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Phosphoproteins of Murine Hepatitis Viruses

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Four strains of the coronavirus murine hepatitis virus were examined for the presence of phosphorylated proteins. The nucleocapsid protein was determined to contain phosphate covalently linked to serine but not to threonine residues. The nucleocapsid protein was the only phosphorylated protein detected in these strains of murine hepatitis virus.

Murine hepatitis viruses (MHV) are members of the Coronaviridae (8, 23). These are enveloped positive-stranded RNA viruses (7) which contain at least three structural proteins (19). The A_{59} strain of MHV (MHV-A_{59}) has a single nucleocapsid protein, VP-50, and at least two glycosylated envelope components, GP-23 and GP-90 (19). An additional virion envelope glycoprotein, GP-180, is a dimer of GP-90 (20). Recently, two reports on the structural proteins of another strain of MHV, the neurotropic JHM strain (MHV-JHM), have appeared. MHV-A_{59} and MHV-JHM are serologically related but distinct (23; J. Childes and S. A. Stohlman, unpublished data) and differ in their tissue tropism in vivo (9). In one report (1), the number of MHV-JHM structural proteins and their molecular weights correspond quite well to those of MHV-A_{59} (19). However, MHV-JHM was also reported to contain six structural proteins with molecular weights of 170,000, 125,000, 97,500, 60,800, 24,800, and 22,700, respectively (25). The biological functions and chemical structure of these viral proteins are not quite understood.

We are interested in the chemical modification, particularly phosphorylation, of the structural proteins of MHV since many animal and plant viruses contain phosphorylated proteins as components of the mature virion. Phosphoproteins have been demonstrated in a variety of enveloped viruses, e.g., Sindbis virus (24), RNA tumor viruses (6, 10), rhabdoviruses (17), influenza virus (11), vaccinia virus (13), and equine herpesvirus (12), and in some nonenveloped DNA viruses, e.g., adenovirus (5, 14) and simian virus 40 (22). On the other hand, human coronavirus strain 229E had no detectable phosphorylated proteins (4). However, the method used in that study may not have been sensitive enough to detect phosphoproteins.

We therefore examined MHV for the possible presence of phosphoproteins. MHV were propagated in DBT cells, labeled with $^{32}P_i$ (20 μCi/ml, ICN Pharmaceuticals, Inc.) or ^{3}H-amino acids (20 μCi/ml, ICN) for 10 to 14 h, and then purified as previously described (7). Viruses labeled with $^{32}P_i$ and ^{3}H-amino acids were purified separately, disrupted with 1% sodium dodecyl sulfate, and then co-electrophoresed in polyacrylamide gels (18). Preliminary studies using unfractionated ^{32}P-labeled MHV-A_{59} showed that a phosphorylated protein comigrated with VP-50 (data not shown). To characterize this phosphorylated protein, we extracted [^{32}P]phosphate-labeled virus with phenol twice and recovered the proteins from the phenol phase by precipitation with 5 volumes of ethanol (6). Before electrophoresis, the sample was treated with 5 μg of RNase A per ml for 30 min at 37°C, followed by ethanol precipitation. Co-electrophoresis of ^{3}H-amino acid-labeled proteins and ^{32}P-labeled proteins of MHV-A_{59} is shown in Fig. 1. A single species of ^{32}P-labeled protein comigrates with VP-50, the nucleocapsid protein of the virion (19). To rule out the possibility that this comigration of ^{32}P-labeled protein and VP-50 is due to nonspecific association of RNA or phospholipid, we subjected the ^{32}P-labeled protein preparation to various chemical treatments. Incubation with RNases A (20 μg/ml) and T_1 (20 U) at 37°C for 30 min, extraction with a methanol-chloroform mixture (1:1), or incubation with 10% trichloroacetic acid at 50°C for 15 min removed less than 10% of the [^{32}P]phosphate label associated with the viral protein. In contrast, treatments with pronase (100 μg/ml)

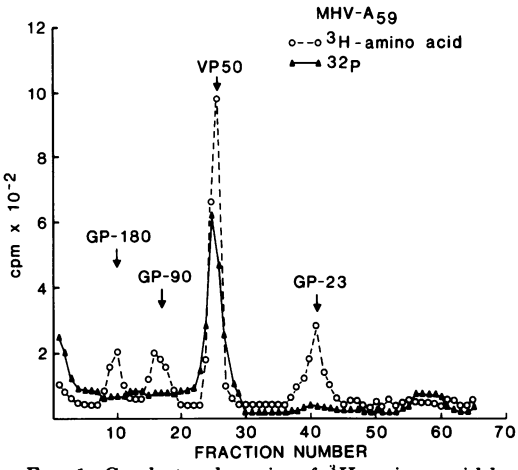

FIG. 1. *Co-electrophoresis of ^{3}H-amino acid-labeled MHV-A_{59} with ^{32}P-labeled MHV-A_{59} proteins. The ^{32}P-labeled protein preparation was predigested with RNase before electrophoresis on 6% neutral polyacrylamide gels as previously described (18). The electrophoresis was run at 40 V for 7 h.*

at 37°C for 30 min or with 1 N NaOH at 100°C for 15 min solubilized more than 90% of the ^{32}P counts. These results prove that the phosphate groups are covalently linked to the viral protein (6, 10). We also determined the phosphoamino acid linkage of the phosphorylated VP-50. The ^{32}P-labeled VP-50 was isolated by polyacrylamide gel electrophoresis, hydrolyzed with 6 M HCl for 1.5 h, and then separated by paper electrophoresis at pH 1.9 (6). As shown in Fig. 2, the hydrolyzed ^{32}P-labeled amino acids from VP-50 comigrated with phosphoserine but not with phosphothreonine. This result is similar to those obtained with the single-stranded DNA binding protein of adenovirus (5) and the nucleoprotein of influenza virus (11). It further confirms that the protein moiety of the nucleocapsid, VP-50, of MHV-A_{59} is phosphorylated. We will hence call this protein pp50.

We then examined other strains of MHV, including the hepatotropic MHV-3 and MHV-2 and the neurotropic JHM strains (23, 26), to see whether the phosphorylation pattern of their phosphoprotein is different. This was of interest since strain variation has been noted in the phosphoproteins of Rhabdoviridae (17). As shown in Fig. 3, a single peak of ^{32}P-labeled protein was found to comigrate with the nucleocapsid proteins of MHV-2, MHV-3, and MHV-JHM viruses. Therefore, we conclude that the nucleocapsid proteins in all of the MHV strains examined so far are phosphorylated and that these are the only phosphoproteins present in MHV. It is also interesting to note that the protein profiles of all of these virus strains are very similar although there are minor variations in their molecular weights. Our results, therefore, did not confirm the presence of extra proteins in MHV-JHM (25).

Phosphorylation has a regulatory mechanism for protein function (21). However, the role of phosphorylation in the nucleocapsid protein of MHV remains to be investigated. Several functions have been proposed for the phosphorylation of viral proteins in general. Phosphorylation has been suggested to exert a regulatory role in the selection of the negative-stranded genome found in mature Sendai virions (8) and in the regulation of overall RNA synthesis or the switch from transcription to replication in vesicular stomatitis virus-infected cells (2). Phosphorylation also influences the specific binding of the viral phosphoprotein to the RNA genome in RNA tumor viruses (15, 16). Therefore, it is conceivable that the degree of phosphorylation in the nucleocapsid protein of MHV could regulate its interaction with the viral RNA and, in turn, regulate the maturation of viral particles.

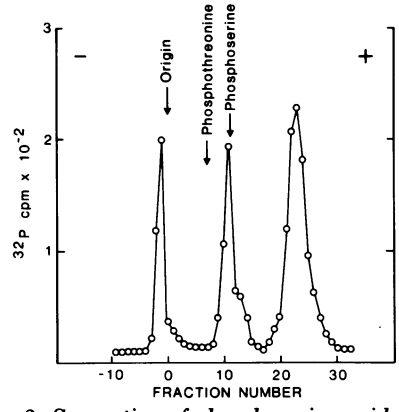

FIG. 2. *Separation of phosphoamino acids by paper electrophoresis. ^{32}P-labeled viral proteins purified by phenol extraction were separated by electrophoresis on a 7% polyacrylamide gel (6). The gel was wrapped with Saran Wrap and exposed to a Kodak NS film for 12 h. The ^{32}P-labeled protein band corresponding to VP-50 was cut out and eluted with a buffer containing 0.1 M ammonium bicarbonate and 0.1% sodium dodecyl sulfate at room temperature for 24 h. The eluted proteins were precipitated with ethanol and partially hydrolyzed in 5.7 M HCl at 110°C for 1.5 h. The hydrolysate was electrophoresed in formic acid-acetic acid-water (1:4:100, pH 1.9) at 1,000 V for 2 h (6). Phosphoserine and phosphothreonine (Sigma Chemical Co.) were included at 50 mg as markers and were identified with ninhydrin spray. The paper was cut into 1-cm strips and counted in toluene-based scintillation fluid (6). The ^{32}P peak near the origin represents undigested peptides, and that closest to the anode represents P_i or other contaminants.*

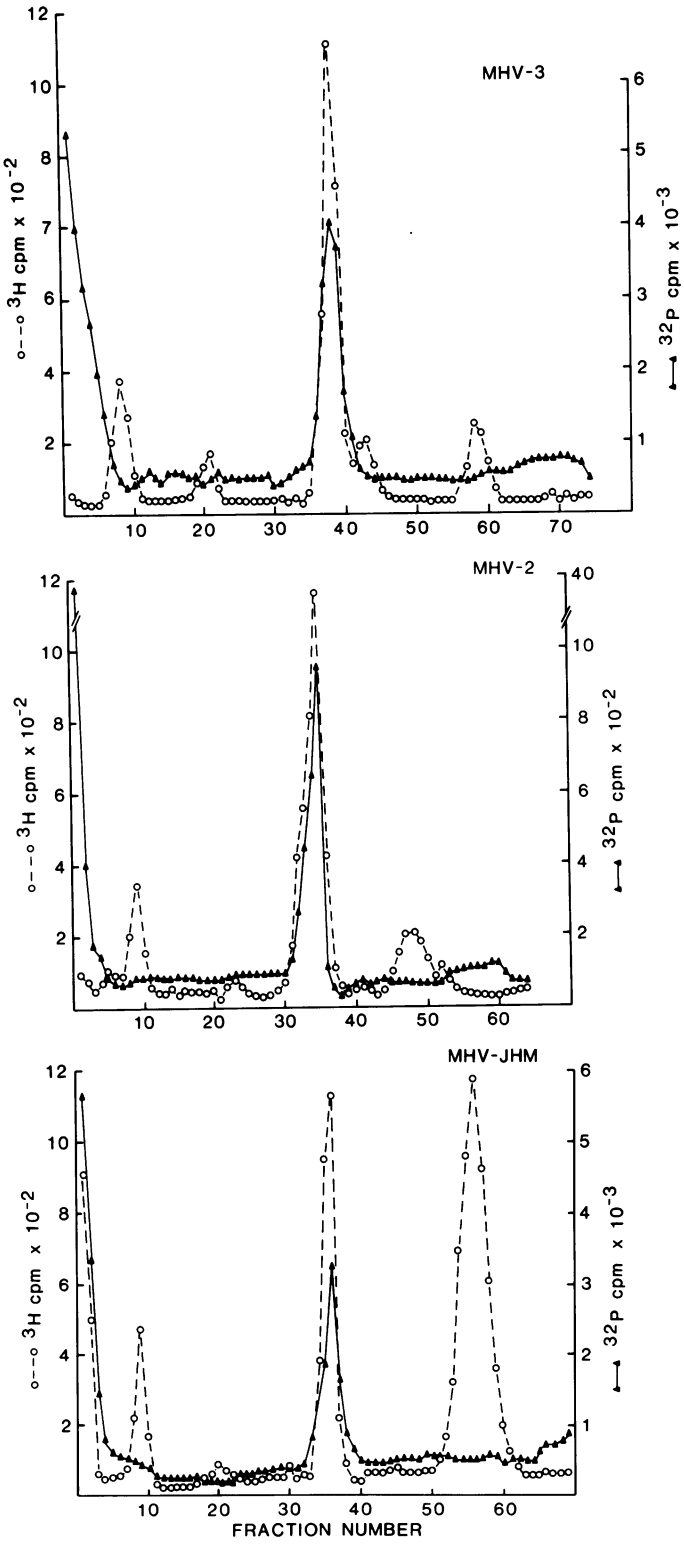

FIG. 3. *Electrophoresis of ^{3}H-labeled amino acids and ^{32}P-labeled proteins from MHV-3, MHV-2, and MHB-JHM, respectively. Conditions were identical to those described in the legend to Fig. 1.*

It remains to be investigated whether there are multiple forms of pp50 with different degrees of phosphorylation. The presence of multiple forms of phosphoproteins could suggest some regulatory functions for the protein.

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LITERATURE CITED

1. **Bond, C., J. Leibowitz, and J. Robb.** 1979. Pathogenic murine coronaviruses. II. Characterization of virus-specific proteins of murine coronaviruses JHMV and A59V. Virology **94**:379-384.
2. **Clinton, G. M., B. W. Burge, and A. S. Huang.** 1978. Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. J. Virol. **27**:340-346.
3. **Guy, J. S., and D. A. Brian.** 1979. Bovine coronavirus genome. J. Virol. **29**:293-300.
4. **Hierholzer, J.** 1976. Purification and biophysical properties of human coronavirus 229E. Virology **75**:155-165.
5. **Jeng, Y.-H., W. S. M. Wold, K. Sugawara, Z. Gilead, and M. Green.** 1977. Adenovirus type 2 coded single-stranded DNA binding protein: in vivo phosphorylation and modification. J. Virol. **22**:402-411.
6. **Lai, M. M. C.** 1976. Phosphoproteins of Rous sarcoma viruses. Virology **74**:287-301.
7. **Lai, M. M. C., and S. A. Stohlman.** 1978. RNA of mouse hepatitis virus. J. Virol. **26**:236-242.
8. **Lamb, R. A., and P. W. Choppin.** 1977. The synthesis of Sendai virus polypeptides in infected cells. III. Phosphorylation of polypeptides. Virology **81**:382-397.
9. **McIntosh, K.** 1973. Coronaviruses: a comprehensive review. Curr. Top. Microbiol. Immunol. **68**:85-129.
10. **Pal, B. K., R. M. McAllister, M. B. Gardner, and P. Roy-Burman.** 1975. Comparative studies on the structural phosphoproteins of mammalian type C viruses. J. Virol. **16**:123-131.
11. **Privalsky, M. L., and E. E. Penhoet.** 1977. Phosphorylated protein component present in influenza virions. J. Virol. **24**:401-405.
12. **Randall, C. C., H. W. Rogers, D. N. Downer, and G. A. Gentry.** 1972. Protein kinase activity in equine herpesvirus. J. Virol. **9**:216-222.
13. **Rosemond, H., and B. Moss.** 1973. Phosphoprotein component of vaccinia virions. J. Virol. **11**:961-970.
14. **Russell, W. C., J. J. Skehel, R. Machado, and H. G. Pereira.** 1972. Phosphorylated polypeptides in adenovirus-infected cells. Virology **50**:931-934.
15. **Sen, A., and G. J. Todaro.** 1976. Specificity of in vitro binding of primate type C viral RNA and homologous virus p12 core protein. Science **193**:326-328.
16. **Sen, A., and G. J. Todaro.** 1977. The genome-associated, specific RNA binding proteins of avian and mammalian type C viruses. Cell **10**:91-99.
17. **Sokol, F., and H. F. Clark.** 1973. Phosphoproteins, structural components of rhabdoviruses. Virology **52**:246-263.
18. **Stohlman, S. A., C. L. Wisseman, Jr., O. R. Eylar, and D. J. Silverman.** 1975. Dengue virus-induced modifications of host cell membranes. J. Virol. **16**:1017-1026.
19. **Sturman, L. S.** 1977. Characterization of a coronavirus. I. Structural proteins: effects of preparative conditions on the migration of protein in polyacrylamide gels. Virology **77**:637-649.
20. **Sturman, L., and K. Holmes.** 1977. Characterization of a coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. Virology **77**:650-660.
21. **Taborsky, G.** 1974. Phosphoproteins, p. 1-187. *In* C. B. Anfinsen and J. Edsall (ed.), Advances in protein chemistry, vol. 28. Academic Press Inc., New York.
22. **Tan, K. B., and F. Sokol.** 1972. Structural proteins of simian virus 40: phosphoproteins. J. Virol. **10**:985-994.
23. **Tyrrell, D., J. Almeida, C. Cunningham, W. Dowdle, M. Hofstad, K. McIntosh, M. Tajima, L. Zakstelskaya, B. Esterday, A. Kapikian, and R. Bingham.** 1975. Coronaviridae. Intervirology **5**:76-82.
24. **Waite, M. R. F., M. Lubin, K. J. Jones, and H. R. Bose.** 1974. Phosphorylated proteins of Sindbis virus. J. Virol. **13**:244-246.
25. **Wege, H., H. Wege, K. Nagashima, and V. ter Meulen.** 1979. Structural peptides of the murine coronavirus JHM. J. Gen. Virol. **42**:37-48.
26. **Weiner, L. P.** 1973. Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). Arch. Neurol. **28**:298-303.