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NOTES

Monoclonal Antibodies to the Peplomer Glycoprotein of Coronavirus Mouse Hepatitis Virus Identify Two Subunits and Detect a Conformational Change in the Subunit Released under Mild Alkaline Conditions

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Monoclonal antibodies (MAbs) directed against the E2 glycoprotein of mouse hepatitis virus (MHV) have been classified according to their ability to bind to either of the two purified 90,000-molecular-weight subunits (90K subunits) of the 180K peplomeric glycoprotein E2. Correlation with previously reported information about these MAbs suggests that both of the subunits of E2 are important for viral infectivity and cell fusion. Incubation of trypsin-treated virions at pH 8.0 and 37°C released only the $E2_N$ subunit from virions. The pattern of MAb reactions suggested that a conformational change occurred in the $E2_N$ subunit in association with its release from virions under mildly alkaline conditions at 37°C, the same conditions which are optimal for coronavirus-induced cell fusion.

The spikes or peplomers on the envelope of mouse hepatitis virus strain A59 (MHV-A59), a murine coronavirus, are composed of a multifunctional glycoprotein called E2 (or S) (18). This glycoprotein is responsible for attachment to virus-specific receptors on plasma membranes of susceptible murine cells, virus-induced cell fusion, and induction of neutralizing antibody and of cell-mediated immune response (1, 5–7, 9, 10, 22–24). The gene for this glycoprotein has been cloned and sequenced (12, 17). Several laboratories have prepared and characterized panels of monoclonal antibodies (MAbs) directed against E2, mapped their antigenic regions, and identified functions inhibited by the MAbs (5, 6, 23). There is considerable interest in identifying the functional domains of the E2 molecule.

In this report, 28 anti-E2 MAbs are assigned to either of the two subunits of E2. The E2 subunits of MHV-A59, designated $E2_N$ (the amino-terminal subunit of E2) and $E2_C$ (the carboxy-terminal subunit of E2), are generated within virus-infected cells by protease cleavage of the 180,000-molecular- weight E2 protein (180K E2 protein) or can be produced by treatment of isolated virions with trypsin (7, 18). Highly purified preparations of the two subunits were prepared by treating gradient-purified virions with trypsin to quantitatively convert the 180K E2 to the two 90K subunits, solubilizing the subunits with Triton X-114 or Nonidet P-40, purifying the 90K subunits by size exclusion chromatography, and separating the $E2_N$ and $E2_C$ subunits by chromatography on hydroxyapatite (16, 18). The $E2_N$ and $E2_C$ subunits could be distinguished by their incorporation of different ratios of radiolabeled precursors and by the observation that only $E2_C$ had covalently bonded palmitic acid (12, 19).

Two panels of MAbs directed against MHV E2 prepared at Scripps Clinic and Research Foundation and at the University of Southern California were selected for use, as they had already been characterized extensively and some information was available on the E2 functions inhibited by these MAbs and on mapping of some antigenic regions by competitive binding assays. The Scripps Clinic MAbs were prepared from animals infected with MHV-JHM (MHV-4), and their antigen specificities were identified by immunofluorescence on fixed virus-infected cells and by immunoprecipitation of radiolabeled viral proteins (2, 5, 22). The University of Southern California MAbs were prepared by infection of mice with MHV-JHM or MHV-A59, followed by selection by solid-phase immunoassay and characterization by immunoprecipitation of radiolabeled viral proteins (6, 8). Biological activities of the MAbs, including virus neutralization, passive protection of mice from virus infection, and inhibition of virus-induced cell fusion, were determined as described previously (5, 6).

To determine which of the E2 subunits each MAb reacted with, solid-phase immunoassays were performed. The reactivities of each MAb with intact MHV-A59 virions or with purified $E2_N$ or $E2_C$ were determined, and representatives of each type of specificity observed are illustrated in Fig. 1. The specificities of all of the MAbs tested are summarized in Tables 1 and 2. Of the 11 MAbs from Scripps Clinic which could be unequivocally assigned to one subunit of E2, all but

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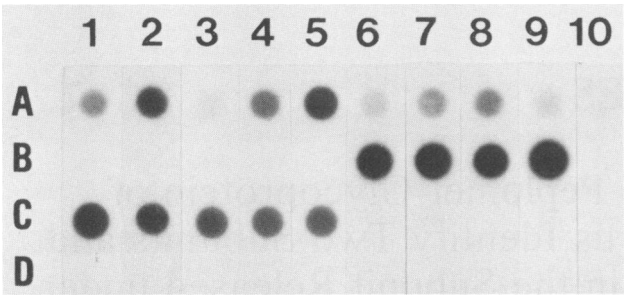

FIG. 1. Specificity of anti-E2 MAbs for the $E2_N$ and $E2_C$ subunits of the MHV peplomer glycoprotein E2. The solid-phase immunoassay compares the reactivities of antigens on virions (row A) with SDS-treated, high-pressure liquid chromatography-purified subunits $E2_C$ (row B) and $E2_N$ (row C) from trypsin-treated virions and buffer control (row D) with MAbs in the following columns: 1, J.7.1; 2, A.2.5; 3, A.1.3; 4, A.1.12; 5, A.3.10; 6, 5B170.3; 7, 5B21; 8, 5B207.3; 9, 5B15; 10, buffer control. Nitrocellulose sheets in a 96-well apparatus were coated with 2 μg of viral or control antigens per well, removed from the apparatus, blocked for 1 h with 2% bovine serum albumin in dilution buffer (DB) (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, 0.1% bovine serum albumin), and incubated overnight at 4°C with a 1:50 dilution of supernatant medium from hybridoma cultures containing MAbs (approximately 10 to 40 μg of immunoglobulin per ml). The panels were washed in DB three times for 5 min each, and samples 6 to 10 were incubated for 1 h with affinity-purified rabbit anti-mouse immunoglobulin G (heavy and light chain), diluted 1:1,000 in DB, and washed three times in DB. Finally, bound antibody in all samples was detected by incubation for 1 h with radioiodinated staphylococcal protein A (specific activity, approximately 8 μCi/μg, 10^5 cpm/ml; 2.5 ml/1.5 cm^2). Panels were washed in DB three times and analyzed by autoradiography.

one were directed against the $E2_C$ subunit. In our assay, most of these required amplification with rabbit anti-mouse immunoglobulin G for detection by radioiodinated staphylococcal protein A. In contrast, 10 of the 13 MAbs from the University of Southern California group which could be assigned to one subunit of E2 were directed against the $E2_N$ subunit, and most of these did not require amplification with rabbit anti-mouse immunoglobulin G for detection in our solid-phase assay system. The difference in the repertoires of the MAbs from the two laboratories is of considerable interest and may result from differences in immunization or MAb screening strategies (5, 6).

Five of the MAbs (4B11.6, J.1.2, J.2.2, J.7.2, and 5A13.5) could not be assigned unequivocally to either E2 subunit of MHV-A59. One of these MAbs, 4B11.6, previously mapped to antigenic region C of MHV-JHM (MHV-4), apparently recognizes a conformationally determined antigenic region since it does not bind to MHV-JHM (MHV-4) E2 on an immunoblot (21, 22). Four of these MAbs, 5A13.5, J.1.2, J.2.2, and J.7.2, show strong neutralizing activity for MHV-JHM (University of Southern California) but did not react with MHV-A59 in a radioimmunoassay (6).

Makino et al. (13) concluded, on the basis of analysis of recombinants between MHV strains, that the domain of E2 involved in cell fusion may be located in the carboxy-terminal third of E2, which would lie within $E2_C$. This conclusion was based on the lack of reactivity with fusion-inhibiting MAb A.3.10 of recombinant viruses RL-1 and EL-6, which were believed by oligonucleotide mapping to contain only the carboxy-terminal third of E2 from one parental virus. However, our study shows that MAb A.3.10 identifies an antigenic region in $E2_N$. Both of these recombinants also react with MAbs A.2.3 and J.7.5 (13), which we have localized as binding to the $E2_N$ subunit. It is therefore possible that recombinants RL-1 and EL-6, which have the smallest amount of MHV-A59 oligonucleotide sequence in the E2 gene, contain deletions within this gene, rather than having a single recombination as previously supposed. Consequently, it may be premature to use data from recombinants to identify the domain(s) of E2 responsible for cell fusion. A complete panel of MAbs which identify all antigenic regions of both subunits would be required to characterize recombinants in the E2 gene, with the understanding that recombination or deletions within the E2 gene could result in conformational changes in the glycoprotein which could affect antigenic regions distant from the site of genetic alteration.

Evidence was obtained for recognition of conformationally masked antigenic regions in E2 by several MAbs. For example, in our immunoassay, MAbs A.1.3 and A.1.9 (Fig.

TABLE 1. MAbs specific for the $E2_N$ subunit of MHV-A59

MAb[a]	Antigenic region[b]	Isotype	Biological activity[c]			Binding specificity		
			Neutralization	Passive protection	Inhibition of cell fusion	$E2_N$	$E2_C$	Virus
A.3.10	A	IgG2a	−	ND[d]	+	+	−	+
A.1.3	A	IgG3	−	ND	−	+	−	+/−
A.1.9	A	IgG3	−	ND	−	+	−	−
A.1.12	A	IgG2a	−	ND	−	+	−	+
A.2.3	A	IgG3	+	ND	−	+	−	+
J.2.5	A	IgG2a	−	+	+	+	−	+
J.7.5	A	IgG2b	+	+	−	+	−	+
J.7.6	A	IgG2b	+	ND	ND	+	−	+
A.1.4	B	IgG2a	−	ND	−	+	−	+
J.7.1	ND	IgG2a	−	ND	−	+	−	+
5B62	ND	ND	−	−	ND	+	+/−	+

[a] MAbs are from references 5 and 6. MAbs beginning with letters are from the University of Southern California (6, 8), and MAbs beginning with numbers are from the Scripps Clinic and Research Foundation (2, 5, 22).

[b] Designated in references 8 and 13 or shown to compete with MAbs which identify the designated antigenic region. Note that these antigenic regions are different from those on $E2_C$ in Table 2.

[c] Determined previously (6) or by the same methods.

[d] ND, Not done.

TABLE 2. MAbs specific for the $E2_C$ subunit of MHV-A59

MAb[a]	Antigenic region[b]	Isotype	Biological activity[c]			Binding specificity		
			Neutralization	Passive protection	Inhibition of cell - fusion	$E2_N$	$E2_C$	Virus
5B19.2	A	IgG1	++	+	+	−	+	+
5B170.3	A	IgG1	+	+	+	−	+	+
5B21	D	IgG1	+	−	ND[d]	−	+	+
5B207.3	E	IgG2b	−	−	ND	−	+	+
5B216.8	E	IgG2a	−	−	ND	−	+	+
5B4	ND	ND	ND	ND	ND	−	+	+
5B15	ND	ND	ND	ND	ND	−	+	+
5B81	ND	ND	ND	ND	ND	−	+	+
5B93.7	ND	ND	+	−	ND	−	+	+
J.7.18	ND	IgG3	+	ND	+	−	+	+
J.1.16	ND	IgG2b	−	ND	−	−	+	+
J.2.6	ND	IgG2b	+	ND	+	−	+	+

[a] MAbs are from references 5 and 6. MAbs beginning with letters are from the University of Southern California (6,8), and MAbs beginning with numbers are from the Scripps Clinic and Research Foundation (2, 5, 22).
[b] Designations are from reference 5 or the regions were shown to compete with MAbs which identify the designated antigenic region.
[c] Determined previously (2, 6, 22) or by the same methods.
[d] ND, Not done.

1, column 3, and Table 1) did not recognize E2 in its native configuration on virions, whereas they did bind well to an antigenic region on sodium dodecyl sulfate (SDS)-denatured, purified $E2_N$. This MAb-binding domain may be hidden within the spike on undenatured virions. Several MAbs specific for antigenic regions A and B of E2, including 5B170.3 and 5B13.5, were assigned to $E2_C$, the subunit which is anchored in the lipid bilayer of the viral envelope (12). In our solid-phase immunoassay, both of these MAbs and MAb 5B15 recognized the SDS-denatured, purified $E2_C$ subunit better than the intact virion (Fig. 1, columns 6 and 9). This observation suggested that some antigenic regions on $E2_C$ are at least partially hidden on the intact virus. In contrast, most of the antigenic regions of $E2_N$ were recognized by MAbs equally well on both virions and the isolated, denatured $E2_N$ subunit. This may reflect the structure of the N-terminal subunit or its greater accessibility on the peplomer.

These results show that both the $E2_N$ and the $E2_C$ subunits contain determinants which will bind MAbs that neutralize virus or block virus-induced cell fusion. In infectious bronchitis virus, both neutralizing and hemagglutination-inhibiting MAbs were initially believed to be directed against only the S1 ($E2_N$) subunit (4), but some MAbs which neutralize virus have more recently been located on the S2 ($E2_C$) subunit (11). The finding that both subunits of E2 may be important for coronavirus infectivity and cell fusion emphasizes the complexity of the E2 glycoprotein. The E2 subunits may be assembled in a configuration in which certain MAbs to either subunit may interfere with functional domains of the E2 glycoprotein. Thus, localization to either subunit of MAbs which inhibit biological functions does not necessarily indicate which of the two subunits contains those functional domains.

Identification of the E2 subunit released from virions under mildly alkaline conditions. The infectivity of MHV-A59 is rapidly and irreversibly inactivated by brief treatment at pH 8.0 and 37°C. Virus-induced cell fusion also occurs optimally under mildly alkaline conditions (20). These functional changes are associated with the aggregation of E2 peplomers and the release of some cleaved 90K E2 from virions (20). MAbs assigned to the two subunits of E2 were used to identify this released material. A panel of MAbs was tested in solid-phase immunoassays with the 90K E2 which had been released from virions following trypsin treatment and incubation at pH 8.0 at 37°C for 18 h and purified by removal of virions by pelleting at 35,000 rpm for 60 min at 4°C in an SW41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). No reaction was observed with MAbs specific for $E2_C$, but several MAbs specific for $E2_N$ reacted with the released

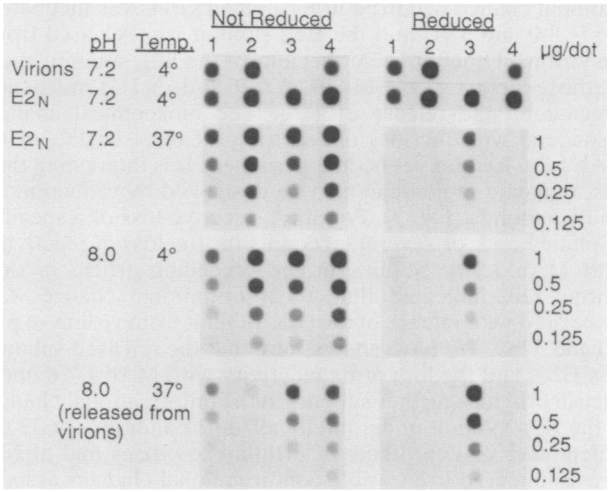

FIG. 2. Effects of temperature and reduction upon antigenic domains of MHV-A59 E2 subunit $E2_N$. This solid-phase immunoassay compares the antigenic domains on virions and on $E2_N$ prepared from trypsin-treated virions as described for Fig. 1, with $E2_N$ prepared as for Fig. 1 from virions treated either at pH 7.2 and 37°C for 30 min or at pH 8.0 and 4°C for 30 min, or with $E2_N$ released from virions by incubation at pH 8.0 and 37°C for 30 min followed by ultracentrifugation to remove virions. The amounts of antigen per dot are indicated at the right. Antigens in the panels on the left were unreduced, and antigens in the panels on the right were reduced with 5% β-mercaptoethanol at 100°C for 5 min before application to the nitrocellulose. After being blocked with bovine serum albumin, antigens were reacted either with goat antibody directed against MHV-A59 peplomers purified by gradient ultracentrifugation from Nonidet P-40-disrupted virus (columns 1) or with anti-$E2_N$ MAb J.7.6 (columns 2), A.1.9 (columns 3), or A.1.4 (columns 4).

material (Fig. 2). Therefore, mild alkaline treatment at 37°C specifically liberated $E2_N$, the amino-terminal subunit of E2, from virions. This is similar to data obtained with infectious bronchitis virus which showed that the S1 subunit (corresponding to $E2_N$ of MHV) can be released from virions by treatment with urea (3, 4). In MHV-A59, urea treatment does not release a subunit from the virions (L. S. Sturman, unpublished observation). The biological significance of the release of the amino-terminal subunit of peplomers from several serologically unrelated coronaviruses remains to be determined.

MAb detects a conformational change of $E2_N$ at pH 8.0 and 37°C. Several MAbs specific for $E2_N$ were tested for reactivity with reduced or unreduced, SDS-treated $E2_N$ isolated from trypsin-treated virions incubated at pH 7.2 and 37°C or pH 8.0 and 4°C for 16 h and also with $E2_N$ released from trypsin-treated virions by treatment at pH 8.0 and 37°C for 16 h (Fig. 2). Reduction of disulfide bonds in $E2_N$, regardless of the manner in which it was prepared, resulted in loss of reactivity with polyclonal anti-E2 antibody and with MAbs J.7.6 and A.1.4, indicating that these antibodies recognized conformationally determined antigenic regions of $E2_N$. In contrast, MAb A.1.9 reacted equally well with reduced or unreduced purified $E2_N$ but did not recognize E2 on MHV-A59 virions. Thus, this MAb probably recognized a linear antigenic region which was apparently unmasked when virions were disrupted.

MAb J.7.6 reacted with undenatured virus and with SDS-denatured but unreduced $E2_N$ prepared from virions incubated at pH 7.2 and 37°C or pH 8.0 and 4°C, but MAb J.7.6 failed to react with unreduced $E2_N$ released from virions by incubation at pH 8.0 and 37°C. This suggests that a conformational change occurred in $E2_N$ when virus was incubated at pH 8.0 and 37°C and the $E2_N$ subunit was released from the virions. Other antigenic regions of the $E2_N$ subunit, such as those detected by MAbs A.1.9 and A.1.4, were not affected by the release of $E2_N$. The biochemical change associated with the loss of reactivity of released $E2_N$ with MAb J.7.6 has not yet been determined. It is interesting that this antigenic region can also be destroyed by reduction of disulfide bonds (Fig. 2). Possibly, selective loss of a specific disulfide bond, or several, accounts for the loss of reactivity with MAb J.7.6. Studies in the preceding article in this journal (20) indicated that a conformational change was associated with release of the 90K subunit from virions at pH 8.0 and 37°C. We have shown here that the released subunit was $E2_N$, and the loss of its reactivity with MAb J.7.6 upon release provides further support for a conformational change of the $E2_N$ subunit under mildly alkaline conditions at 37°C.

Peplomer glycoproteins of orthomyxoviruses and alphaviruses undergo irreversible conformational changes associated with lowering the pH to 5.5 (14). This change is believed to be associated with activation of the fusion activity of the viral glycoproteins, permitting fusion of the viral envelopes with endosomal membranes after intracellular acidification of the endosomes. Fusion of the viral envelopes with the plasma membrane can occur if the pH of the medium is briefly lowered to 5.5 to activate the fusion glycoprotein of the virus. Our data show that the coronavirus E2 glycoprotein, which is responsible for both attachment to receptors and cell fusion, requires both proteolytic cleavage and pH-dependent conformational change for activation. The coronavirus E2 glycoprotein, like those of paramyxoviruses and human immunodeficiency virus (14, 15), showed a conformational change associated with alkaline, rather than acidic, conditions. It is therefore possible that coronaviruses, like paramyxoviruses and human immunodeficiency virus, initiate infection by fusion of the viral envelope with the plasma membrane rather than with endosomal membranes.

Conformational changes in the protease-cleaved influenza virus hemagglutinin are triggered by low pH and are associated with rapid and irreversible loss of viral hemolytic activity and infectivity (25). Following treatment of virions at low pH, certain MAbs showed greatly increased or decreased binding to each of the four major antigenic regions on both subunits of the influenza virus hemagglutinin glycoprotein. We have demonstrated that treatment of coronavirus E2 at pH 8.0 and 37°C leads to analogous changes in virus infectivity and in reduced reactivity with MAb J.7.6. These observations suggest that conformational changes in the E2 glycoprotein of MHV may be associated with the initiation of fusion and infectivity.

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