 1995), G protein of the rabies virus (Gaudin et al., 1991, 1993), and E1 protein of SFV (Kielian and Helenius, 1985; Gibbons et al., 2000). In the presence of detergents VSV G protein was fully digested with trypsin at pH 7.4, but resistant to trypsin at acid pH (Fredericksen and Whitt, 1996; Shokralla et al., 1998, 1999). This altered proteolytic sensitivity was, therefore, used to monitor the change in conformation of G protein induced by urea or heat treatment at neutral pH. Samples of VSV were preincubated at 37°C at pH 7.4, 6.4, 5.6, or 5.0, digested with trypsin at the indicated pH at 37°C for various times, and analyzed by SDS-PAGE (Fig. 5). At neutral pH the G protein is fully digested by trypsin. In contrast, G protein is resistant to trypsin digestion at pH 6.4, 5.6, or 5.0. Addition of SDS to the samples at acidic pH showed complete digestion of G protein, indicating that the observed resistance of G protein to trypsin disgestion at acidic pH was not due to lack of activity of trypsin at low pH. It may be noted that both L and M proteins are cleaved by trypsin due to the presence of Triton in the assay mixture while the N protein present as a nucleocapsid complex is resistant to the proteolytic digestion.

Studies with the soluble ectodomain of G protein showed that exposure to acidic pH triggers a conformational change which is reversible (Crimmins et al., 1983). The activation of fusion by VSV and insertion of G protein into liposomes were also shown to be reversible (Puri et al., 1988; Blumenthal et al., 1987; Pak et al., 1997; Gaudin, 2000). To test if the conformational change monitored by protease sensitivity is also reversible, samples of VSV preincubated at pH 5.6 were neutralized to pH 7.5 and digested with trypsin. Results presented in Fig. 5C show that G protein becomes sensitive to digestion by trypsin upon neutralization of pH 7.5. Conversely, G protein preincubated at pH 7.5 upon acidification to pH 5.6 becomes fully resistant to tryptic digestion (Fig. 5D). These results show that conformational change of VSV G protein as monitored by trypsin digestion is also fully reversible. Thus, it should be noted that it is not possible to incubate VSV with trypsin at neutral pH after a pretreatment in acidic pH, because reneutralization would result into recovery of the native conformation.

Earlier we showed that denaturants, such as urea or heat, failed to induce fusion activity of G protein at neutral pH. The lack of fusion activity of G protein at neutral pH in the presence of destabilizing agents could be due to the fact that the acid-induced conformational change could not be induced at neutral pH in the presence of urea or by heat. The effect of urea or heat on the conformational change in G protein was, therefore, evaluated by using the protease sensitivity assay. Exposure of VSV, at neutral pH, to either urea (3 or 5 M) or heat (55 or 60°C) did not change the proteolytic sensitivity of G protein. In all of these cases, similar to the untreated sample at pH 7.4, G protein was completely digested (Figs. 6A and B). Control experiments show that at acid pH, in the presence of urea or after heating, G protein is still resistant to proteolysis (Figs. 6C and D). Our results indicated that the low-pH-induced conformational change of G protein could not be induced by urea or heat at neutral pH.

In an effort to show that the denaturants, urea or heat, could destabilize the native structure of G protein, we digested VSV at neutral pH at either 0 or 37°C. As reported earlier, native G protein was fully sensitive to trypsin at 37°C. In contrast, at 0°C native G protein was resistant to tryptic digestion. M protein present in the reaction mixture was, however, digested by trypsin at 0°C either in the

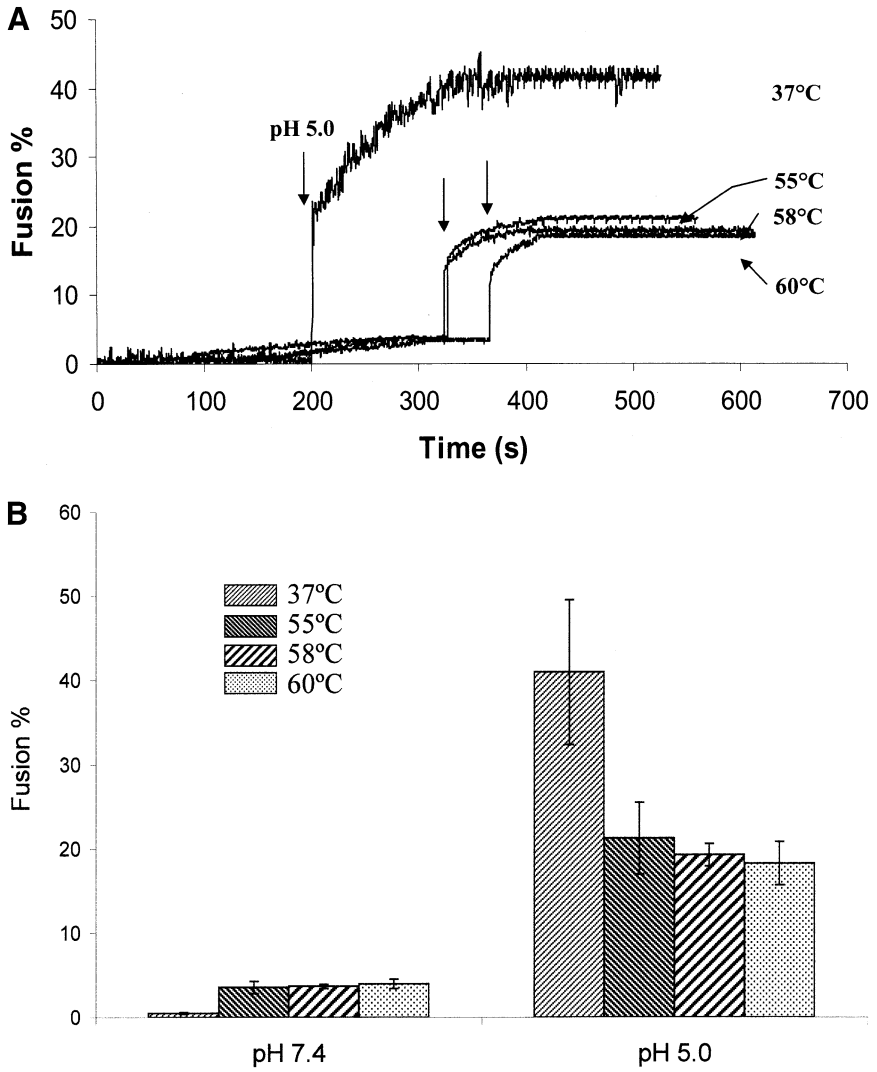

Fig. 4. Effect of heat treatment on VSV-induced membrane fusion. Liposomes were added to the buffer (pH 7.4) at 37, 55, 58, or 60°C and VSV was added. The fluorescence was recorded at the indicated temperature at pH 7.4. After 5 min Triton X-100 was added to induce complete loss of FRET. (A) Kinetics of liposome fusion as a function of increasing temperatures. The pH of the liposome-virus mixture was lowered to pH 5.0 at the time points indicated on the curve by arrows and the fluorescence was monitored. (B) Virus-liposome mixtures at pH 7.5 or 5.0 were incubated at the indicated temperature and the extents of fusion after 5 min were calculated. Average data and standard deviation of three experiments are shown.

absence or in the presence of SDS, showing that trypsin was active (Fig. 7). This difference in protease sensitivity was used to demonstrate conformational change of native G protein following urea or heat treatment. Pretreatment of G protein with 5 M urea or heating at 60°C for either 15 or 60 min followed by digestion with trypsin at 0 or 37°C showed that the G protein exposed to 5 M urea or heat at neutral pH was completely digested by trypsin at 0°C, indicating that the denaturants do destabilize the native structure of the G protein (Fig. 7). Similar destabilization of the native spike proteins of SFV by treatment with urea or heat at neutral pH was also observed (Gibbons, et al., 2000). As in the case of G protein, the untreated spike proteins of SFV were resistant to trypsin digestion at 0°C but the urea- and heat-treated spike proteins were very sensitive to trypsin at 0°C.

Discussion

The results presented in this report show that the mechanism of VSV G protein-mediated acid-pH-induced membrane fusion is different from that used by two well-studied classes of viral glycoproteins, class I viral glycoproteins exemplified by the fusion proteins of influenza, HIV, SIV, MLV, Ebola, and SV5 viruses (Skehel and Wiley, 1998; Weissenhorn et al., 1999), and the class II fusion proteins of SFV and TBE virus (Gibbons et al., 2000; Lescar et al., 2001; Stiasny et al., 2001). Class I fusion proteins use α-helical coiled-coil motif-based structures for fusion and contain hydrophobic fusion peptides at or near the N-terminus. Most of the class I fusion proteins are generated by proteolytic cleavage of a precursor. Class II fusion proteins

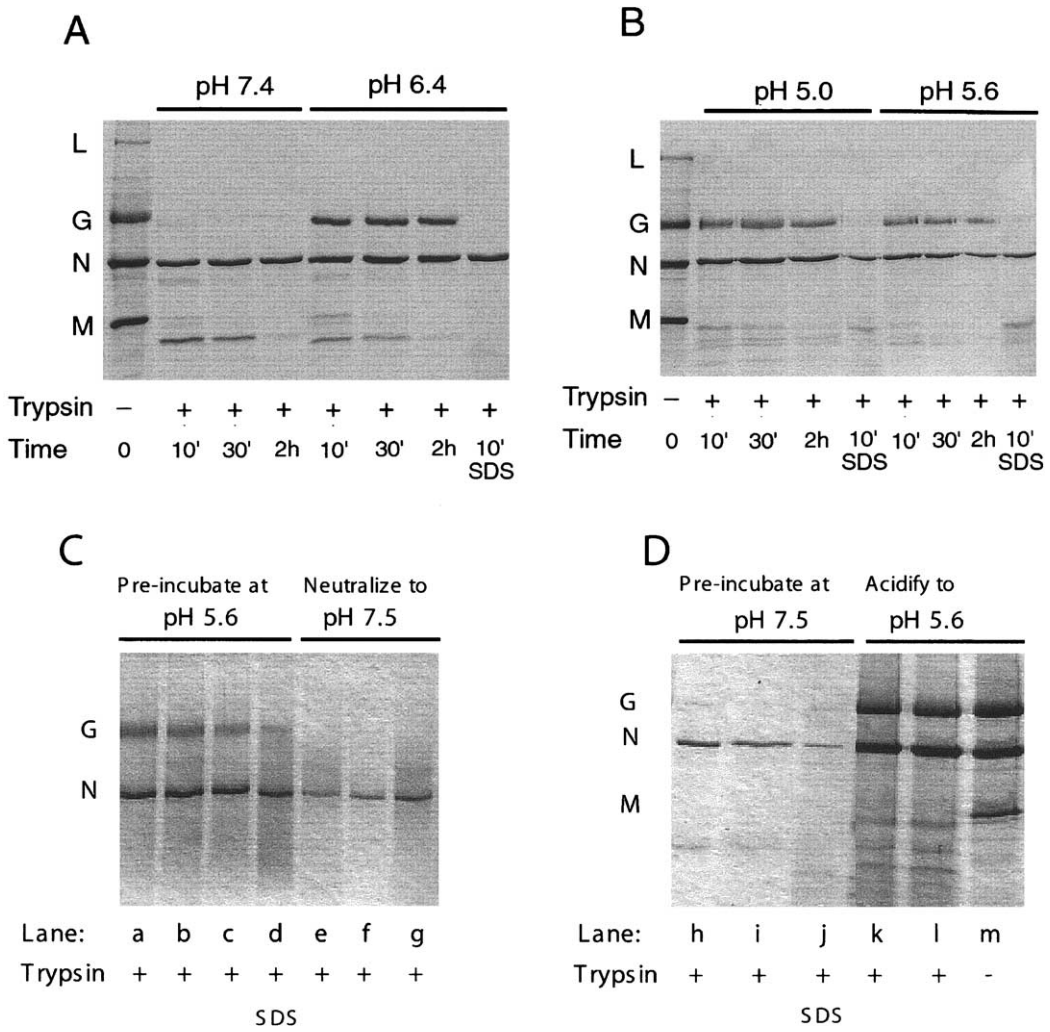

Fig. 5. Low-pH-induced conformational change of VSV G protein. Samples of VSV were preincubated at 37°C for 10 min in buffers containing 20 mM Mes, 30 mM Tris, 200 mM NaCl, and 1% Triton X-100 at the indicated pH (A and B). VSV was then digested with trypsin at a ratio of VSV: trypsin (25:1 wt/wt) for 0, 10, 30, and 120 min at 37°C. Reaction was stopped with aprotinin and samples were analyzed on a 10% SDS-polyacrylamide gel. Protein was visualized by Coomassie blue staining. The gels shown are representative of three experiments. Reversibility of the acid-pH-induced conformational change was monitored by sensitivity to trypsin digestion of G protein. (C) G protein was preincubated at pH 5.6 at 0°C for 10 (lanes a and d), 30 (lane b), or 120 (lane c) min. Aliquots of the preincubated samples were then adjusted to pH 7.5 and incubated at 0°C for 10(lane e), 30(lane f), or 120 (lane g) min. Samples were digested with trypsin at 37°C for 30 min. (D) G protein was preincubated at 0°C at pH 7.5 for 30(lanes h and j) or 120(lane i) min. Aliquots of the samples were then adjusted to pH 5.6 and incubated at 0°C for 30(lane k) or 120 min(lane l). Samples were digested with trypsin at 37°C for 30 min. Samples present in lanes d and j were digested in the presence of SDS. Sample in lane m was not treated with trypsin.

are not proteolytically cleaved and do not contain the predictable coiled-coil motif, and the fusion peptide is internal. In both of the cases, the conformational change induced during activation from nonfusogenic to the fusogenic form is irreversible (Carr et al., 1997; Lescar et al., 2001; Stiasny et al., 2001). Rhabdoviral fusion protein G is not proteolytically cleaved from a precursor, contains an internal nonhydrohobic fusion peptide, and lacks a predictable coiled-coil motif, and the conformational change triggered by low pH during fusion activation is fully reversible, suggesting that viruses belonging to this family may use a fusion mechanism functionally distinct (Class III) from the other two types (Gaudin, 2000).

Trp fluorescence-melting profiles of the G protein at neutral and acidic pH indicated that the nonfusogenic and fusogenic forms of the G protein do not differ in their thermal stabilities. In contrast, marked change in the Trp fluorescence-melting profile of influenza BHA or HA at neutral and acidic pH was reported (Ruigrok et al., 1986; Remeta et al., 2002). In the case of the E protein of TBEV, marked difference in the thermal profile of Trp fluorescence between the native and the low-pH form of the E protein was observed, suggesting that acidic pH induces a change to more thermally stable form (Stiasny et al., 2001). Thermal denaturation of the G protein at neutral and acidic pH, as monitored by CD, also showed small changes in the molar ellipticity. However, the thermal denaturation was irreversible at either pH. In the case of BHA a marked difference in

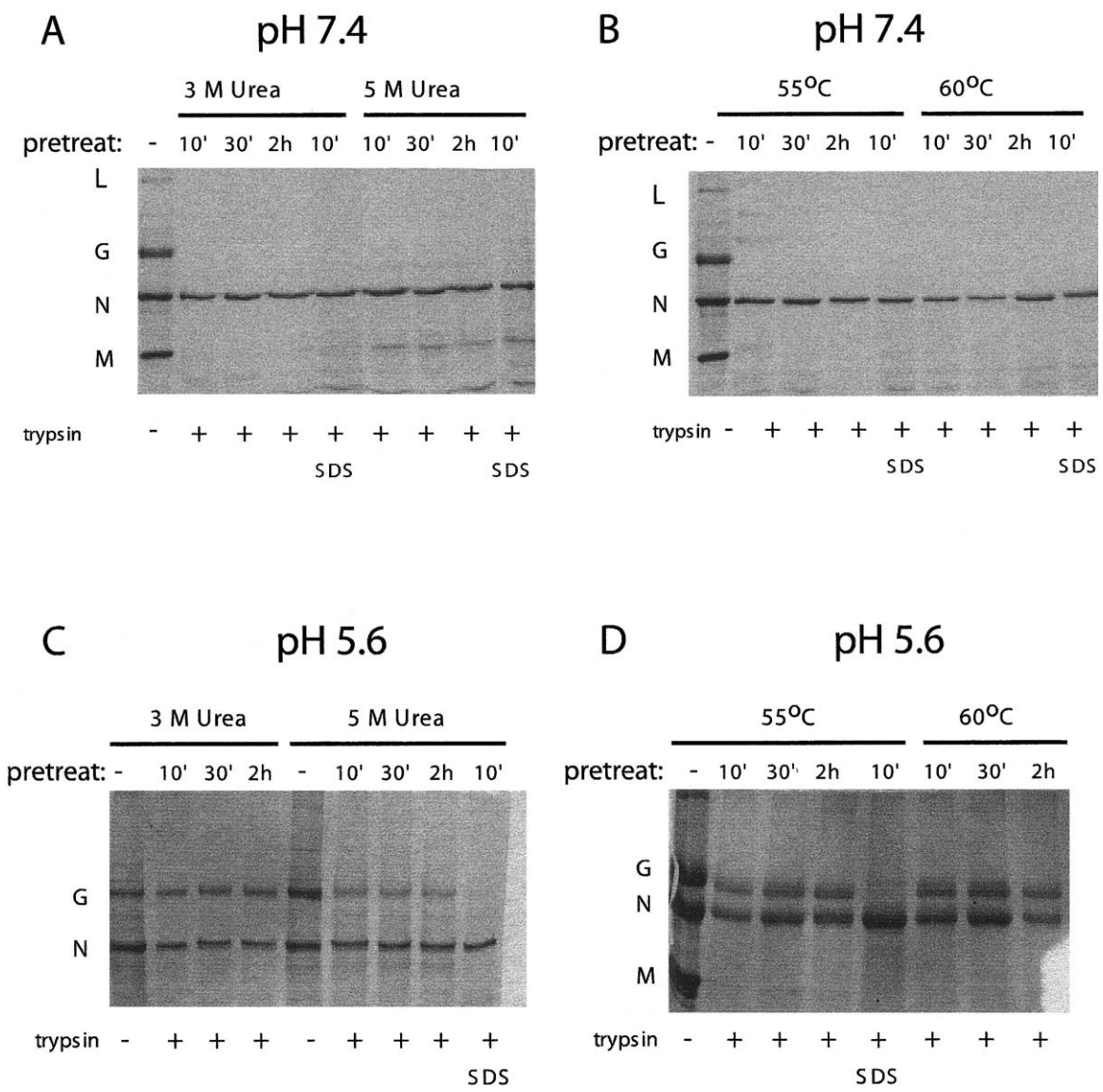

Fig. 6. Effects of denaturants on the conformational change of VSV G protein. (A) Conformational change following urea treatment was monitored by incubating VSV at pH 7.4 in the presence of 3 or 5 M urea at 37°C for 10, 30, or 120 min. The samples were then digested with trypsin for 30 min at 37°C. (B) Conformational change following heat treatment was determined by incubating samples at 55 or 60°C for 10, 30, or 120 min at pH 7.4. The samples were then digested with trypsin for 30 min at 37°C. Control experiments to monitor trypsin sensitivity of G protein at pH 5.6, in the presence of 3 or 5 M urea (C) or heating at 55°C or 60°C (D), are also shown. The gels are representative of three experiments.

the thermal denaturation profile monitored by CD at neutral and acidic pH was observed (Ruigrok et al., 1986). At neutral pH CD intensities decreased as a single transition at a denaturation temperature of 63°C. At pH 5, however, only small reversible changes in ellipticity were observed at temperatures above 80°C. Biochemical studies based on protease sensitivity of the G protein at neutral and acidic conditions showed that the native protein is less stable to protease digestion than the low-pH form. SFV fusion proteins also showed increase stability to protease digestion in the low-pH-induced state (Kielian and Helenius, 1985). In contrast, the native forms of influenza virus HA (Carr et al., 1997; Skehel et al., 1982) and rabies virus G protein (Gaudin et al., 1991, 1993) were more stable to protease digestion than the fusogenic form. The observed differential sensitivities of the native and the fusogenic forms of viral fusion proteins may, therefore, not be due to metastability of the native form but due to conformational changes affecting the accessibility of the protease.

The fact that denaturants such as urea or heat failed to activate fusion at neutral pH further suggests that the G protein may not be in a conformation at neutral pH that can be converted into a fusion active state by denaturants. In contrast, HA-induced membrane fusion at neutral pH could be triggered by urea or high-temperature treatments. The inability of denaturants to induce fusion of membranes by VSV G protein is also in accord with the fact that activation of rhabdorvirual fusion proteins by acid pH is reversible.

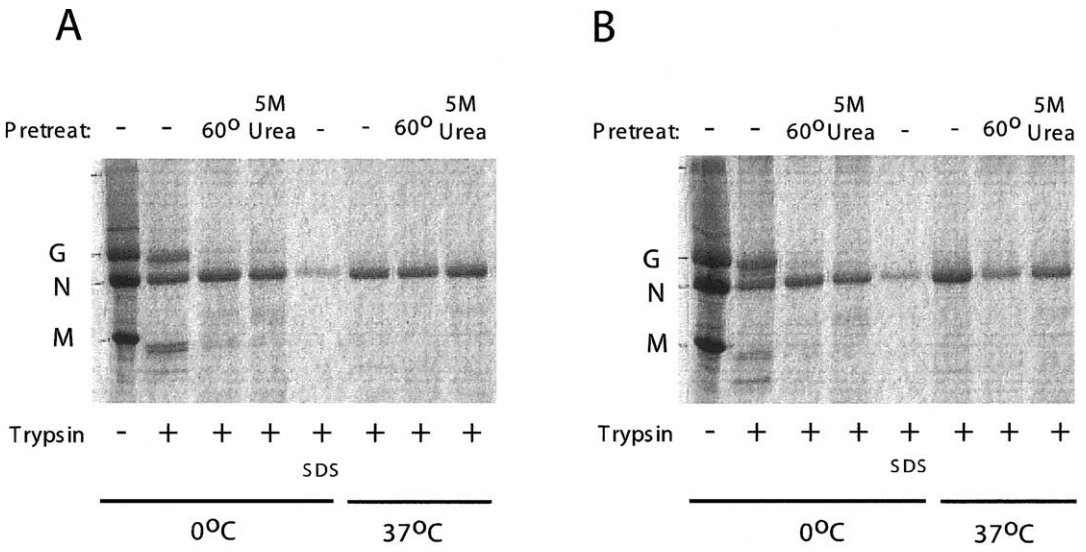

Fig. 7. Destabilization of G protein structure by urea or heat as monitored by protease sensitivity at 0°C. G protein at pH 7.4 was pretreated at 0°C in the presence of 5 M urea or at 60°C for either 15 min (A) or 60 min (B). Samples of untreated and treated G protein at pH 7.4 were then digested with trypsin for 60 min at 0 or 37°C. The gels shown are representative of four separate experiments.

Results presented here further show that conformational changes induced by acidic pH as monitored by protease sensitivity are also reversible. Similar reversibility of the sensitivity to protease digestion of rabies virus G protein was also reported by Gaudin et al. (1991). In fact, not only the conformational changes but also the insertion of VSV G protein into the target membrane was shown to be reversible (Pak et al., 1997). In the case of the fusion protein E1 of SFV, the presence of urea or heat similarly failed to induce fusion at neutral pH (Gibbons et al., 2001). The fusion protein E of TBEV also could not be converted to the fusogenic form at neutral pH by heating (Stiasny et al., 2001). Thus, the acid-induced change(s) in conformation of the fusion proteins of VSV, SFV, or TBE viruses cannot be triggered at neutral pH by destabilization with denaturants or with heat. These observations, along with the reversibility of the pH effects, indicate that there is no kinetically trapped high-energy state of the VSV G protein at neutral pH.

Our results further show that destabilizing agents such as urea or heat failed to induce conformational change, as detected by trypsin sensitivity, of VSV G at 37°C. However, urea or heat affected the structural integrity of native G protein as shown by the altered trypsin sensitivity at 0°C. In the case of SFV E1, denaturants such as heat or urea also could not induce any homotrimer formation or membrane fusion by the native fusion protein. However, heat or urea induced major structural changes in the native structure of E1 protein as measured by increased trypsin sensitivity at 0°C. Also pretreatment of SFV or soluble E1 protein with urea failed to trigger E1 homotrimer formation at acid pH but E1 homotrimers formed in acid pH were stable to urea or heat (Gibbons et al., 2000). The fusion protein E of TBE virus is irreversibly converted by acidic pH to a homotrimeric form which is more thermostable than the native dimer. The transition from the native to the fusogenic state was triggered only by low pH. Similar to SFV E1, heat treatment of the native E protein of TBE virus also failed to induce fusion activity or homotrimer formation (Stiasny et al., 2001). Structural changes induced by low pH in these class II fusion proteins could not be substituted by simply destabilizing the protein structure with denaturants as observed in the case of influenza HA (Carr et al., 1997). Thus, the structural change(s) required for fusion activation by low pH of VSV G, SFV E1, and TBEV E fusion proteins differs from that used by the class I fusion proteins. Such a conclusion is in agreement with the finding that none of these three fusion proteins contain sequences predicted to form an α-helical coiled-coil structure used by class I fusion proteins (Gibbons et al., 2000; Stiasny et al., 2001). X-ray structure analyses further showed that the structural arrangements of the class II fusion proteins, TBE virus E (Rey et al., 1995) and SFV E1 (Lescar et al., 2001), differed strikingly from those of the class I fusion protein, influenza HA (Wilson et al., 1981; Bullough et al., 1994).

Most viral fusion proteins studied form trimers; however, in the case of VSV the trimer structure is more stable at low pH. At neutral pH the trimer is in a dynamic equilibrium with the monomer, both in vitro and in vivo, and increased acidity stabilizes the trimeric form (Doms et al., 1987; Lyles et al., 1990; Zagouras and Rose, 1993). Trimerization was also shown to be reversible and the pH dependence of the oligomerization of G protein was correlated to the reversible conformational change triggered by low-pH activation of fusion (Doms et al., 1987). The reversibility of the trypsin sensitivity of VSV G protein reported here demonstrate that conformational change induced by low pH is also reversible. It may be noted that heating to 95°C at neutral or acid

pH resulted into irreversible structural changes of VSV G protein. Results of analyses of the far-UV CD spectra showed that changes in secondary structure of VSV G protein induced by low pH differed from the secondary structure changes observed due to heating at neutral or acid pH. Thus exposure to acid pH shows a decrease in the β-structure content of VSV G protein while heating resulted into a marked increase in β-structure.

A three-state model for VSV G protein based on kinetics of fusion and photosensitized labeling (Clague et al., 1990; Puri et al., 1988; 1992; Pak et al., 1997) was formulated to relate the different stages of conformational changes occurring during viral fusion. Studies with rabies virus using a number of biochemical, biophysical, immunological, and electron microscopic data (Gaudin, 2000; Gaudin et al., 1991; 1993; 1999) also suggested the involvement of three different states of RV G protein during low-pH-induced fusion process. At neutral pH G protein is in a "native" ("tense") state. At slightly acidic pH the G protein in the "native" state is transformed to an "activated" ("relaxed") state, which is fusion competent. A second step of protonation is necessary for fusion to occur. It is possible that a conformational change, not yet identified, occurs when the fusion-competent G protein interacts with the target membrane via the fusion peptide. Finally, the "fusion" state is reached and fusion between the two membranes proceeds. Prolonged exposure to acid pH, however, induces conformational changes in the G protein leading to an "inactive" ("desensitized") state. The fact that the conformational change in the G protein is reversible also suggests that the different states are in a pH-dependent equilibrium. Formation of a fusion complex containing multiple trimers of the fusion protein is generally believed to be an intermediate step in membrane fusion. In the case of VSV G, it was shown by using radiation-inactivation that about 5 trimers of G are required to form a fusion complex (Bundo-Morita et al., 1988). Recent studies with rabies virus glycoprotein G showed the existence of a pH-dependent thermodynamic equilibrium between the different states of G and the requirement of a large number of trimers of G for stable interaction with the target membrane (Roche and Gaudin, 2002). The reversibility of the conformational change involved in rhabdovirus fusion further suggests that the energy barrier between the native and the fusogenic glycoprotein may be smaller compared to the class I and II fusion protein. A large fusion complex of G protein could overcome the small energy barrier existing between the nonfusogenic and the fusogenic forms. Thus, the fusion of membranes by the rhabdorivus fusion protein may be driven by the conformational change(s) induced by acidic pH by a mechanism distinct from that used by class I or class II fusion proteins. Elucidation of the 3-D structure of a rhabdovirus fusion glycoprotein will facilitate our understanding of mechanism of membrane fusion by class III fusion proteins.

Materials and methods

Virus, cells, and antibodies

BHK-21 cells were cultured at 37°C in Delbucco's modified Eagle medium containing 5% fetal calf serum, 100 U penicillin/ml, 100 μg streptomycin/ml. The VSV used in these experiments was a well-characterized, plaque-purified isolate of Indiana serotype (Kotwal et al., 1983). Virus grown in BHK-21 cells was purified through two consecutive discontinuous sucrose gradients (Kotwal et al., 1983). A polyclonal rabbit anti-G antibody raised in our laboratory (Ghosh and Ghosh, 1999) was used for detection of G protein by immunoblot analysis. Gel electrophoresis of the G protein was performed as described previously (Li et al., 1993).

Liposomes

N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (RHO-PE), *N*-(7-nitro-2,1,3-benzoxa-diazol-4-yl)phosphatidylethanolamine (NBD-PE), phosphatidylcholine (PC; bovine brain), phosphatidylethanolamine (PE; bovine brain), and phosphatidylserine (PS; bovine brain) were purchased from Avanti Polar Lipids, Inc. Cholesterol was supplied by Sigma-Aldrich. The lipids, PC, PE, PS and cholesterol, dissolved in chloroform, were mixed at a molar ratio of 1:0.5:0.5:1, respectively, and dried under N_2 to form a film. The resulting lipid films were further dried under vacuo. The lipid film was suspended in 1 ml of buffer A (10 mM Hepes, pH 7.4, 145 mM NaCl), vortexed, frozen in liquid N_2, thawed under tap water, and vortexed again. The freeze-thaw-vortex steps were repeated 3 times. The mixture was sonicated in a bath-sonicator in ice water until a transparent solution containing small unilamellar vesicles (SUV) was obtained. Liposomes were stored on ice and used the same day. The labeled liposomes contained in addition 1 mol% each of RHO-PE and NBD-PE.

Lipid-mixing assay for membrane fusion

The resonance energy transfer assay of Struck et al. (1981) was used to monitor membrane fusion. Fluorescence was recorded at excitation and emission wavelengths of 465 and 530 nm, respectively, using a 490-nm cutoff filter located between the cuvette and the emission monochromator, on either a SLM Aminco Bowman AB-2 spectrofluorimeter or a Perkin-Elmer LS fluorescence spectrophotometer. Each assay mixture contained 2 ml of buffer A containing liposomes and virus at the indicated pH in a siliconized glass cuvette with continuous stirring in a thermostated cuvette holder. The initial fluorescence intensity, F_0, was taken as zero. The maximum fluorescence, F_{max}, was obtained by dilution of the labeled lipids in the presence of 0.1% Triton

X-100. Percentage lipid mixing at time t is given by $[(F_t - F_0)/(F_{max} - F_0)]100$. In a typical assay SUV at a lipid concentration of 150 μM was added to 2 ml of buffer A in the cuvette at 37°C and then virus (50 μg protein) was added. After recording the fluorescence at pH 7.4, the pH in the cuvette was lowered by addition of predetermined amounts of 0.1 M citric acid. After recording the fluorescence for several minutes, 10% Triton X-100 was added to a final concentration of 0.1% to induce complete loss of fluorescence resonance energy transfer (FRET). Fusion of lipsomes by VSV at neutral pH under destabilization conditions in the presence of urea or heat was measured as follows: liposomes were added to buffer A (pH 7.4) containing 3, 4, or 5 M urea and the mixture was incubated at 37°C. VSV (50 μg) was added and the fluorescence was recorded at pH 7.4. After several minutes the pH of the sample was lowered to 5.0 by addition of 0.1 M citric acid. After recording the fluorescence for several minutes Triton X-100 was added to a final concentration of 0.1%. Virus preincubated at 3.75 M urea for 1 h at 37°C before addition to the vesicles showed no fusion activity at either pH 7.4 or pH 5.0. For heat-induced fusion experiments, liposomes in buffer A were incubated at 37, 55, 58, or 60°C. Virus (50 μg) was added and fluorescence was recorded at the indicated temperature at pH 7.4. After several minutes the pH of the sample was lowered to 5.0 by addition of 0.1 M citric acid. After recording the fluorescence for several minutes Triton X-100 was added to 0.1% to obtain complete loss of FRET.

Proteolysis assay for the conformation change

Conformational change at low pH of VSV G was monitored by measuring the proteolytic resistance of the glycoprotein at the pH of fusion as described earlier (Odell et al., 1997). In the presence of detergent VSV G protein becomes increasingly resistant to digestion with trypsin as the pH is lowered from 7.4. VSV samples were preincubated at 37°C for 10 min in buffers containing 20 mM Mes, 30 mM Tris, 200 mM NaCl, and 1% Triton X100 at pH 7.4–5.0 and then digested with trypsin (TPCK treated, Worthington) at a ratio of VSV:trypsin of 25:1 for 0, 20, 30, or 120 min at 37°C. The reaction was stopped by adding 10 U of aprotinin and then heated with sample buffer at 100°C for 3 min. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized by either Coomassie brilliant blue staining or by immunoblotting using a polyclonal anti-G antiserum. For measuring protease resistance in the presence of urea, the digestion mixture was adjusted with neutralized urea solution to a final concentration of 3 or 5 M and incubated at 37°C for 10, 30, or 120 min at pH 7.4. Heat-induced conformational change was determined by incubating samples at 55 or 60°C for 10, 30, or 120 min at pH 7.4. The preincubated samples were then digested with trypsin for 30 min at 37°C.

Computational analyses of G protein sequence for coiled-coil prediction

VSV Indiana G protein sequence (Rose and Gallione, 1981) was analyzed for probability of forming α-helical coiled-coil structures using four different programs. Coils (Lupas et al., 1991; Lupas, 1996; www.ch.embnet.org/software/COILS_form.html), Paircoil (Berger et al., 1995; nightingale.lcs.mit.edu/cgi-bin/score), Multicoil (Wolf et al. 1997; nightingale.lcs.mit.edu/cgi-bin/multicoil), and Learn-Coil-VMF (Singh et al. 1999; web.wi.mit.edu/kim) programs were used by submitting sequences to a World Wide Web server hosting the programs. Different cutoff values were used in searching for coiled-coil regions.

Fluorescence spectroscopy

Isothermal and temperature-dependent fluorescence spectra were acquired on a SLM Aminco Bowman AB-2 spectrofluorimeter equipped with a programmable thermoelectrically controlled bath with circulating fluid pumped into a jacketed sample cell compartment. Fluorescence spectra were recorded at 300–500 nm employing an excitation wavelength of 285 nm in quartz cells. Temperature-dependent changes in intrinsic Trp fluorescence as a function of pH were measured by heating the samples at a programmed rate of 1°C per min over the range of 25–80°C. The fluorescence emission intensity at 340 nm was recorded at 0.1°C intervals.

Circular dichroism spectroscopy

The CD spectra were recorded using a AVIV Model 215 instrument equipped with a programmable thermoelectrically controlled sample compartment. The thermal stability of the G protein secondary structure was measured by heating samples at a programmed rate of 0.5°C/min. The mean residue ellipticity at 222 nm was recorded at 0.1°C intervals, in quartz cells. Estimation of secondary structure from far-UV CD spectra was done using a method based on neutral network theory (Bohm et al., 1992).

Acknowledgments

We thank Amarpreet Cheema for computational help, V.S. Ananthanarayanan for allowing us to use his spectrofluorimeter, Y. Gaudin for comments, D.A. Andrews for critical reading of the manuscript, S. Culp-Stewart for assistance and M.M. Strong for manuscript preparation. This work was supported by the Canadian Institute of Health Research.

References

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D., 1994. The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology 202, 586–605.

Baker, K.A., Dutch, R.E., Lamb, R.A., Jardetzky, T.S., 1999. Structural basis for paramyxovirus-mediated membrane fusion. Mol. Cell 3, 309–319.

Berger, B., Wilson, D., Wolf, E., Toucher, T., Milla, M., Kim, P.S., 1995. Predicting coiled-coil by use of pairwise residue correlations. Proc. Natl. Acad. Sci. USA 92, 8259–8263.

Blacklow, S.C., Lu, M., Kim, P.S., 1995. A trimeric subdomain of the simian immunodeficiency virus envelope glycoprotein. Biochemistry 34, 14955–14962.

Blumenthal, R., Bali-Puri, A., Walter, A., Covell, D., Eidelman, O., 1987. pH-dependent fusion of vesicular stomatitis virus with Vero cells. Measurement by dequenching of octadecyl rhodamine fluorescence. J. Biol. Chem. 262, 13614–13619.

Bohm, G., Muhr, R., Jaenicke, R., 1992. Quantitative analysis of protein far UV circular dichroism spectra by neutral networks. Protein Eng. 1, 191–195.

Bullough, P.A., Hughson, F. M., Skehel, J.J., Wiley, D.C., 1994. Structure of influenza hemagglutinin at the pH of membrane fusion. Nature 371, 37–43.

Bundo-Morita, K., Gibson, S., Lenard, J., 1988. Radiation inactivation analysis of fusion and hemolysis by vesicular stomatitis virus. Virology 163, 622–624.

Carneiro, F.A., Ferradosa, A.S., Poian, A.T.D., 2001. Low pH-induced conformational changes in vesicular stomatitis virus glycoprotein involve dramatic structure reorganization. J. Biol. Chem. 276, 62–67.

Carr, C.M., Chaudhry, C., Kim, P.S., 1997. Influenza hemagglutinin is spring-loaded by a metastable native configuration. Proc. Natl. Acad. Sci. USA 94, 14306–14313.

Carr, C.M., Kim, P.S., 1993. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell 73, 823–832.

Chambers, P., Pringle, C.R., Easton, A.J., 1990. Heptad repeat sequences are located adjacent to hydrophobic regions in several types of virus fusion glycoproteins. J. Gen. Virol. 71, 3075–3080.

Chen, J., Lee, K.H., Steinhauer, D.A., Stevens, D.J., Skehel, J.J., Wiley, D.C., 1998. Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of labile conformation. Cell 95, 409–417.

Clague, M.J., Schoch, C., Zech, L., Blumenthal, R., 1990. Gating kinetics of pH-activated membrane fusion of vesicular stomatitis virus with cells: stopped flow measurements by dequenching of octadecylrhodamine fluorescence. Biochemistry 29, 1303–1308.

Coll, J.M., 1995a. The glycoprotein G of rhabdoviruses. Arch. Virol. 140, 827–851.

Coll, J.M., 1995ab. Heptad repeat sequences in the glycoprotein of rhabdoviruses. Virus Genes 10, 107–114.

Concannon, P., Cummings, J.W., Salser, W.A., 1984. Nucleotide sequence of the influenza virus A/USSR/90/77 hemagglutinin gene. J. Virol. 49, 276–278.

Crimmins, D., Mehard, W., Schlesinger, S., 1983. Physical properties of a soluble form of the glycoprotein of vesicular stomatitis virus at neutral and acidic pH. Biochemistry 22, 5790–5796.

Doms, R.W., Keller, D.S., Helenius, A., Balch, W.E., 1987. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. J. Cell Biol. 105, 1957–1969.

Douglas, N.W., Munro, G.H., Daniels, R.S., 1997. HIV/SIV glycoproteins: structure-function relationships. J. Mol. Biol. 273, 122–149.

Durrer, P., Gaudin, Y., Ruigrok, R.W., Graf, R., Brunner, J., 1995. Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. J. Biol. Chem. 270, 17575–17581.

Eckert, D.M., Kim, P.S., 2001. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. 70, 777–810.

Fass, D., Harrison, S.C., Kim, P.S., 1996. Retrovirus envelope domain at 1.7Å resolution. Nature Struct. Biol. 3, 465–469.

Fredericksen, B.L., Whitt, M.A., 1995. Vesicular stomatitis virus glycoprotein mutations that affect membrane fusion activity and abolish virus infectivity. J. Virol. 69, 1435–1443.

Fredericksen, B.L., Whitt, M.A., 1996. Mutations at two conserved acidic amino acids in the glycoprotein of vesicular stomatitis virus affect pH-dependent conformational changes and reduce the pH threshold for membrane fusion. Virology 217, 49–57.

Florkiewicz, R.Z., Rose, J.K., 1984. A cell line expressing vesicular stomatitis virus glycoprotein fuses at low pH. Science 225, 721–723.

Gaudin, Y., 2000. Reversibility in fusion protein conformational changes: the intriguing case of rhabdovirus-induced membrane fusion. Subcell. Biochem. 34, 379–408.

Gaudin, Y., Tuffereau, C., Durrer, P., Brunner, J., Flamand, A., Ruigrok, R., 1999. Rabies virus-induced membrane fusion. Mol. Mem. Biol. 16, 21–31.

Gaudin, Y., Tuffereau, C., Segratain, D., Knossow, M., Flamand, A., 1991. Reversible conformational changes and fusion activity of rabies virus glycoprotein. J. Virol. 65, 4853–4859.

Gaudin, Y., Ruigrok, R., Knossow, M., Flamand, A., 1993. Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. J. Virol. 69, 1435–1443.

Gaudin, Y., Ruigrok, R.W.H., Brunner, J., 1995. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. J. Gen. Virol. 76, 1541–1556.

Gaudin, Y., Raux, H., Flamand, A., Ruigrok, R.W.H., 1996. Identification of amino acids controlling the low-pH-induced conformational change of rabies virus glycoprotein. J. Virol. 70, 7371–7378.

Ghosh, K., Ghosh, H.P., 1999. Role of the membrane anchoring and cytoplasmic domains in intracellular transport and localization of viral glycoproteins. Biochem. Cell Biol. 77, 165–178.

Gibbons, D.I., Ahn, A., Chatterjee, P.K., Kielian, M., 2000. Formation and characterization of the trimeric form of the fusion protein of Semliki forest virus. J. Virol. 74, 7772–7780.

Heinz, F.X., Allison, S.L., 2001. The machinary for flavivirus fusion with host cell membranes. Curr. Opin. Microbiol. 4, 450–455.

Hernandez, L.D., Hoffman, L.R., Wolfsberg, T.G., White, J.M., 1996. Virus-cell and cell-cell fusion. Annu. Rev. Cell Dev. Biol. 12, 627–661.

Hughson, F.M., 1995. Molecular mechanisms of protein-mediated membrane fusion. Curr. Opin. Struct. Biol. 5, 507–513.

Hughson, F.M., 1997. Enveloped viruses: a common mode of fusion? Curr. Biol. 7, R565–R569.

Kielian, M.C., Helenius, A., 1985. pH induced alterations in the fusogenic spike protein of Semliki Forest virus. J. Cell Biol. 101, 2284–2291.

Kotwal, G.J., Capone, J., Irving, R.A., Rhee, S.H., Bilan, P., Toneguzzo, F., Hoffman, T., Ghosh, H.P., 1983. Viral membrane glycoproteins: comparison of amino terminal amino acid sequences of the precursor and mature glycoproteins of three serotypes of vesicular stomatitis virus. Virology 129, 1–11.

Kreis, T.E., Lodish, H.F., 1986. Oligomerization is essential for transport of vesicular stomatitis virus glycoprotein to the cell surface. Cell 46, 929–937.

Lescar, J., Roussel, A., Wien, M.W., Navaza, J., Fuller, S.D., Wengler, G., Wengler, G., Rey, F.A., 2001. The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell 105, 137–148.

Li, Y., Drone, C., Sat, E., Ghosh, H.P., 1993. Mutational analysis of the vesicular stomatitis virus glycoprotein G for membrane fusion domains. J. Virol. 67, 4070–4077.

Lu, M., Blacklow, S.C., Kim, P.S., 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nature Struct. Biol. 2, 1075–1082.

Lupas, A., 1996. Prediction and analysis of coiled-coil structures. Methods Enzymol. 266, 513–525.

Lupas, A., van Dyke, M., Stock, J., 1991. Predicting coiled-coils from protein sequences. Science 252, 1162–1164.

Lyles, D.S., Varela, V.A., Parce, J.W., 1990. Dynamic nature of the quaternary structure of the vesicular stomatitis virus envelope glycoprotein. Biochemistry 29, 2442–2449.

Odell, D., Wanas, E., Yan, J., Ghosh, H.P., 1997. Influence of membrane anchoring and cytoplasmic domains on the fusogenic activity of vesicular stomatitis virus glycoprotein G.J. Virol. 71, 7996–8000.

Pak, C.C., Puri, A., Blumenthal, R., 1997. Conformational changes and fusion activity of vesicular stomatitis virus glycoprotein: [^{125}I] iodonaphthylazide photolabeling studies in biological membranes. Biochemistry 36, 8890–8896.

Pal, R., Barenholz, Y., Wagner, R.R., 1988. Pyrene phospholipid as a biological fluorescent probe for studying fusion of virus membrane with liposomes. Biochemistry 27, 30–36.

Paterson, R.G., Russell, C.J., Lamb, R.A., 2000. Fusion protein of the paramyxovirus SV5: destabilizing and stabilizing mutants of fusion activation. Virology 270, 17–30.

Puri, A., Grimaldi, S., Blumenthal, R., 1992. Role of viral envelope sialic acid in membrane fusion mediated by vesicular stomatitis virus envelope glycoprotein. Biochemistry 31, 10108–10113.

Puri, A., Winick, J., Lowy, J.R., Covell, D., Eidelman, O., Walter, A., Blumenthal, R., 1988. Activation of vesicular stomatitis virus fusion with cells by pretreatment at low pH. J. Biol. Chem. 263, 4749–4753.

Reidel, H., Kondor-Koch, C., Garoff, H., 1984. Cell surface expression of fusogenic vesicular stomatitis virus G protein from cloned cDNA. EMBO J. 3, 1477–1483.

Remeta, D.P., Krumbiegel, M., Minetti, C.A.S.A., Puri, A., Ginsberg, A., Blumenthal, R., 2002. Acid-induced changes in thermal stability and fusion activity of influenza hemagglutinin. Biochemistry 41, 2044–2054.

Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., Harrison, S.C., 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2Å resolution. Nature 375, 291–298.

Roche, S., Gaudin, Y., 2002. Characterization of the equilibrium between the native and fusion-inactive conformation of rabies virus glycoprotein indicates that the fusion complex is made of several trimers. Virology 297, 128–135.

Rose, J.K., Gallione, C.J., 1981. Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J. Virol. 39, 519–528.

Ruigrok, R.W.H., Martin, S.R., Wharton, S.A., Skehel, J.J., Bayley, P.M., Wiley, D.C., 1986. Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. Virology 155, 484–497.

Sakaguchi, T., Toyoda, T., Gotoh, B., Inocencio, N.M., Kuma, K., Miyata, T., Nagai, Y., 1989. Newcastle disease virus evolution. I. Multiple lineages defined by sequence variability of the hemagglutinin-neuraminidase gene. Virology 169, 260–272.

Schlegel, R., Tralka, M., Willingham, M.C., Pastan, I., 1983. Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV binding site? Cell 32, 639–646.

Shokralla, S., Chernish, R., Ghosh, H.P., 1999. Effects of double-site mutations of vesicular stomatitis virus glycoprotein G on membrane fusion activity. Virology 256, 119–129.

Shokralla, S., He, Y., Wanas, E., Ghosh, H.P., 1998. Mutations in a carboxy-terminal region of vesicular stomatitis virus glycoprotein G that affect membrane fusion acitivity. Virology 242, 39–50.

Singh, M., Berger, B., Kim, P.S., 1999. Learn coil-VMF: computational evidence for coiled-coil-like motifs in many viral fusion proteins. J. Mol. Biol. 290, 1031–1041.

Skehel, J.J., Bayley, P.M., Brown, E.B., Martin, S.R., Waterfield, M.D., White, J.M., Wilson, I.A., Wiley, D.C., 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA 79, 968–972.

Skehel, J.J., Bizebard, T., Bullough, P.A., Hughson, F.M., Knossow, M., Steinhauer, D.A., Wharton, S.A., Wiley, D.C., 1995. Membrane fusion by influenza hemagglutinin. Cold Spring Harbor Symp. Quant. Biol. 60, 573–580.

Skehel, J.J., Wiley, D.C., 1998. Coiled coils in both intracellular and viral membrane fusion. Cell 95, 871–874.

Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Ann. Rev. Biochem. 69, 531–569.

Stiasny, K., Allison, S.I., Mandl, C.W., Heinz, F.X., 2001. Role of metastability and acidic pH in membrane fusion by tick-borne encephalitis virus. J. Virol. 75, 7392–7398.

Struck, D.C., Hoekstra, D., Pagano, R.E., 1981. Use of resonance energy transfer to monitor membrane fusion. Biochemistry 20, 4093–4099.

Wagner, R.R., Rose, J.K. 1996. Rhabdoviridae: the viruses and their replication, in: Fields (Ed), Fundamental Virology, Lippincott-Raven, Philadelphia., pp 561–575.

Weissenhorn, W., Dessen, A., Calder, L.J., Harrison, S.C., Skehel, J.J., Wiley, D.C., 1999. Structural basis for membrane fusion by enveloped viruses. Mol. Membr. Biol. 16, 3–9.

Weissenhorn, W., Carfi, A., Lee, K.H., Skehel, J.J., Wiley, D.C., 1998. Crystal structure of the Ebola virus membrane fusion subunit GP2, from the envelope glycoprotein ectodomain. Mol. Cell 2, 605–616.

Weissenhorn, W., Dessen, A., Harrison, S.C., Skehel, J.J., Wiley, D.C., 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature 387, 426–430.

Wharton, S.A., Skehel, J.J., Wiley, D.C., 2000. Temperature dependence of fusion by Sendai virus. Virology 271, 71–78.

White, J., Maitlin, K., Helenius, A., 1981. Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. J. Cell Biol. 89, 674–679.

White, J.M., 1992. Membrane fusion. Science 258, 917–924.

Wilson, I.A., Skehel, J.J., Wiley, D.C., 1981. Structure of hemagglutinin membrane glycoprotein of influenza virus at 3Å resolution. Nature 289, 366–373.

Wolf, E., Kim, P.S., Berger, B., 1997. Multicoil: a program for predicting two- and three-stranded coiled-coils. Protein Science 6, 1179–1189.

Yamada, S., Ohnishi, S.-I., 1986. Vesicular stomatitis virus binds and fuses with phospholipid domain in target membranes. Biochemistry 25, 3703–3708.

Zagouras, P., Rose, J.K., 1993. Dynamic equilibrium between vesicular stomatitis virus glycoprotein monomers and trimers in the Golgi and at the cell surface. J. Virol. 67, 7533–7538.

Zhang, L., Ghosh, H.P., 1994. Characterization of the putative fusogenic domain in vesicular stomatitis virus glycoprotein G. J. Virol. 68, 2186–2193.

Zhao, X., Singh, M., Malashkevich, V.N., Kim, P.S., 2000. Structural characterization of the human respiratory syncytial virus fusion protein core. Proc. Natl. Acad. Sci. USA 97, 14172–14177.