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Unique N-linked glycosylation of murine coronavirus MHV-2 membrane protein at the conserved O-linked glycosylation site[☆]

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Abstract

The membrane (M) proteins of murine coronavirus (MHV) strains have been reported to contain only O-linked oligosaccharides. The predicted O-glycosylation site consisting of four amino acid residues of Ser–Ser–Thr–Thr is located immediately adjacent to the initiator Met and is well conserved among MHV strains investigated so far. We analyzed the nucleotide sequence of a highly virulent strain MHV-2 M-coding region and demonstrated that MHV-2 had a unique amino acid, Asn, at position 2 at the conserved O-glycosylation site. We also demonstrated that this substitution added N-linked glycans to MHV-2 M protein resulting in increment of molecular mass of MHV-2 M protein compared with JHM strain having only O-linked glycans. © 2000 Elsevier Science B.V. All rights reserved.

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Murine coronavirus (MHV) is an enveloped virus with single-stranded, positive-sense genomic RNA of about 30 kb. MHV has three to four proteins on the membrane depending on strains. The spike (S) protein is a large glycoprotein and forms surface peplomers which are characteristic

of coronaviruses. The membrane (M) protein plays an important role in viral assembly together with the small membrane (E) protein. The hemagglutinin-esterase (HE) protein is contained in some MHV strains and in other species of serogroup 2 coronaviruses (reviewed in Siddell, 1995; Holmes and Lai, 1996).

The M protein is the integral membrane protein spanning the lipid bilayer three times. A short NH₂-terminal region (about 25 amino acids) is exposed on the virion surface, whereas most of it resides inside the virion (Armstrong et al., 1984;

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Krijnse Locker et al., 1992b). The M protein of the MHV-A59 strain has been reported to contain only O-linked oligosaccharides (Niemann et al., 1984; Krijnse Locker et al., 1992a). The O-glycosylation site is considered to be located at a cluster of four hydroxylamino acids at the NH₂-terminus: Ser–Ser–Thr–Thr immediately adjacent to the initiator Met (reviewed in Rottier, 1995). The M proteins of four other MHV strains so far sequenced also contained a well conserved hydroxylamino acid cluster forming potential O-glycosylation sites (Pfleiderer et al., 1986; Homberger, 1994). Recently de Haan et al. (1998b) clearly showed that Thr at position 5 is exclusively O-glycosylated by expressing a wide variety of mutant M proteins in mammalian cells.

Coronaviruses are separated into three groups based on their antigenic properties. MHV according to this classification belongs to group 2. The evidence that the M protein contains only O-linked oligosaccharides has been found in other members in group 2: bovine coronavirus (BCV) and human coronavirus (HCV)-OC43 (Lapps et al., 1987; Mounir and Talbot, 1992). Unlike the M proteins of group 2, the M proteins of porcine transmissible gastroenteritis virus (TGEV) and avian infectious bronchitis virus (IBV) of groups 1 and 3, respectively, have been demonstrated to be

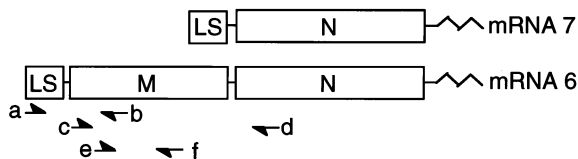


Fig. 1. Primer locations on mRNA 6 of MHV. LS, leader sequence. M, the M protein coding region. N, the N protein coding region. (a) 5'-TATAAGAGTGATTGGCGTCC-3' [genomic sense of JHM leader sequence (Skinner and Siddell, 1983)]. (b) 5'-CACATTATTTAGCGCAT-3' [complementary sense of the JHM M coding region, nt. 230 to 246 (Pfleiderer et al., 1986)]. (c) 5'-CATTATGAATAGTACCACTC-3' [genomic sense of the MHV-2 M coding region, nt. -4 to 16]. (d) 5'-TTTAAACCCGCGCTCGGTTT-3' [complementary sense of the JHM N coding region, nt. 97 to 116 (Skinner and Siddell, 1983)]. (e) 5'-CATACTACAGTTCGGTT-3' [genomic sense of the JHM M coding region, nt. 117 to 133 (Pfleiderer et al., 1986)]. (f) 5'-GACTTCACATAAACAGC-3' [complementary sense of the JHM M coding region, nt. 601 to 617 (Pfleiderer et al., 1986)].

N-glycosylated (Stern and Sefton, 1982; Kapke et al., 1988).

The MHV-2 strain is a highly virulent strain of MHV (Hirano et al., 1981). The molecular mass of the M protein of MHV-2 has been reported to be larger than that of MHV-A59 (Keck et al., 1988), although the molecular basis for this difference has not been addressed as yet. In this paper, we have demonstrated the presence of N-linked oligosaccharides attaching to MHV-2 M protein, in addition to conserved O-glycans, by means of nucleotide sequencing and biochemical analysis. The results indicate that the additional N-linked glycans make the molecular mass of MHV-2 M protein larger than other MHV M proteins with only O-linked oligosaccharides.

Total cellular RNA was extracted from DBT cells infected with MHV-2 or other MHV strains namely: MHV-1, -3, -S, -D, and -NuU, at 16 h post inoculation. After extraction, 10 µg of RNA was reverse transcribed into cDNA using oligo(dT) as a primer, as described previously (Yamada et al., 1993). The cDNA transcripts (5 µl) were amplified by PCR using 2.5 U Takara Ex Taq (Takara Biochemicals), in the presence of 0.25 µM each of dATP, dCTP, dTTP, and dGTP (dNTPs) and 0.2 µM each of adequate primers (Fig. 1). The amplified samples were electrophoresed on a 1.0% agarose gel and purified by the prep-A Gene DNA purification kit (Bio-Rad). Sequencing of PCR products was performed by a dideoxy termination labeling method (Applied Biosystems; Model 373A-18 DNA sequencing system).

The first PCR was performed to amplify a part including non-coding leader sequence of about 74 nucleotides and one-third of the M protein coding region using primers a and b (Fig. 1) from the cDNA of MHV-2-infected DBT cells, since the presence of abundant N protein-coding mRNA 7 (which shares sequences at 5'- and 3'-terminal sequences with M protein-coding mRNA 6), hampered amplification of the entire M gene using primers a and d. After determining directly the nucleotide sequence of this PCR product, the cDNA spanning the entire MHV-2 M coding region and 117 nucleotides of N coding region was amplified using primers c and d. The am-

plified PCR products were directly sequenced using primers c to f. Sequences of a part including the initiation site of MHV-1, -3, -S, -D, and -NuU were also analyzed on PCR products amplified using primers a and b.

Because the amino acid substitution was found at the conserved O-glycosylation site in the M protein of MHV-2 compared with other strains, the entire M gene of MHV-2 and a mutated MHV-2 M gene with the nucleotide sequence of JHM at the O-glycosylation site were subjected to vaccinia virus expression system. The entire coding region of the M gene of MHV-2 was amplified using primers 2M-PH: 5'-CGAAGCTTCATTATGAATAGTACCACTC-3' and 2M-NB: 5'-CGGGATCCTAGATTAGATTCTCAACAAT-3'. In order to introduce a mutation at nucleotide 5, 4M-PH primer: 5'-CGAAGCTTCATTATGAGTAGTACCACTC-3' was used instead of 2M-PH primer. PCR products were inserted into the *Hind*III and *Bam*HI sites of pGEM4Z (Promega). The resulting plasmids were designated pM-2 and pM-4/2, respectively. Nucleotide sequences of inserts were confirmed to be correct by sequence analysis. RK cells were infected with a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3, kindly provided by Dr B.H. Moss) and then transfected with plasmids, pM-2, and -4/2 by electroporation as described previously (Ohtsuka et al., 1996). At 16 h post transfection, transfected cells were washed with PBS and lysed with PBS containing 0.65% Nonidet P-40. The lysed samples were centrifuged at 15000 rpm for 10 min. The supernatants were collected and stored at -20°C .

To deglycosylate N-linked sugars, 5 μl of RK cell lysate expressing MHV-2 M protein was denatured by boiling in a buffer containing 0.5% SDS and 50 mM β -mercaptoethanol. It was then treated with 0.3 U N-glycosidase F (natural N-glycanase, Genzyme) for 1 h at 37°C . As a control, expressed MHV-2 M protein was treated in the same way without N-glycanase.

For the analysis of viral M protein, DBT cells were infected with MHV-2 or JHM (MOI 1). At 12 h post inoculation, culture fluids were clarified by spinning at 3000 rev./min for 10 min. Ten microlitres of supernatants of virus-infected cell

culture, lysates of plasmid-transfected cells following vaccinia virus inoculation or MHV-2 M protein-expressing cell lysates with or without N-glycanase treatment were subjected to SDS-polyacrylamide gel electrophoresis using a 10% gel. Samples were then analyzed by Western blotting as described previously (Kubo et al., 1994) using polyclonal rabbit anti-MHV antiserum (kindly provided by Dr T. Itoh) and horseradish peroxidase-conjugated anti-rabbit IgGs (Cappel Organon Teknik). The bands were visualized with enhanced chemiluminescence (Amersham).

The M protein gene of MHV-2 was sequenced directly from RT-PCR products amplified from the template RNA of virus-infected cells. In the M protein coding region, 45 nucleotides were different as compared with A59, resulting in 11 amino acid substitutions (Fig. 2). Among these replacements, the first was found at the hydroxylamino acid cluster forming a potential O-glycosylation site. The amino acid immediately adjacent to the initiator Met was Asn in MHV-2 instead of Ser in A59. Since it was previously reported that the potential O-glycosylation site was well conserved among MHV strains (Hombberger, 1994), the nucleotide sequences of this site of additional MHV strains that had not been investigated, were analyzed. As shown in Fig. 3, amino acid sequences of this site were Ser-Ser-Thr-Thr in all of five newly analyzed MHV strains in this paper namely: MHV-1, -3, -S, -D, and -NuU, in addition to five strains reported previously. MHV-2 was, therefore, the only strain with an amino acid substitution at this site.

The molecular mass of the M protein of MHV-2 strain has been reported to be larger than that of MHV-A59 (Keck et al., 1988). We confirmed this evidence with Western blot analysis using MHV-2 and JHM viral particles. The M protein of JHM (Fig. 4, lane 1) was detected as a broad band of 23–25 kDa. On the other hand, MHV-2 M protein (lane 2) appeared as minor 23, major 28 and 30 kDa species. Among these bands, 23 kDa was considered as an unglycosylated form (de Haan et al., 1998b).

To demonstrate the role of a second amino acid substitution in increased molecular mass of the M protein in MHV-2, a mutated M protein in which

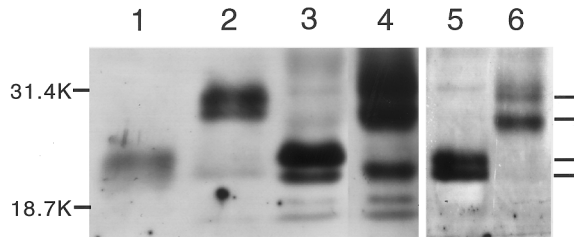


Fig. 4. Western blot analysis of the M protein. Viral M proteins of JHM (lane 1) and MHV-2 (lane 2), lysates of RK cells transfected with pM-4/2 (lane 3) and pM-2 (lane 4) following vaccinia virus inoculation, MHV-2 M protein-expressing cell lysates with (lane 5) or without (lane 6) N-glycanase treatment were electrophoresed followed by Western blotting. Marker sizes are indicated on the left. Four major bands of the M protein are marked on the right.

second amino acid Asn in MHV-2 M protein actually accepted N-linked glycans, resulting in increment of the molecular mass of MHV-2 compared with other MHV strains with only O-linked glycans.

Sequence analysis of MHV-2 M protein revealed that MHV-2 had a unique amino acid, Asn, at position 2 at the O-glycosylation site which was conserved among murine coronaviruses. We have demonstrated biochemically that Asn at position 2 was N-glycosylated and that these N-glycans made the molecular mass of MHV-2 M protein larger than those of other MHV strains. de Haan et al. (1998a) previously demonstrated that an experimentally created M mutant of A59 strain with Asn at position 2 made the molecular mass of the M protein larger than that of parental A59. They also showed that Thr at position 5 and Pro at position 8 were required for O-glycosylation and that both N- and O-linked glycans were present in the mutant M protein of A59 with Asn at position 2 (de Haan et al., 1998b). Since Thr at position 5 and Pro at position 8 were conserved in MHV-2, MHV-2 M protein is considered to have both N- and O-linked oligosaccharides like mutant M protein of A59 with Asn at position 2. This paper is the first report demonstrating that an MHV strain has both N- and O-linked oligosaccharides on the M protein resulting in an increment of the molecular mass.

Although the molecular mechanisms of N-linked glycosylation are well understood, those of O-linked glycosylation have yet to be studied. In this sense, the M proteins of coronaviruses in group 2 seem to provide a good tool for elucidation of the mechanisms of O-glycosylation, since they have been reported to carry only O-linked oligosaccharides. As we have demonstrated the presence of additional N-linked sugars, careful attention should be paid when using MHV-2 M protein for this purpose.

It is not clear whether the presence of extra sugars affects the function of the M protein or not. Viral assembly, an event in which the M protein plays an important role, is not interfered with by the additional N-glycosylation, since equal efficiency was displayed by both the mutant M protein of A59 with N-linked oligosaccharides and the original A59 M protein lacking N-glycan in assembling in virion-like particles (de Haan et al., 1998a). MHV-2 does not induce cell-to-cell fusion on cultured DBT cells forming syncytium, as opposed to most MHV strains. However, it seems unlikely that MHV-2 M protein is responsible for non-syncytium formation, since the S protein has been demonstrated to be the only element that determines the phenotype of MHV strain with regard to syncytium formation (Yamada et al., 1997). The M protein of MHV-2 does not seem to have sufficient role in changing pathogenicity or tissue tropism, since an A59-based recombinant virus containing the entire M gene of MHV-2 produced encephalitis and hepatitis similar to parental A59 following intracerebral inoculation (Lavi et al., 1998). Further investigation is required to elucidate whether the differently modified M protein of MHV-2 is somehow involved in determining the biological characteristics of this particular strain.

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