DeGrado, P. Leder, *Science*, **225**, 687 (1984).
8. H. Persson and P. Leder, in preparation.
9. C. Prives, Y. Bech, H. Shore, *J. Virol.* **33**, 689 (1980).
10. M. J. Hayman *et al.*, *Cell* **32**, 579 (1983); M. L. Privalsky, L. Sealy, J. M. Bishop, J. P. McGrath, A. D. Levinson, *ibid.*, p. 1257.
11. H. Land, L. F. Parada, R. A. Weinberg, *Nature (London)* **304**, 596 (1980).
12. L. T. Feldman and J. R. Nevins, *Mol. Cell Biol.* **3**, 829 (1983).
13. T. R. Stewart, A. R. Bellve, P. Leder, *Science*, in press.
14. J. Battey *et al.*, *Cell* **34**, 779 (1983); W. W. Colby, E. Y. Chen, D. H. Smith, A. D. Levinson, *Nature (London)* **301**, 722 (1983); R. Watt *et al.*, *ibid.* **303**, 725 (1983); O. Bernard, S. Cory, S. Gerondakis, E. Webb, J. M. Adams, *EMBO J.* **2**, 2375 (1983).
15. R. T. Sauer, R. R. Yocum, R. F. Doolittle, M. Lewis, C. O. Pabo, *Nature (London)* **298**, 447 (1983).
16. Reviewed in T. J. Kelly, Jr., *Organization and Replication of Viral DNA*, A. Kaplan, Ed. (CRC Press, Boca Raton, Fla., 1982), pp. 115–146.
17. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
18. H.P. acknowledges a fellowship from the European Molecular Biology Organization. We are also grateful to E. I. du Pont de Nemours & Co., Inc., and American Business for Cancer Research Foundation for their support.

16 April 1984; accepted 26 June 1984

A Cell Line Expressing Vesicular Stomatitis Virus Glycoprotein Fuses at Low *p*H

Abstract. *A stable cell line expressing a complementary DNA clone encoding the vesicular stomatitis virus glycoprotein fused and formed polykaryons at* p*H 5.5. The formation of polykaryons was dependent on the presence of glycoprotein anchored at the cell surface and could be prevented by incubation of cells with a monoclonal antibody to the glycoprotein. Fusion occurred at a* p*H 0.5 unit lower than that observed for cells infected with vesicular stomatitis virus.*

A virus particle must enter the host cell to grow. There are two ways in which enveloped viruses are known to enter the cell (*1*). Paramyxoviruses such as Sendai virus can enter through direct fusion (in a *p*H-independent manner) of the virion envelope with the plasma membrane of the cell (*1, 2*). The second path of entry, which is used by influenza virus (*1, 3*), Semliki Forest virus (SFV) (*1, 3, 4*), and vesicular stomatitis virus (VSV) (*1, 3, 5, 6*), is the internalization of virus particles in coated vesicles. The internalized vesicle is acidified, possibly after fusing with other intracellular vesicles (*1, 3*). The low *p*H in the vesicles containing virus particles causes fusion of the viral envelope with the membrane of that vesicle (*3*), and the viral nucleocapsids are released into the cytoplasm.

Direct evidence for a membrane fusion activity of viral glycoproteins has been obtained by expressing cloned complementary DNA's (cDNA's) encoding the SFV glycoproteins E1 and E2 (*7*) and the influenza hemagglutinin glycoproteins HA1 and HA2 (*8*). In each case the respective glycoproteins, when expressed transiently in eukaryotic cells, were shown to promote cell-to-cell fusion at low *p*H. It is believed that the hydrophobic amino terminus of HA2 of influenza virus is required to promote membrane fusion (*8*). The E2 protein of SFV does not promote fusion alone, but when both E1 and E2 are present on the cell surface, fusion will occur at low *p*H (*7, 9*). It has been suggested that a hydrophobic amino acid sequence near the amino terminus of E1 might play a crucial role in fusion (*7, 9*).

The VSV glycoprotein (G protein) is a single polypeptide chain that is held in the viral membrane by a domain of hydrophobic amino acids near the carboxyl terminus (*10*). More than 95 percent of each protein molecule is exposed on the surface of the virion. It has been observed that VSV-infected cells will fuse at low *p*H and that virus particles alone will promote cell-to-cell fusion at low *p*H (*1*). Cell fusion was thought to be mediated by G protein at the cell surface.

In the study reported here we attempted to determine whether G protein, in the absence of other VSV proteins, will promote cell fusion at low *p*H. We previously described a mouse cell line (CG1) that expresses VSV G protein at the cell surface (*11*). These cells are ideal for investigating the role of G protein in cell fusion because they express this protein in the absence of other viral proteins, for example the VSV matrix protein, which could affect fusion by interacting with G protein. The matrix protein may interact with the cytoplasmic domain of G protein during virus maturation (*12*). Stable expression of G protein in CG1 cells has been established with a hybrid expression vector that includes the SV40 early promoter (*13*), cDNA sequences encoding normal G protein (*10*), the SV40 small t intron, SV40 early polyadenylation signals (*13*), and the 69 percent subgenomic DNA transforming fragment

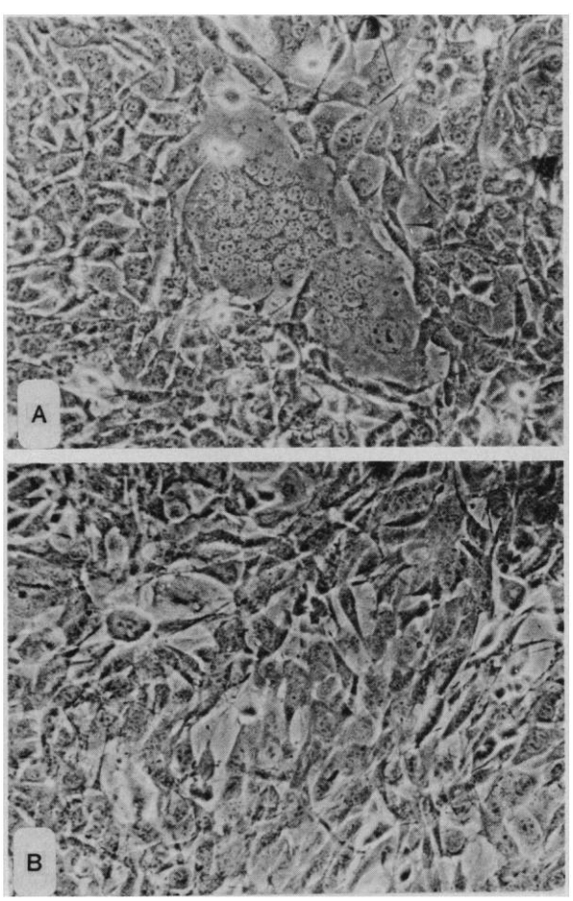

Fig. 1. Formation of polykaryons as a result of cell fusion at low *p*H. CG1 or CTG1 cells (1×10^6) were plated in 50-mm tissue culture dishes. The cells were grown for 16 hours in Dulbecco's modified Eagle's medium (DMEM) plus 5 percent fetal bovine serum in an atmosphere containing 10 percent CO_2. The culture medium was removed and replaced with 2 ml of prewarmed (37°C) fusion medium (1.85 m*M* $NaH_2PO_4 \cdot H_2O$, 8.39 m*M* $NaHPO_4 \cdot 7H_2O$, 2.5 m*M* NaCl), 10 m*M* Hepes, and 10 m*M* 2-(*N*-mopholino)ethanesulfonic acid). This buffer was adjusted to a final *p*H of 5.25. The cells were incubated in this for 1 minute; then the fusion medium was removed and replaced with DMEM plus 5 percent fetal bovine serum. In this experiment the cultures were returned to an incubator (37°C and 10 percent CO_2) and examined for fusion 12 hours later as a convenience, but it should be noted that fusion could be detected within 2 hours after incubation in fusion medium. (A) Polykaryon formation in CG1 cells. (B) Typical field of CTG1 cells, showing the absence of polykaryon formation. The photographs were taken with Polaroid film (type 55) and an Olympus inverted microscope.

of bovine papilloma virus (*14*). A hybrid SV40-VSV G protein messenger RNA (mRNA) is transcribed from the SV40 early promoter. The mRNA encodes authentic G protein, which is processed, glycosylated, and transported normally to the cell surface (*11*).

We incubated CG1 cells in fusion medium at *p*H 5.2 for 1 minute and then in normal medium for 2 to 12 hours. Polykaryons with as many as 30 nuclei were then observed (Fig. 1A), but were not seen in the nontransformed parental (C127) cell line, which does not express G protein.

To determine whether fusion requires G protein to be "anchored" at the cell surface or can be induced by a secreted form of G protein, we looked for polykaryon formation with the transformed cell line CTG1. CTG1 cells express a truncated (TG) form of G protein that lacks the transmembrane anchor sequence and is secreted (*11*). Cells that express TG protein did not fuse at low *p*H (Fig. 1B). This implies that G protein must be anchored in the cell membrane to promote cell fusion and that low *p*H–induced fusion is not a consequence of cell transformation by the vector. A lack of fusion activity for a secreted form of influenza HA protein has also been reported (*8*).

Since low *p*H causes fusion of VSV-infected cells (*1*), we compared the low *p*H–induced fusion of VSV infected CG1, CTG1, and C127 cells with that of uninfected CG1, CTG1, and C127 cells. To do this we incubated VSV-infected and uninfected cells in fusion medium of *p*H 5.0 to 6.5. When uninfected CG1 cells were examined, the *p*H at which maximum formation of polykaryons occurred was 5.5 (Fig. 2). However, after VSV infection, CG1, C127, and CTG1 cells all showed maximum fusion at *p*H 6.0 (Fig. 2). In addition, unlike virus-infected CG1 cells, in which the entire monolayer fused after incubation at low *p*H, polykaryon formation in uninfected CG1 cell cultures was limited to defined regions of the monolayer.

Treatment of CG1 cells with a monoclonal antibody (I3) against G protein (*15*), which neutralizes virus infectivity, prevented their fusion at low *p*H. This inhibition was specific for G protein because fusion was not prevented in parallel experiments with a monoclonal antibody against the VSV matrix protein. This demonstrates that G protein plays a role in the fusion of CG1 cells. Whether this represents an interaction with a domain on G protein involved in fusion or is simply a result of steric hindrance is unknown.

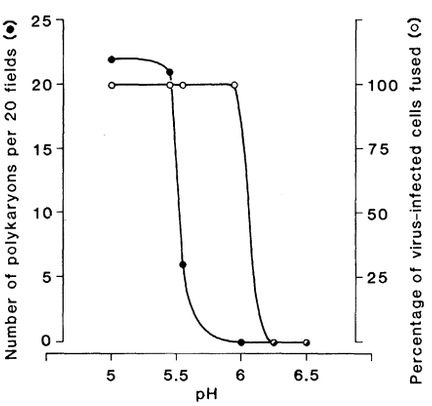

Fig. 2. The *p*H-dependent fusion of VSV-infected and uninfected CG1 cells. Twelve plates of CG1 cells were prepared as described in the legend to Fig. 1, except that six plates were first incubated with VSV (Indiana serotype; multiplicity of infection, approximately ten) for 30 minutes at 37°C. The virus inoculum was removed and replaced with DMEM plus 5 percent fetal bovine serum. Sixteen hours later the cells were incubated with fusion medium at *p*H 4.7 to 6.5. Six parallel plates of CG1 cells were treated similarly except that they were not incubated with virus. Fusion of uninfected CG1 cells and virus-infected cells was determined 2 hours after incubation in fusion medium by counting the number of polykaryons with more than four nuclei in 20 random fields of approximately 350 cells each. Fusion of VSV-infected cells is shown as the percentage of fused cells visible.

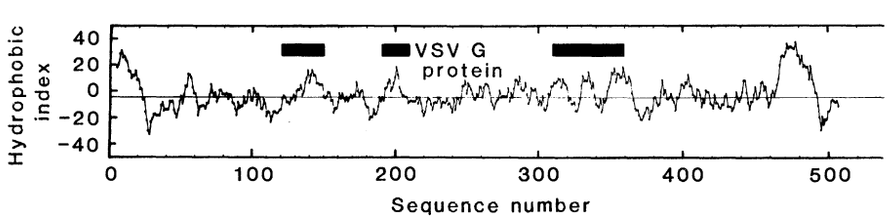

Fig. 3. Relative hydrophobic index of G protein. The continuous average hydrophobicity along the length of VSV G protein (including the signal sequence) was determined as described by Kyte and Doolittle (*18*). Continuous average hydrophobicity was determined from the average hydrophobicity of a moving segment of seven amino acids, starting at the amino terminus and proceeding to the carboxyl terminus. Bars appear over the stretches of amino acids with a marked hydrophobic character.

Unlike the HA2 G protein of influenza, mature G protein has no obvious hydrophobic amino acid sequence at or near its amino terminus that could mediate fusion (*8, 16, 17*). However, by analysis of the hydrophobic index of G protein with the method of Kyte and Doolittle (*18*), several stretches of amino acids, in addition to the carboxyl terminal transmembrane domain and the amino terminal signal sequence, were located in G protein which have a marked hydrophobic character (Fig. 3). These stretches of amino acids, between residues 120 to 150, 190 to 210, and 300 to 360, could be involved in promoting membrane fusion, possibly after low *p*H–induced conformational changes. Site-specific mutagenesis in these regions may allow us to identify a specific domain involved in fusion.

Why does the maximum fusion of CG1 cells happen at *p*H 5.5 while the fusion of the same cells infected with VSV occurs at *p*H 6.0; and why is the extent of fusion limited to a small percentage of the total population of cells and not the entire monolayer? A difference has not been found between the *p*H at which cells expressing only influenza virus glycoproteins HA1 and HA2 or SFV glycoproteins E1 and E2 fuse and the *p*H at which the respective virus-infected cells fuse (*1*). We have reported (*11*) that >95 percent of CG1 cells express levels of G protein on their cell surface that can be detected by immunofluorescence. However, the amount of this protein varied from cell to cell in the population. Analysis of CG1 cells by flow cytometry (*11*) indicated considerable variation in the amount of G protein present on the surface of cells in the population. It seems likely that only the small fraction of cells with large amounts of G protein at the cell surface can initiate fusion. If this is true, then the extent of polykaryon formation and the shift in the *p*H at which fusion occurs may simply reflect the difference in the amount of G protein at the cell surface of virus-infected cells as opposed to stably expressing CG1 cells. Alternatively, the ability of VSV-infected cells to fuse at *p*H 6 could reflect an interaction between G protein and another VSV protein, for example the matrix protein, which then serves to alter the distribution of G protein at the cell surface and thereby affect low *p*H–induced fusion. Investigators using temperature-sensitive mutants of VSV have obtained contradictory results with respect to the involvement of VSV matrix protein in the formation of polykaryons at neutral *p*H (*19, 20*).

In conclusion, cells that express VSV

G protein on their surfaces can, in the absence of other virus proteins, be used to investigate the ability of VSV G protein to cause membrane fusion. Fusion was observed at low *p*H and in the absence of any other virus-specific proteins. The formation of polykaryons required that G protein be anchored at the cell surface and was specifically inhibited by monoclonal antibodies to G protein.

R. Z. FLORKIEWICZ
J. K. ROSE

Molecular Biology and Virology Laboratory, Salk Institute, San Diego, California 92138

References and Notes

1. J. White, M. Kielian, A. Helenius, *Q. Rev. Biol. Phys.* **16** (No. 2), 151 (1983).
2. M. Homma and M. Ohuchi, *J. Virol.* **12**, 1457 (1973).
3. M. Marsh *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **156**, 835 (1982).
4. A. Helenius, J. Kartenbeck, K. Simons, E. Fries, *J. Cell Biol.* **84**, 404 (1980).
5. J. E. Dahlberg, *Virology* **58**, 250 (1974).
6. D. P. Fan and B. M. Sefton, *Cell* **15**, 985 (1978).
7. C. Kondor-Koch, B. Burke, H. Garoff, *J. Cell Biol.* **97**, 644 (1983).
8. J. White, A. Helenius, M.-J. Gething, *Nature (London)* **300**, 658 (1982).
9. H. Garoff, A.-M. Frischauf, K. Simons, H. Lehrach, H. Delius, *ibid.* **288**, 236 (1980).
10. J. K. Rose and J. E. Bergmann, *Cell* **30**, 753 (1982).
11. R. Z. Florkiewicz, A. Smith, J. Bergmann, J. K. Rose, *J. Cell Biol.* **97**, 1381 (1983).
12. H. Fraenkel-Conrat and R. R. Wagner, *Comprehensive Virology* (Plenum, New York, 1975), pp. 1–93.
13. R. C. Mulligan and P. Berg, *Science* **209**, 1422 (1980).
14. D. R. Lowy *et al.*, *Nature (London)* **287**, 72 (1980).
15. L. Lefrancois and D. Lyles, *Virology* **121**, 157 (1982).
16. J. J. Skehel and M. D. Waterfield, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 93 (1975).
17. J. J. Skehel, P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, D. C. Wiley, *ibid.* **79**, 968 (1982).
18. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982).
19. F. Chany-Fournier, C. Chany, F. Lafay, *J. Gen. Virol.* **34**, 305 (1977).
20. K. Handa, F. Chany-Fournier, S. Rousset, C. Chany, *Biol. Cell* **44**, 261 (1982).
21. We thank J. White for providing advice on the protocol for fusion: D. Lyles for monoclonal antibodies; R. Doolittle for performing the computer analysis of the VSV G protein; and L. Zokas, C. Machamer, B. Sefton, and T. Hunter for helpful suggestions concerning the manuscript. Supported by grants from the Public Health Service (AI15481) and the National Cancer Institute (CA 14195) and by a Public Health Service fellowship (1 F32 AI06911-01) to R.Z.F.

17 February 1984; accepted 6 June 1984

Immunologically Induced Alterations of Airway Smooth Muscle Cell Membrane

Abstract. *Active and passive sensitization, both in vivo and in vitro, caused significant hyperpolarization of airway smooth muscle cell preparations isolated from guinea pigs. An increase in the contribution of the electrogenic Na^+ pump to the resting membrane potential was responsible for this change. Hyperpolarization, as induced by passive sensitization, was not prevented by agents that inhibit specific mediators of anaphylaxis but was abolished when serum from sensitized animals was heated. The heat-sensitive serum factor, presumably reaginic antibodies, appears to be responsible for the membrane hyperpolarization of airway smooth muscle cells after sensitization.*

A number of respiratory diseases, including bronchial asthma, are characterized by an increased bronchoconstrictive response to numerous stimuli such as histamine or methacholine inhalation. The physiological factors underlying this so-called airway hyperreactivity are poorly understood. One idea is that fundamental changes may occur in the excitability and contractile properties of the airway smooth muscle itself (*1*). We showed that, in guinea pigs, sensitization with ovalbumin is associated with hyperpolarization of airway smooth muscle and that this hyperpolarization is, in turn, related to an increase in the contribution of the electrogenic Na^+ pump to the resting membrane potential. Further, hyperpolarization of the airway smooth muscle could be produced by passive sensitization in vitro and was not prevented by agents that inhibit mediators of anaphylaxis. However, heating serum obtained from sensitized animals prevented the change in resting membrane potential. These findings suggest that the airway response induced by sensitization to antigen involves a direct interaction between specific serum antibodies and the airway smooth muscle cell membrane. Thus, in addition to the role of the vagal reflex (*2*), release of mediators from mast cells (*3*), and possible alteration of specific membrane receptors (*4*), changes in airway smooth muscle membrane can be responsible for the phenomenon of airway hyperreactivity.

Segments of the middle portion of trachea isolated from male guinea pigs of the Camm-Hartley strain were studied in a temperature-controlled chamber as described (*5*). Single smooth muscle cells of tracheal muscle were impaled with glass microelectrodes made of borosilicate glass filled with 3*M* KCl and having a tip potential less than 10 mV and resistance of 80 to 90 megaohms. The tip potential and the resistance of each electrode were measured after each impalement. Successful impalement of a cell was indicated by a prompt negative deflection of the oscilloscope trace and maintenance of a steady potential (within 5 mV) for at least 10 seconds (*6*). Simultaneously with the measurement of resting membrane potential (E_m), the isometric force developed by tracheal segments was measured by means of a special

Table 1. The effect of active sensitization, active sensitization and resensitization, passive in vivo and in vitro sensitization on the resting membrane potential of guinea pig airway smooth muscle cells, and the response of airway smooth muscle preparations to ovalbumin, ouabain ($10^{-5}M$) and K^+-free solution. Values are means ± standard error; N.R., no response; N.D., not done.

Condition	E_m (mV)	Peak response to ovalbumin		E_m (mV)	
		E_m (mV)	Peak isometric force (g)	After $10^{-5}M$ ouabain	After K^+-free solution
Controls	−61.3 ± 0.5	N.R.	N.R.	−49.3 ± 0.6	−50.7 ± 0.4
Active sensitization	−72.7 ± 0.6*	−56.3 ± 0.3	3.8 ± 0.3	−51.8 ± 0.5	−51.9 ± 0.6
Active sensitization and resensitization	−78.1 ± 0.5*	−53.7 ± 0.8	7.8 ± 0.4	−49.9 ± 0.9	−51.4 ± 0.5
Controls	−60.5 ± 0.4	N.R.	N.R.	N.D.	N.D.
Passive sensitization in vivo	−69.5 ± 0.3*	−51.2 ± 1.3	5.3 ± 1.1	−50.5 ± 0.8	N.D.
Controls	−60.7 ± 0.6	N.R.	N.R.	−49.5 ± 5	N.D.
Passive sensitization in vitro	−68.5 ± 0.4*	−53.0 ± 0.9	4.7 ± 0.7	−50.2 ± 0.6	N.D.

*$P < 0.001$ compared to control.