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PHOSPHORYLATION OF THE MOUSE HEPATITIS VIRUS NUCLEOCAPSID PROTEIN

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SUMMARY: Analysis of the radiolabeled tryptic peptides derived from the nucleocapsid proteins of two serotypes of mouse hepatitis virus showed each to have a small number of unique peptides; however, two biologically distinct variants of the JHM strain appeared identical. Analysis of [^{32}P]-labeled nucleocapsid-derived peptides showed that phosphorylation occurs at only a few sites and that all three viruses differed in the sites of phosphorylation. No differences in the sites of phosphorylation were found between the nucleocapsid proteins derived from purified virions and the membranes or the cytosol of infected cells, suggesting that post-translational phosphorylation plays no role in the regulation of viral assembly. These data show unequivocal evidence that the nucleocapsid proteins of mouse hepatitis virus strains differ in the sites of phosphorylation.

Mouse hepatitis viruses (MHV) are members of the Coronaviridae and are composed of only three structural proteins (9,18). Two of these proteins, E1 and E2, are envelope glycoproteins which form, respectively, the matrix protein and peplomers on the external surface of the virions. The third protein, designated N, is the phosphorylated nucleocapsid protein associated with the virion positive-stranded genomic RNA, which together form the helical nucleocapsid (8,12). The only post-translational modification of the N protein is rapid phosphorylation following synthesis, which occurs exclusively on serine residues (8,13,14). In the prototype non-neurovirulent strain (A59), this protein has a molecular weight of approximately 50,000 daltons determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a molecular weight of 49,660

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Abbreviations used are: HPLC, high pressure liquid chromatography; MHV, mouse hepatitis virus; N, nucleocapsid protein, SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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predicted from the nucleotide sequence (1,13,15). In contrast, the neurovirulent strain (JHM) has an N protein with a molecular weight of approximately 60,000 determined by SDS-PAGE and a molecular weight of 49,700 predicted from the nucleotide sequence (9,11,13). The cause of this disparity is unknown at present.

In addition, we have previously reported the isolation of two plaque morphology variants, designated DS for small plaque and DL for large plaque, from the JHM strain of MHV (13). These two strains have only minor variations in their oligonucleotide fingerprints; however, they differ in their neurovirulence for mice (13). The DS variant produces predominantly demyelination while the DL variant produces predominantly encephalitis following infection of mice.

Oligonucleotide fingerprint analysis of the mRNA (number 7) which encodes the N protein (7,10) and the SDS-PAGE of the N proteins from both DL and DS suggested that the sequences of the genes encoding N in these two strains are indistinguishable (data not shown). However, in contrast to DL, the nucleotide sequence of the gene encoding the N protein of DS has not been determined. In addition to differences in their pathogenicity, of the ten monoclonal antibodies specific for the N protein of the DL variant of JHM, four do not recognize the N protein of the DS variant or A59, while the remaining six react strongly with A59 and show only weak reactivity to DS (3). Conversely, none of the seven monoclonal antibodies specific for the N protein of MHV-A59 distinguish the DL and DS variants of JHM (Gilmore, personal communication). These studies suggest that the N protein differs among these three closely related strains of MHV.

To address this question we examined the relatedness of the N proteins from the JHM variants DL and DS and compare them to the N protein of the prototype A59 strain by comparing the [^{3}H]-leucine- and [^{3}H]-phenylalanine-labeled tryptic peptides using high-pressure liquid chromatography (HPLC). In addition, we examined the [^{32}P]-labeled tryptic peptides to determine whether phosphorylation plays a role in the assembly of MHV.

MATERIALS AND METHODS

The N protein was prepared from MHV-infected DBT cells as previously described (12,13,14). At 3h postinfection, 100 uCi/ml [^{3}H] -leucine or [^{3}H] -phenylalanine or 200 uCi/ml [^{32}P]-orthophosphate was added. At confluent cytopathology, which occurred approximately 7h postinfection, the cells were lysed in 10mM Tris-HCl buffer, pH7.4,

containing 150mM NaCl, 0.5% NP-40 and 200 ug/ml phenylmethylsulfonyl fluoride. The N proteins were isolated by immunoprecipitation using monoclonal antibody J.3.3 as previously described (3,14). The radiolabeled N protein was purified by electrophoresis on 6-18% gradient polyacrylamide gels, eluted into 0.01% SDS in water, mixed with 0.5 mg of bovine gammaglobulin and lyophilized. The protein was then reduced, alkylated, precipitated with trichloroacetic acid and digested with trypsin as described (5). The resulting tryptic peptides were analysed at room temperature on a Perkin-Elmer Series 3B HPLC system using a Waters Z module system fitted with a reverse-phase Radial-PAK CN cartridge. The solvent system consisted of an aqueous phase of 0.01M sodium phosphate/0.04M NaCl, pH2.0, and an increasing gradient of acetonitrile. Fractions of 0.5 ml were collected every 30 sec and counted. The reproducibility of the maps, ascertained by monitoring the separation of immunoglobulin peptides at 210nm, proved to be within 1 min (2 fractions) for all the maps.

RESULTS AND DISCUSSION

The [^{3}H] -labeled tryptic peptide maps shown in Figure 1 (Panels A,B and C) indicate that the N proteins from DL and DS are virtually indistinguishable from each other and are closely related to the N protein of A59. This is in agreement with the 94% homology between the N proteins of JHM and A59 predicted from their nucleotide sequences (1,11).

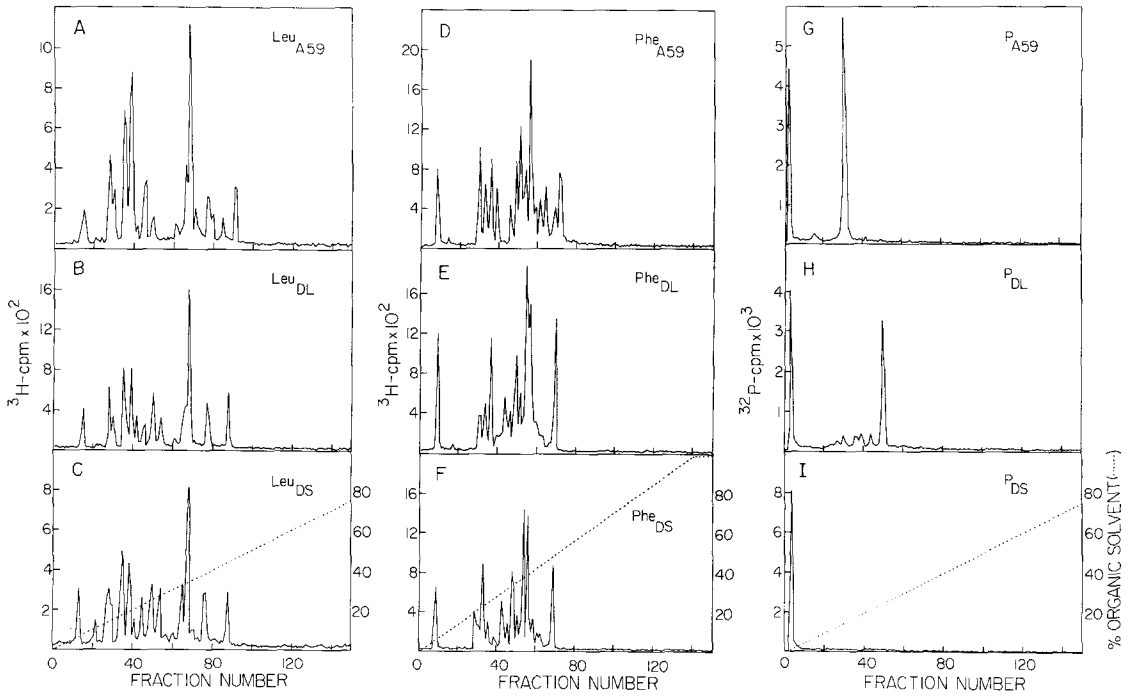

Figure 1. HPLC tryptic peptide maps of the [^{3}H]-leucine (panels A, B and C), [^{3}H]-phenylalanine (panels D,E and F) and [^{32}P] -orthophosphate (panels G, H and I) labelled peptides of the N proteins of MHV-A59 (panels A,D and G), MHV-JHM variant DL (panels B,E and H) and MHV-JHM variant DS (panels C, F and I) viruses.

[^{3}H]-leucine was used initially because it incorporates well into cells and labelled 14 out of a possible 43 tryptic peptides predicted from the nucleotide sequence of the N proteins of A59 and JHM (1,12). [^{3}H] -phenylalanine labelling (panels D, E and F) was used to examine nine additional peptides. Thus, the use of both [^{3}H] -phenylalanine and [^{3}H] -leucine resolved the majority of the phenylalanine and leucine containing tryptic peptides in the N protein. The [^{3}H] -leucine and [^{3}H] -phenylalanine maps from DL and DS are virtually indistinguishable, in agreement with the oligonucleotide fingerprints; however, there are small but significant differences between the two JHM variants and A59.

The analysis of the tryptic peptides of the [^{32}P]-labeled N proteins derived from infected cells is shown in Figure 1, panels G, H and I. Very few [^{32}P] -peptides were observed. DL and A59 could be clearly distinguished by the elution profile of the two phosphopeptides. Only a single peak of [^{32}P] -label was detected for DS. The [^{32}P] -labelled peak at fraction 2 does not correspond to any [^{3}H] -labeled peptides and may represent either a hydrophilic unresolved peptide or free phosphate. Although we cannot completely rule out the possibility that the peak at fraction 2 represents free phosphate, it is more likely an unresolved peptide for three reasons. First, no acid-catalyzed decomposition of phosphopeptide to free phosphate occurred during our isolation procedure because identical maps were obtained when acetone or ethanol were substituted for trichloroacetic acid during protein isolation. Second, the equivalence between the counts eluted at fraction 2 and those retained on the column for [^{32}P] -labeled A59 and DL strongly suggests that each peak contains a peptide. Third, examination of the nucleic acid sequence of the genes encoding the N proteins suggests that twelve serine-containing peptides lacking leucine and phenyalanine could be derived by tryptic digestion. Of these, four would contain six amino acids or less. The data suggest that A59 and JHM unequivocably differ in the phosphorylation site on the peptide retained on the column. We believe that the N protein of all three viruses are also phosphorylated on a second small hydrophilic peptide not retained on the column. We cannot at present determine if these peptides are identical. Irrespective of this, we conclude that the three viruses differ in at least one site of phosphorylation on the N protein.

Since phosphorylation of N is known to occur on serine residues (8,12), and since the nucleic acid sequence predicts the presence of 22 possible serine-containing tryptic

peptides (1,11), the paucity of [^{32}P]-labeled peptides indicates that phosphorylation occurs at only one or at most a very few of the possible sites on the N protein. We conclude from our data that the phosphorylation of the MHV N protein is exceedingly specific and that the phosphorylation sites on the N proteins derived from the three virus strains are different. Particularly interesting is the finding that the N proteins of the two plaque morphology variants of JHM differ in phosphorylation pattern. This observation might account for the difference in the antigenic determinants of the N proteins of DL and DS detected by monoclonal antibodies specific for the DL variant (3). Although the sequence of the N protein of DS is not yet known, comparison of the deduced amino acid sequences of DL and A59 suggest that there is only one nonhomologous region containing a serine residue (between amino acids Ala-136 and Ala-162). Analysis of the sequence divergence in this region suggest that tryptic digestion would yield a hexapeptide from A59 and an octapeptide from DL. Based on predictions of hydrophilicity the A59 peptide would be expected to elute faster from the HPLC than the JHM peptide (6). Although direct proof is lacking, these data suggest that a site of phosphorylation of A59 occurs at serine-135 and of JHM at serine-136. Our data predicts that an analogous serine residue will be absent from DS. Confirmation will await complete sequence analysis of the N gene from DS.

It has been suggested that, in addition to possible roles in regulating viral gene expression and genome replication (2), phosphorylation of viral proteins may play a critical role in the assembly of enveloped viruses (4). To determine if there is indeed a difference in the phosphorylation of the N protein which could be correlated with viral maturation, we compared the [^{32}P]-labeled tryptic peptide maps of A59 N proteins derived from purified virions, cellular membranes and the cytosol of virus-infected cells. No difference could be detected (data not shown), suggesting that complete phosphorylation of the N protein occurs immediately following the synthesis of the protein, in agreement with the previous kinetic studies of the phosphorylation of the N protein (14).

These results indicate that phosphorylation of the N protein of murine coronaviruses is a very specific process, since only one or at most two of the 22 serine-containing tryptic peptides are phosphorylated. The sequences around the phosphorylation sites on the N protein from different MHVs were found to be different from each other. The

biological significance of such variation of phosphorylation sites is not clear, but it may contribute to the antigenic differences in the N proteins of these viruses demonstrated with monoclonal antibodies (3; Gilmore, personal communication).

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