Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 118-124

PHASE TRANSITIONS IDENTIFIED IN THE LIPID ENVELOPE OF FRIEND MURINE LEUKEMIA VIRUS

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Received March 27,1981

SUMMARY

Electron spin resonance spectroscopy on Friend murine leukemia virus spin-labelled with 5-doxyl stearic acid has revealed, for the first time in any virus studied to date, the presence of phase transitions corresponding to a shift between a gel phase and a liquid crystalline state within the viral lipid bilayer. Transitions were identified at 19.6°C and 33.7°C. Similar studies on the Friend virus-producing Eveline cell line have shown that the viral envelope is significantly more rigid than the host plasma membrane from which it is derived. Evidence is presented which suggests that the envelope-associated proteins of the virus mediate this enhanced rigidity.

INTRODUCTION

The technique of electron spin resonance spectroscopy has been extensively utilized in studies of the lipid bilayers of cells and of synthetic lipid vesicles (1,2,3,4), but ESR studies on the lipid bilayers of enveloped viruses have been fewer in number (5,6,7). The technique as it is usually employed involves the insertion of a nitroxide ring-containing stearic acid or phospholipid spin probe into the bilayer, and monitoring of the resultant ESR spectra. The mobility of the environment probed by the spin label is reflected in the splitting ($2T_{||}$, actually twice the hyperfine splitting constant) between the low field and high field extrema of the first derivative ESR spectrum. Smaller values of $2T_{||}$ imply greater mobility. Plots of the splitting constant as a function of temperature may show discontinuities or inflections corresponding to phase transitions within the bilayer under study (2). While phase transitions have been described in cell membranes and in synthetic lipid vesicles, studies on a number of enveloped viruses, including SV5, VSV, and influenza

Abbreviations: ESR, electron spin resonance; VSV, vesicular stomatitis virus; SV5, simian virus 5; FLV, Friend murine leukemia virus; BSA, bovine serum albumin; MDBK cells, Madin Darby bovine kidney cells; BHK cells, baby hamster kidney cells.

0006-291X/81/090118-07$01.00/0
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virions, have failed to demonstrate the presence of phase transitions in the envelope of a virus (7). Using refined techniques, including a FORTRAN computer program designed especially for the identification of phase transitions (8), we are able to report the existence of two phase transitions within the lipid envelope of Friend murine leukemia virus. While these transitions are subtle compared to those obtained in cell and liposome systems, they enjoy a high degree of statistical significance. In addition, our findings have shown that the envelope of FLV is much more rigid than the plasma membrane of the FLV-producing Eveline cell line from which it is derived.

METHODS

Tissue culture. The Eveline line of FLV-producing mouse embryo cells was grown in roller bottles in a fortified Dulbecco modified Eagle's medium containing 10% newborn calf serum as previously described (9). For harvesting of virus, cells were first pelleted by centrifugation for 30 minutes in a Sorvall GSA rotor at 8000 rpm. The clarified supernatant was then layered over 10 ml of 10% sucrose and centrifuged in a Beckman Type 19 rotor for 90 minutes at 19,000 rpm to pellet FLV.

Spin labelling and ESR spectroscopy. A spin label working solution was prepared which contained 1 mg/ml of either 5-doxyl stearic acid or 16-doxyl stearic acid (Syva, Palo Alto, Cal.) plus 50 mg/ml BSA (Sigma, St. Louis, Mo.) as carrier. One mg of FLV and 200 µl of spin label working solution were mixed and brought to a volume of 1 ml by the addition of phosphate-buffered saline. The mixture was incubated at 4°C for 2 hours after which unbound spin label was removed by centrifugation on a 25-60% sucrose gradient for 2 hours in an SW 50.1 rotor at 45,000 rpm. The viral band was removed from the centrifuge tube with a syringe and transferred to a quartz aqueous sample ESR flat cell. Eveline cells were spin labelled by incubating approximately 20 x 10^6 cells with 200 µl of spin label working solution. Spin labelled cells were washed several times in Eagle's medium and pelleted in a clinical centrifuge. The pelleted cells were resuspended in Eagle's medium and transferred to an ESR flat cell. To prevent sedimentation of cells during scanning, the cell suspension was circulated through the flat cell using a peristaltic pump. All ESR spectra were obtained on a Varian E-109 ES EPR Spectrometer. Temperature was regulated to within 0.1°C using a temperature control device of our own design. Temperature measurements were made with a chromel-constantan thermocouple (Omega Engineering, Stamford, Conn.).

Phospholipid and cholesterol determinations. Aliquots of FLV and Eveline cells were extracted with 2:1 (v:v) chloroform:methanol and the resulting organic phases containing total lipids were subjected to the following analyses. Phospholipid was determined as inorganic phosphate by the method of Duck-Chong (10). Cholesterol was determined with the Enzymatic Cholesterol assay kit purchased from Calbiochem-Behring (San Diego, Cal.).

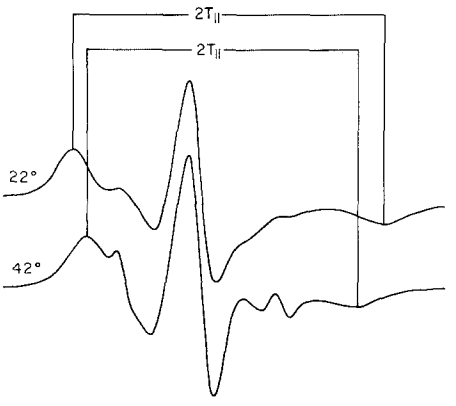

Figure 1. Representative electron spin resonance spectra of Friend murine leukemia virus spin labelled with 5-doxyl stearic acid. Scans shown were obtained at 22°C and 42°C.

RESULTS

The ESR spectra of nitroxide ring-containing spin labels reflect the mobility of the environment into which these probe molecules are inserted (11). Figure 1 shows the spectra obtained at two different temperatures when 5-doxyl stearic acid is inserted into the lipid envelope of FLV. The distance between the low and high field extrema, $2T_{||}$, varies inversely with the degree of mobility within the bilayer (11). As the temperature increases, the "fluidity" of the bilayer increases and the distance $2T_{||}$ contracts. Spectra obtained with 16-doxyl stearic acid spin labelled FLV (not shown) were characterized by very low values of $2T_{||}$ indicating that the motion of fatty acyl chains deep within the FLV bilayer was highly isotropic. No change in splitting constant could be detected with 16-doxyl stearic acid labelled virus over the temperature range of 4-50°C.

Figure 2 illustrates phase transition plots obtained with 5-doxyl stearic acid spin labelled FLV and FLV-infected Eveline mouse cells. Each study was conducted so as to obtain at least 100 temperature points per plot. All phase transition data were then subjected to analysis by a FORTRAN computer program designed to localize phase transitions and evaluate their statistical significance (8). Briefly, a B-spline is used to provide a smooth fit for the ESR data and points of inflection are used to group data. Regression lines are calculated for each group and then plotted, allowing for the determination of

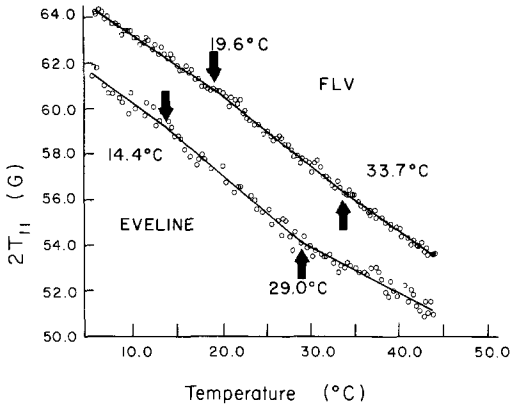

Figure 2. Phase transition plots of Friend murine leukemia virus (upper plot) and FLV-infected Eveline cells (lower plot) spin labelled with 5-doxyl stearic acid. Arrows indicate positions of phase transitions.

break points which correspond to lipid phase transitions (3,8). The statistical significance of these transition points are evaluated using the F-test. Phase transition studies on spin-labelled FLV, run in triplicate and reproducible to $\pm$ 0.2°C, reveal two phase transition temperatures, the first at 19.6°C and the second at 33.7°C. Both of these transition temperatures, upon F-test analysis, achieved confidence levels of better than 99.9% certainty ($p < 0.001$). The phase transition temperatures detected for the Eveline cells were equally significant, and occurred at 14.4°C and 29.0°C.

Examination of the two plots reveals that the phase transition plot of FLV is displaced upward by the equivalent of at least 10° relative to the phase transition plot of FLV-infected Eveline cells. Thus, for a given value of the splitting constant, the viral envelope requires an ambient temperature 10° higher than the cell membrane in order to achieve the same degree of mobility. The lipid bilayer of FLV, then, is substantially more rigid than the plasma membrane of the host Eveline cell. In addition, the first phase transition temperature of FLV is approximately 5° higher than the first phase transition temperature of the plasma membrane, a finding which is consistent with the enhanced rigidity of the viral bilayer.

To address the question of why the lipid bilayer of FLV is more rigid than the plasma membrane of the host Eveline cell we have determined the phospholipid and cholesterol compositions of the two

bilayers. Our results yield a phospholipid/cholesterol ratio of 1.25 for the envelope of FLV and 1.27 for the plasma membrane of the Eveline cells. Since these ratios are identical within experimental error, a result which would be expected for a virus which buds from the plasma membrane of its host cell, we suggest that the enhanced rigidity of the FLV bilayer is due to the stiffening action of one or more of the envelope-associated proteins of the virus (9,12).

DISCUSSION

The failure of past workers to observe phase transitions in the bilayers of enveloped viruses may be accounted for by one of two possible explanations. The viruses previously studied (7) may have lacked phase transitions over the temperature range studied, or more probably, the viral bilayer transitions may have escaped detection owing to their subtlety as compared with the phase transitions of cell membranes and lipid vesicles. In our studies, we have collected in excess of 100 temperature points per phase transition plot, and have had the advantage of a sophisticated computer program (8) which is capable of identifying phase transitions associated with very subtle plot inflections. It must be stressed that while the phase transitions of the FLV envelope are subtle, they reflect a statistical level of certainty in excess of 99.9% and are reproducible to within 0.2°C in studies run over several months. The first inflection in the phase transition plots of FLV and of FLV-infected Eveline cells presumably corresponds to a transition between lipid in the gel (solid) phase and lipid in the first liquid crystalline state. At the temperature corresponding to this inflection fatty acyl chains in the all-trans conformation begin to incorporate gauche conformers (13,14). At the second inflection in the phase transition plot (the second phase transition temperature) no further gauche conformations may be added to the fatty acyl chains of the bilayer. It is believed that the actual transition between the gel and liquid crystalline phases of the fatty acyl chains spans the temperature range between the first and second inflections of the phase transition plot (13,14).

The rigidity of the FLV envelope agrees with findings of other workers who have demonstrated that the envelopes of VSV, SV5, and influenza virus are likewise more rigid than the plasma membranes of their respective host cells (5,7,15,16). This rigidity appears to

reflect the stiffening action of viral envelope-associated proteins, but is capable of modulation by the lipid components of the viral bilayer as well (7). Electron spin resonance studies have been conducted on 5- and 16-doxyl stearic acid spin labelled VSV (5) SV5, and influenza virions (15), each grown in two different cell lines: MDBK cells, which had a lower phospholipid/cholesterol ratio and BHK cells, which had a higher molar ratio of phospholipid to cholesterol and which were, therefore, more fluid. All three viruses were shown to contain more fluid envelopes when grown in BHK cells (5,15). Additional studies have revealed that protease digestion of the glycoprotein spikes of influenza virions does not affect viral bilayer fluidity (16), whereas the same treatment elicited an increase in the fluidity of the VSV envelope (5). This increased fluidity was not large enough, however, to significantly compensate for the rigidity of the viral bilayer. Likewise, changes in envelope fluidity associated with growth of the virus in a less or more fluid cell line could not account for the rigidity of the viral bilayer as compared with the plasma membrane of the host. It has been suggested for influenza virus that the M-protein which lies beneath and in close proximity to the viral envelope may be the principle determinant of envelope rigidity (7). In FLV, envelope-associated proteins have been identified and characterized (9,12). FLV surface projections appear to consist of heavily glycosylated, hydrophilic gp 71 molecules disulfide linked to hydrophobic p15E protein, which may span the lipid bilayer. The FLV phosphoprotein, designated pp12, is evidently located immediately beneath the lipid envelope forming an inner coat structure analogous to the M-protein of influenza virions. Thus certain ordered, repetitive interactions of these FLV envelope-associated proteins with the lipid bilayer may induce a rigidity not possible with the complex cellular plasma membrane. Now that our ESR techniques are capable of detecting the subtle phase transitions in FLV envelopes, determination of the contributions of gp71, p15E, and pp12 to the rigidity of the viral envelope will be greatly facilitated.

ACKNOWLEDGEMENTS

The authors wish to extend their special thanks to John C. Hogan, Department of Chemistry, Louisiana State University, without whose many contributions this project would not have been possible. We also thank Donald G. Brunder, Richard T. Coughlin, and Estelle J. McGroarty of Michigan State University for providing the computer program for transition point analysis, and D. Bolognesi of Duke University Medical

Center for providing the Eveline cell line. This project is supported in part by funds from the Louisiana Agricultural Experiment Station, American Cancer Society Grant MV-110 and N.I.H. Grant 1-RO1-AI 17594 from the Institute of Allergy and Infectious Diseases.

REFERENCES

1. Kleemann, W., and McConnell, H. M. (1976) Biochim. Biophys. Acta 419:206-222.
2. Hubbell, W. L., and McConnell, H. M. (1971) J. Amer. Chem. Soc. 93:314-326.
3. Janoff, A. S., Coughlin, R. T., Racine, F. M., McGroarty, E. J., and Vary, J. C. (1979) Biochem. Biophys. Res. Comm. 89:565-570.
4. Sackmann, E., and Trauble, H. (1972) J. Amer. Chem. Soc. 94:4482-4491.
5. Landsberger, F. R. and Compans, R. W. (1976) Biochem. 15:2356-2360.
6. Landsberger, F. R., Compans, R. W., Paxton, J., and Lenard, J. (1972) J. Supramol. Struct. 1:50-54.
7. Landsberger, F. R., Lyles, D. S., and Choppin, P. W. In: Mahy, B. W. J., and Barry, R. D., eds. Negative Strand Viruses and the Host Cell pp. 787-800. Academic Press, London (1978).
8. Brunder, D. G., Coughlin, R. T., and McGroarty, E. J. Computers in Biology and Medicine, in press.
9. Montelaro, R. C. Sullivan, S. J., and Bolognesi, D. P. (1978) Virology 84:19-31.
10. Duck-Chong, C. G. (1979) Lipids 14:492-497.
11. Schreier, S., Polnaszek, C. F., and Smith, I. C. P. (1978) Biochim. Biophys. Acta 515:375-436.
12. Montelaro, R. C., and Bolognesi, D. P. In: Blough, H. A., and Tiffany, J. M., eds. Cell Membranes and Viral Envelopes pp. 683-707, Academic Press, London (1980).
13. Gruen, D. W. R. (1980) Biochim. Biophys. Acta 595:161-183.
14. Scott, H. L., and Cheng, W. H. (1979) Biophys. J. 28:117-132.
15. Landsberger, F. R., Compans, R. W., Choppin P. W., and Lenard, J. (1973) Biochem. 12:4498-4502.
16. Landsberger, F. R., Lenard, J., Paxton, J., and Compans, R. W. (1971) Proc. Nat. Acad. Sci. USA 68:2579-2583.