CHARACTERIZATION OF A SMALL PLAQUE MUTANT OF THE A59 STRAIN

OF MOUSE HEPATITIS VIRUS DEFECTIVE IN CELL FUSION

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INTRODUCTION

The virions of murine coronaviruses contain nucleocapsid protein and two membrane associated proteins El and E2. E2 or S glycoprotein forms the peplomer or spike protein and, as shown by Lawrence Sturman and Kathryn Holmes and co-workers (Sturman et al., 1985), is synthesized initially as a 180,000 Dalton glycoprotein that is cleaved into two 90,000 Dalton glycoproteins. They have also demonstrated that the cleaved form of E2 is required for cell fusion (Sturman et al., 1985) and that there are host-dependent differences in the proteolytic cleavage of E2 and in cell fusion (Frana et al., 1985). Recently, Stuart Siddell (Siddell, 1986) has sequenced the E2 gene of the JHM strain of mouse hepatitis virus (MHV) and found a protease cleavage site (arg-arg-ser) approximately in the middle of the open reading frame of RNA 3, the messenger RNA encoding E2.

RESULTS

A mutant or variant of the A59 strain of MHV (MHV A59) was isolated from persistently infected seventeen clone one (17CL1) cells which appears to be defective in the proteolytic cleavage of E2. Initially, 17CL1 cells were infected with 10 pfu/cell of MHV A59. The cells surviving the infection were passaged about forty times over a six month period. After six months in continuous passage, the medium from the persistently infected cells was assayed at 37°C for plaque forming virus and found to contain about 100,000 pfu/ml or 1 pfu per cell of mostly small plaque forming virus. Virus was picked from one of the small plaques and plaque purified directly twice more. Figure 1 illustrates the small plaque nature of the variant or mutant of MHV A59 which we call MHV/sp, for small plaque. This figure shows that the A59 virus produced plaques of about 5 mm diameter on 17CL1 cells after two days at 37°C but the small plaque mutant produced only 1 mm diameter plaques after two days. However, plaques produced by the small plaque mutant grew to 5 mm diameter after 3-4 days. In addition to the difference in plaque size, the small plaque mutant did not cause cell fusion at neutral pH in infected 17CL1 and L cells, whereas MHV A59 did (data not shown). Comparison of the kinetics of viral growth demonstrated that the
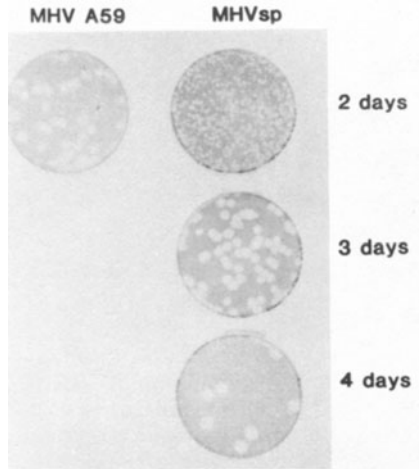
Figure 1. Plaque size of MHV A59 and MHV/sp on 17CL1 cells in 60 mm
           petri dishes at 37°C after 2, 3 or 4 days.

time of virus production, the rate of viral RNA synthesis and the amount
of virus produced by the small plaque mutant was not noticeably
different from that of parental MHV A59. Figure 2 illustrates the
pattern of viral RNA synthesized during a 90 min labeling period with
^{32}P-orthophosphate. Comparable amounts of all seven species of viral
RNA were produced in 17CL1 cells infected with MHV/sp as with MHV A59
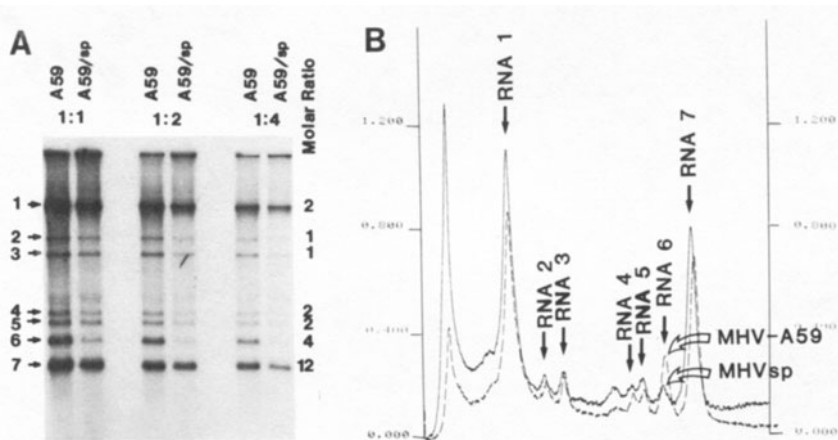

Figure 2. Pattern of viral RNA synthesized in MHV A59 or MHV/sp
           infected 17CL1 cells. A. Cultures of 17CL1 cells were
           infected with 100 pfu/cell of MHV A59 or MHV/sp at 37°C
           and incubated in phosphate-free medium. At 5 h p.i.
           phosphate-free DMEM containing 0.25% BSA, 20 ug/ml
           actinomycin D and 100 uCi/ml of ^{32}P-orthophosphate (ICN,
           Irvine, CA) was added. The cells were labeled for 90
           min and phenol-chloroform extracted. The ethanol
           precipitated material was subjected to electrophoresis
           on 1% agarose gels in 2.2 M formaldehyde as described
           (Sawicki and Sawicki, 1985). B. The fluorogram shown
           in panel A was scanned on a Shimadzu CS-930 dual
           wavelength scanner (Shimadzu Corp., Japan).

virus. The only difference that was noticed was a two-fold relative underproduction of RNA 6 in the MHV/sp infected cells compared to the MHV A59 infected cells. This is illustrated in Figure 2B, which shows a densitometric scan of the fluorogram shown in Figure 2A. Whereas about the same relative quantities of all the other species of viral RNA were produced in MHV A59 and MHV/sp infected cells, RNA 6 was about half the intensity in the MHV/sp infected cells. The mechanism responsible for the underproduction of RNA 6, the messenger RNA encoding the El or M protein, is unknown.

Figure 3 shows a coomassie blue stained polyacrylamide gel of purified virions of MHV A59 and of small plaque mutant. Both viruses were grown on 17CL1 cells under identical conditions and purified by isopycnic banding in potassium tartrate. Virions from both viruses possessed an E1 that migrated at about 23,000 Daltons as several discrete bands with one major band, the nucleocapsid or N protein that migrated at about 50,000 Daltons and a diffuse band that migrated at about 180,000 Daltons. Only the MHV A59 virus possessed a diffuse coomassie blue stained band that migrated at about 90,000 Daltons. Figure 4 shows a fluorogram of a polyacrylamide gel of the purified virions of MHV A59 and MHV/sp that were labeled with ^{14}C-amino acids. Once again, MHV A59 and MHV/sp were grown and purified under identical conditions. None of the 180,000 Dalton form of E2 was cleaved to the 90,000 Dalton form in virus purified from small plaque mutant infected cells. In contrast, virions purified from the MHV A59 infected cells
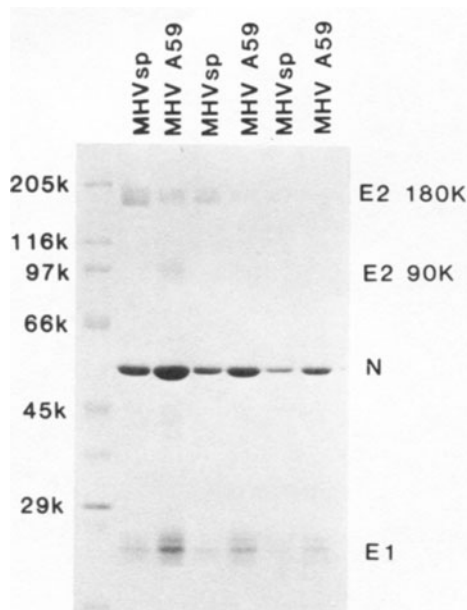

Figure 3.   Purified virions of MHV A59 or MHV/sp were solubilized in sodium dodecyl sulfate (SDS) and subjected to electrophoresis on 8% polyacrylamide SDS gels which were stained with coomassie blue. Two fold dilutions of the purified virions are shown.
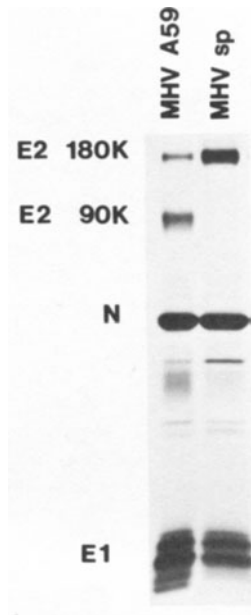
Figure 4.   Fluorogram of ^{14}C-amino acid labeled proteins present in purified virions of MHV A59 or MHV/sp. The proteins were subjected to electrophoresis on 8% polyacrylamide SDS gels before fluorography.

had most of the E2 cleaved to the 90,000 Dalton form. Figure 5 shows a Western blot of the MHV A59 and MHV/sp using goat anti-E2 antibodies (a generous gift of K.V. Holmes) and ^{125}I-labeled Staph A protein. The 90,000 Dalton form of E2 was found in MHV A59 virions but not in virions of the small plaque mutant. Both virions possessed the 180,000 Dalton form of E2 which was detected by anti-E2 antibody. Therefore, the MHV/sp virions were produced without cleavage of E2 and appeared to be as infectious as virions of MHV A59.
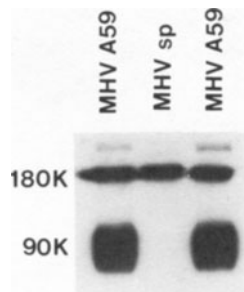

Figure 5.   Western blot of the E2 proteins in purified virions of MHV A59 or MHV/sp subjected to electrophoresis on 8% polyacrylamide SDS gels. The electroblotted proteins were detected after incubation with goat anti-E2 antibodies (the gift of K. V. Holmes) followed by radiolabeled Staph A protein.

Next, I determined if trypsin, chymotrypsin or thermolysin would cleave the 180,000 Dalton form of E2 in virions of the MHV/sp and produce the 90,000 form of E2. Approximately 5ug of purified virions of MHV A59 or MHV/sp in a reaction volume of 4ul was treated with varying concentrations of either trypsin (TPCK treated), chymotrypsin (TLCK treated) or thermolysin for 30 minutes at 37°C at pH 6.5. The samples were solubilized with SDS and subjected to electrophoresis on 8% polyacrylamide SDS gels. The gels were electroblotted onto nitrocellulose which was incubated with anti-E2 and developed with ^{125}I-Staph A protein. Figure 6A shows the results of trypsin treatment. With MHV A59 virions concentrations of 1ug/ml or greater converted the 180,000 Dalton to the 90,000 Dalton form of E2. However, with MHV/sp virions, greater than 100-fold higher concentrations of trypsin were needed to convert the 180,000 Dalton form of E2 to the 90,000 Dalton form. In some experiments, treatment of MHV/sp virions with 1mg/ml of trypsin resulted in complete cleavage of the 180,000 Dalton form of E2 to the 90,000 Dalton form of E2. Changing the pH to 7.4 or 8.0 did not alter the resistance of MHV/sp E2-180K. Figure 6B shows the results of chymotrypsin treatment. Neither the E2-180K protein of MHV A59 nor of
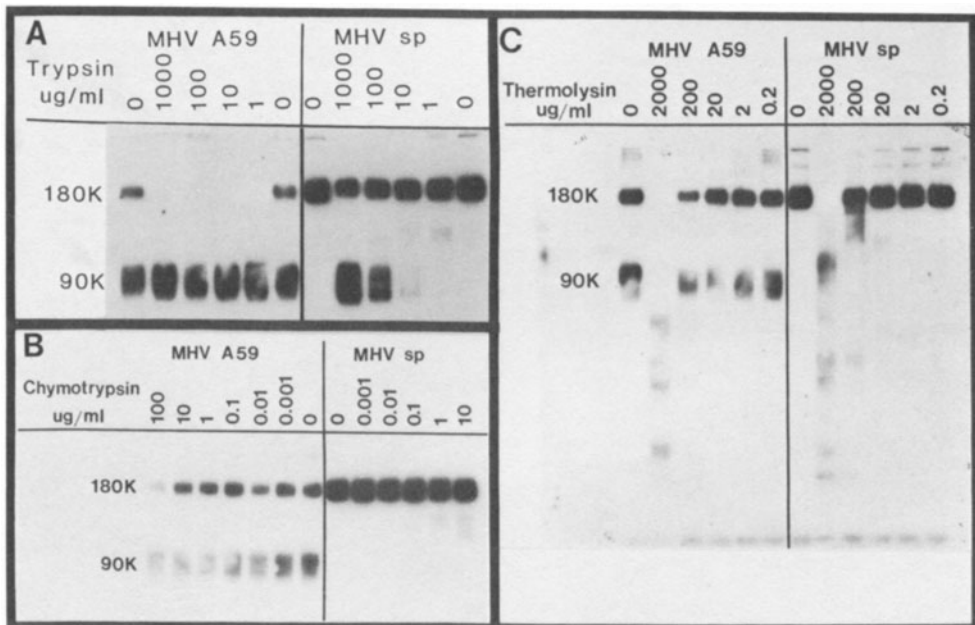

Figure 6. Proteolytic sensitivity of the E2 glycoproteins of MHV A59 or MHV/sp. Equal amounts of purified virions in 2ul were digested for 30 min at 37°C with 2ul of trypsin (A), chymoptrypsin (B) or thermolysin (C) at the concentrations indicated in buffer containing 50 mM Tris-maleate, 1 mM CaCl$_2$, 0.1 M NaCl, pH 6.5, before denaturation, electrophoresis on 8% polyacrylamide SDS gels, and Western blotting.

MHV/sp was cleaved with chymotrypsin. Figure 6C shows that the E2-180K protein of neither virus was sensitive to thermolysin; at very high concentrations (1-2 mg/ml) both the 180,000 Dalton and 90,000 Dalton forms of E2 were degraded to a series of small fragments.

DISCUSSION

These results demonstrate that the small plaque mutant of MHV A59 that was isolated from persistently infected cells is defective in the cleavage of E2. Although trypsin treatment of purified virions of the small plaque mutant will convert the 180,000 Dalton form of E2 to the 90,000 Dalton form, greater than 100 times higher concentrations of trypsin are required for cleavage compared to MHV A59. We do not know if the cleavage of the 180,000 Dalton form of E2 with high concentrations of trypsin will affect infectivity of the small plaque mutant, nor do we know if it will restore cell fusion activity. Sequencing of the E2 gene of the A59 strain and of the small plaque mutant will determine if the small plaque mutant has altered the arg-arg-ser protease cleavage site that was found by Stuart Siddell (1986) in the JHM strain of MHV.

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