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Functional Analysis of Matrix Proteins Expressed from Cloned Genes of Measles Virus Variants That Cause Subacute Sclerosing Panencephalitis Reveals a Common Defect in Nucleocapsid Binding

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We have developed an in vitro nucleocapsid-binding assay for studying the function of the matrix (M) protein of measles virus (MV) (A. Hirano, A. H. Wang, A. F. Gombart, and T. C. Wong, Proc. Natl. Acad. Sci. USA, 89:8745–8749, 1992). In this communication we show that the M proteins of three MV strains that cause acute infection (Nagahata, Edmonston, and YN) bind efficiently to the viral nucleocapsids whereas the M proteins of four MV strains isolated from patients with subacute sclerosing panencephalitis (SSPE) (Biken, IP-3, Niigata, and Yamagata) fail to bind to the viral nucleocapsids. MV Biken (an SSPE-related virus) produces variant M sequences which encode two antigenically distinct forms of M protein. A serine-versus-leucine difference is responsible for the antigenic variation. MV IP-3 (an SSPE-related virus) also produces variant M sequences, some of which have been postulated to encode a functional M protein responsible for the production of an infectious revertant virus. However, the variant M proteins of Biken and IP-3 strains show no nucleocapsid-binding activity. These results demonstrate that the nucleocapsid-binding function is conserved in the M proteins of MV strains that cause acute infection and that the M proteins of MV strains that cause SSPE exhibit a common defect in this function. Analysis of chimeric M proteins indicates that mutations in the amino-terminal, carboxy-proximal, or carboxy-terminal region of the M protein all abrogate nucleocapsid binding, suggesting that the M protein conformation is important for interaction with the viral nucleocapsid.

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative neurological disease caused by persistence of measles virus (MV) in the central nervous system (22). The virus isolated from brains of patients with SSPE by cocultivation with cultured cells is typically defective in virion production. It spreads from cell to cell by inducing cell fusion (24). Early studies revealed that patients with SSPE lacked antibodies against the matrix (M) protein of MV (11, 25), and M protein was not detected in the brains of patients with SSPE by antibodies against MV (9, 10). Since M protein plays an important role in paramyxovirus maturation (8), the apparent absence of M protein was hypothesized to be the cause of nonproductive infection by MV strains that cause SSPE (7).

Subsequent studies showed that the genes encoding M protein of virus strains that cause SSPE are inundated with mutations. The diverse mutations can affect the translation products (1, 5, 6, 21) or the translation process (2, 5). Besides the changes in the M gene, abnormalities and sequence alterations have been observed in other genes of MV strains that cause SSPE, especially those encoding the fusion (F) and hemagglutinin (H) proteins (3, 5, 15, 20). However, the functions of the viral proteins in MV strains that cause SSPE and the biological significance of the protein changes in chronic infection have not been determined.

MV strains that cause SSPE are genetically heterogeneous (5). The IP-3 strain produces variant M sequences, including sequences postulated to encode a partially functional M protein that allows the production of a revertant virus (6). Variant M sequences are also found in the Biken strain (see below). Because the variant M proteins can potentially affect the biological phenotype of the virus, the variant M protein sequences are of great interest.

A major function of the paramyxovirus M protein is to interact with the viral nucleocapsids during virus maturation (13, 17). In the present study, we examined the nucleocapsid-binding function of the M proteins in three MV strains that cause acute infection and four strains that cause SSPE and we examined the M proteins produced by variant M sequences in two of the MV strains that cause SSPE. We studied chimeric M proteins to investigate how mutations in different regions of the M protein might affect the nucleocapsid-binding function.

MATERIALS AND METHODS

Viruses and cells. The Edmonston and Nagahata strains of MV, which cause acute infection, and the Biken, Niigata, and Yamagata strains, which cause SSPE, have been described previously (1, 2, 26, 27). The YN strain, which causes acute infection, was isolated in Japan in 1989 and was kindly provided by S. Ueda, Osaka University. Viruses were propagated in African green monkey kidney CV-1 cells. Biken-HEL cells, generated by cocultivating human embryonic lung (HEL) cells with brain tissues from patients with SSPE (23), and Biken–CV-1 cells, persistently infected with the Biken strain (12), were also kind gifts from S. Ueda. All cells were maintained in Eagle's minimum essential medium supplemented with 10% newborn calf serum.

Plasmids. Full-length cDNAs representing the M genes of Edmonston and Nagahata strains, which cause acute infec-

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tion, and Biken, Niigata, and Yamagata strains, which cause SSPE, have been described previously (1, 2, 26, 27). Full-length cDNAs representing the M gene of the YN strain were selectively cloned with the pTZ-18RX vector system as previously described (1). The same method was used to obtain the full-length M cDNAs BM2, BM5, BM6, BM7, and BM8, which are sibling clones of the previously characterized BM1 clone, from Biken-HEL cells (1). The independent sibling full-length M cDNAs B5M1, B5M2, B5M3, B5M4, and B5M5 were cloned by the same method from a single cell-derived subline of the Biken–CV-1 cells. The M1 and M2 cDNAs, representing the M gene of the IP-3 strain, and the vM2 cDNA, representing the M gene of the 3-1 revertant virus (6, 19), were kindly provided by R. Cattaneo, Universität Zürich, Zurich, Switzerland.

Antisera. The polyclonal M-BE, M-EE, and M-BC antisera against the amino region, carboxyl region, and almost the entire M protein of Edmonston strain (see Fig. 3C), respectively, were described previously (1).

In vitro expression of cloned genes. Plasmids M1 and M2 were linearized at the *Bam*HI site, and vM2 was linearized at the *Xho*I site, downstream of the cDNA inserts. The other plasmids were linearized at the *Hin*dIII site. Equal amounts of the linearized plasmid DNAs were transcribed with either T7 or T3 RNA polymerase and translated in vitro in the reticulocyte lysate-coupled transcription-translation system (Promega) in the presence of [^{35}S]methionine (DuPont-NEN). Equal amounts of trichloroacetic acid-precipitable ^{35}S-labeled M protein were immunoprecipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) (SDS-PAGE).

Nucleocapsid-binding assay. The nucleocapsid-binding assay for M protein has recently been described (13). Briefly, equal amounts of trichloroacetic acid-precipitable ^{35}S-labeled M protein synthesized in vitro were incubated for 2 h on ice with lysates of MV Nagahata-infected CV-1 cells. The viral nucleocapsids were pelleted through 50% glycerol at 35,000 rpm for 150 min at 11°C in a Beckman SW50.1 rotor. The nucleocapsid-associated and free protein fractions were analyzed by SDS-PAGE.

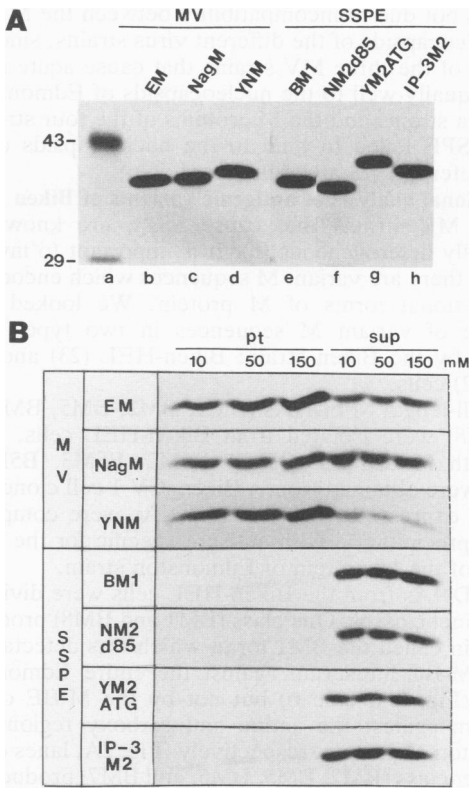

FIG. 1. Binding of M proteins of MV strains that cause acute infection and those that cause SSPE to nucleocapsids in vitro. (A) Full-length M cDNAs of Edmonston, Nagahata, and YN strains (EM, NagM, and YNM, respectively) and Biken, Niigata, Yamagata, and IP-3 strains (BM1, NM2d85, YM2ATG, and IP-3M2, respectively) were transcribed and translated in vitro. The ^{35}S-labeled M proteins were analyzed by SDS-PAGE. (B) Equal and excess amounts of the ^{35}S-labeled M protein were incubated with extracts from Nagahata strain MV-infected CV-1 cells at 10 to 150 mM NaCl. The viral nucleocapsids were pelleted through a 50% glycerol cushion. The nucleocapsid-bound and free M protein was recovered from the pellet (pt) and supernatant (sup) fractions, respectively, and analyzed by SDS-PAGE.

RESULTS

M proteins of MV strains. Full-length cDNAs representing the M genes of the Edmonston, Nagahata, and YN strains (which cause acute infection) and the Biken, IP-3, Niigata, and Yamagata strains (which cause SSPE) were transcribed and translated in vitro. The M proteins of Edmonston and Nagahata strains, which differ by four amino acids (26), have identical (M_r 38,000) electrophoretic mobilities (Fig. 1A, lanes b and c, respectively). The M gene of YN strain, which has not been sequenced, encodes a protein with slightly retarded mobility (lane d). The M proteins of Biken and IP-3 strains, which carry different mutations (1, 6), also have slightly different mobilities (lanes e and h).

The M gene of the Niigata strain is interrupted by a premature termination codon, and the AUG start codon of the M gene of the Yamagata strain is mutated to ACG (2). Nonetheless, the Niigata strain M gene produces low levels of a truncated M protein from a downstream AUG codon and the Yamagata M gene produces low levels of a cryptic M protein by use of the ACG codon (2). To gain insight into the possible function of the cryptic M proteins, a truncated Niigata M gene containing only the downstream protein-coding region (NM2d85) and a Yamagata M gene with a normal AUG codon (YM2ATG) were constructed to produce higher levels of the cryptic M proteins (Fig. 1A, lanes f and g, respectively). The proteins expressed from the NM2d85 and YM2ATG constructs are electrophoretically indistinguishable from the cryptic M proteins produced by the original Niigata and Yamagata M genes (2).

Presence of a common functional defect in the M proteins of MV strains that cause SSPE. We examined the binding of the ^{35}S-labeled M proteins to the viral nucleocapsids as described in Materials and Methods. The M proteins of the three MV strains that cause acute infection bound efficiently to the viral nucleocapsids at physiological as well as low salt concentrations, as judged by the relative amounts of M protein recovered from the nucleocapsid pellet and supernatant fractions (Fig. 1B, MV). The M proteins did not bind to cellular ribonucleoprotein complexes and was not pelleted in the absence of the viral nucleocapsids (reference 13 and data not shown). In sharp contrast, the M proteins of Biken and IP-3 strains and the cryptic M proteins of Niigata and Yamagata strains did not bind efficiently to the viral nucleocapsids and were recovered mainly from the supernatant (Fig. 1B, SSPE). The lack of association between the M proteins of the strains that cause SSPE and viral nucleocap-

sids was not due to incompatibility between the M proteins and nucleocapsids of the different virus strains, since the M proteins of the three MV strains that cause acute infection bound equally well to the nucleocapsids of Edmonston and Nagahata strains and the M proteins of the four strains that cause SSPE failed to bind to the nucleocapsids of Biken strain (reference 13 and data not shown).

Functional analysis of antigenic variants of Biken strain M protein. MV strains that cause SSPE are known to be genetically heterogeneous (5). It is important to investigate whether there are variant M sequences which encode different functional forms of M protein. We looked for the presence of variant M sequences in two types of cells infected by the Biken strain: Biken-HEL (23) and Biken–CV-1 (12) cells.

Six full-length M cDNAs (BM1, BM2, BM5, BM6, BM7, and BM8) were isolated from Biken-HEL cells, and five full-length M cDNAs (B5M1, B5M2, B5M3, B5M4, and B5M5) were obtained from a Biken–CV-1 cell clone. The M proteins expressed from these cDNAs were compared by immunoprecipitation with antisera specific for the different regions of the M protein of Edmonston strain.

The cDNAs from the Biken-HEL cells were divided into two distinct classes. One class (BM1 and BM8) produced an M protein called the BM1 form, which was detectable only by the M-BC antiserum against the entire Edmonston M protein (Fig. 2A, lane b) but not by the M-BE or M-EE antiserum against the amino and carboxy regions of the Edmonston M protein, respectively (Fig. 2A, lanes c and d). The other class (BM2, BM5, BM6, and BM7) produced an M protein called the BM2 form, which was recognized by both the M-BC and the M-BE antisera (Fig. 2A, lanes b and c). All five cDNAs (B5M1 through B5M5) from the clonally derived Biken–CV-1 cells belonged to the latter class (Fig. 2B, lanes b and c).

To understand the basis of the antigenic difference between the two forms of Biken M protein, we completely sequenced the BM2 and B5M1 cDNAs which encoded the BM2 form and compared them with the previously characterized BM1 cDNA, which encodes the BM1 form. There are four nucleotide differences (positions 82, 194, 452, and 1295) between the BM1 and BM2 cDNAs (Fig. 2C), but only the cytosine-versus-uridine difference at position 82 results in an amino acid substitution. The B5M1 cDNA differs from the BM1 clone at three positions (Fig. 2C; positions 82, 194, and 416). Again, only the C-to-U transition at position 82 leads to an amino acid substitution. Therefore, the BM2 and B5M1 cDNAs encode an identical BM2 form Biken M protein, which differs from the previously characterized BM1 form by having a serine instead of a leucine at amino acid 17 (Fig. 2C). Studies of chimeric M genes constructed by swapping the 5′ *Sac*I fragments of the BM1 and BM2 cDNAs (Fig. 2C) confirmed that this single amino acid substitution is solely responsible for the antigenic difference between the two forms of Biken M protein (data not shown). The M proteins of Edmonston and Nagahata MV, which cause acute infection, are recognized by the M-BE antiserum and contain a serine at position 17 (Fig. 2C) (4, 26).

We examined the binding of the BM2 form of Biken M protein to the viral nucleocapsids to see whether this form of M protein was functional. The results showed that the Edmonston M protein bound efficiently to the viral nucleocapsids (Fig. 3A, lane EM) but the BM1 and BM2 forms of Biken M protein expressed from the different variant sequences had the same low affinity for the viral nucleocapsids (lanes BM1, BM2, and B5M1), indicating that both forms of

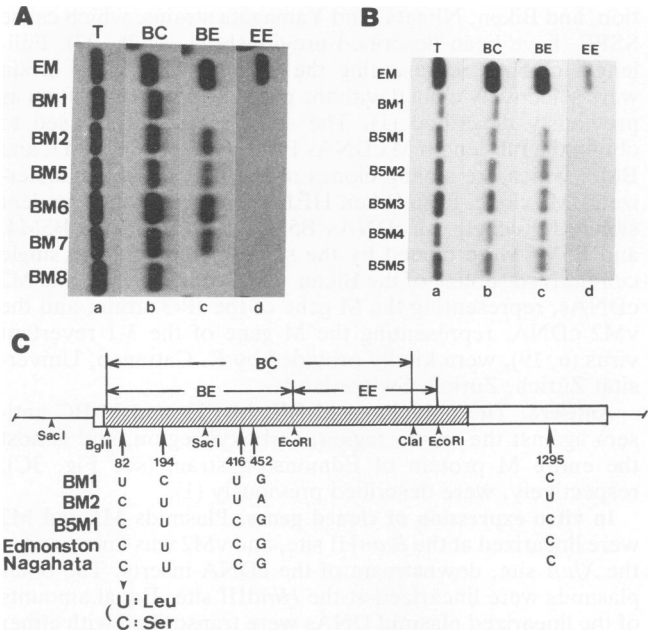

FIG. 2. Biken strain produces two antigenically distinct forms of M protein. (A) Full-length Biken M cDNAs (BM1, BM2, BM5, BM6, BM7, and BM8) from Biken-HEL cells and the Edmonston M (EM) cDNA were transcribed and translated in vitro. Equal counts of the ^{35}S-labeled proteins were immunoprecipitated with the M-BE (lane c), M-EE (lane d), and M-BC (lane b) antisera against the amino region, the carboxyl region, or almost the entire M protein, respectively, of Edmonston strain and analyzed by SDS-PAGE along with the total unprecipitated translation products (lane a). Each lane shows the proteins electrophoresed in the same gel from left to right. (B) Sibling Biken M cDNAs (B5M1, B5M2, B5M3, B5M4, and B5M5) obtained from a Biken–CV-1 cell clone and Edmonston M (EM) cDNA were transcribed and translated in vitro. M proteins were analyzed as described in panel A. (C) Nucleotide and predicted amino acid differences among the BM1, BM2, and B5M1 variant Biken M cDNAs, compared with the corresponding nucleotides in the Edmonston and Nagahata M sequences. For simplicity, the numerous Biken mutations (1, 26) common to BM1, BM2, and B5M1 are not shown. M sequences are depicted as double lines, vector sequences are shown as single lines, and the M protein-coding region are hatched.

Biken M protein are defective in the nucleocapsid-binding function.

Functional analysis of phenotypic variants of the IP-3 strain M protein. Two independent cDNA clones of the IP-3 M gene (M1 and M2) differ at eight positions (6). A biological revertant of the IP-3 virus called 3-1 was found to release infectious particles. Two cDNA clones (vM2 and vM3) obtained from the revertant virus were predicted to encode M proteins with two additional amino acid changes. Since a valine-to-isoleucine substitution was present in both the vM2 and vM3 clones, that substitution was postulated to be responsible for virus production (6).

We tested the nucleocapsid binding of the M proteins produced by the M1 and M2 cDNAs of the IP-3 strain and the vM2 clone of the revertant virus. As expected, the M proteins produced by the M1 and M2 cDNAs did not bind efficiently to the viral nucleocapsids (Fig. 3B). However, the M protein produced by the vM2 clone also failed to bind efficiently to the viral nucleocapsids (Fig. 3B). Therefore,

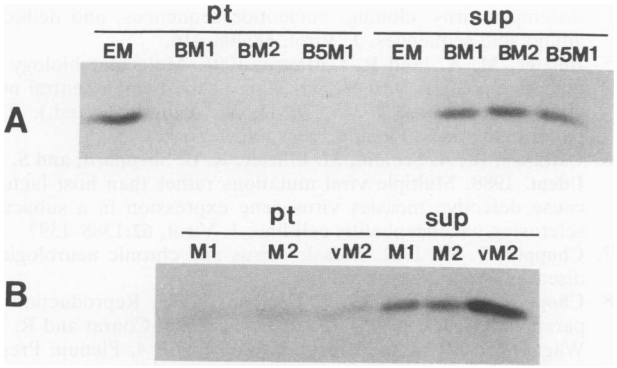

FIG. 3. Nucleocapsid-binding function of variant M proteins of Biken and IP-3 strains. (A) ^{35}S-labeled M protein expressed from M cDNAs of Biken strain (BM1, BM2, and B5M1) and Edmonston strain (EM) was incubated with an extract from Nagahata strain-infected CV-1 cells at 150 mM NaCl. (B) ^{35}S-labeled M protein expressed from M cDNAs of IP-3 strain (M1 and M2) and the 3-1 revertant virus (vM2) was incubated with an extract from Nagahata strain-infected cells at 150 mM NaCl. The nucleocapsid-associated (pt) and free (sup) M proteins were separated and analyzed as described in the legend to Fig. 1B.

the additional mutations in the vM2 M gene cannot explain the productivity of the 3-1 revertant virus.

Functional analysis of chimeric M proteins carrying different Biken mutations. The Biken M protein contains 15 amino acid substitutions and an 8-amino-acid carboxy-terminal extension (1, 26). To determine which of these mutations affect the interaction with the viral nucleocapsids, chimeric M genes which carry the mutations affecting the amino-terminal (pTZ-EMW/Ba), carboxy-proximal (pTZ-EMW/Bb), or carboxy-terminal (pTZ-EMW/Bc) region of the Biken M protein were constructed from the Edmonston (pTZ-EMW) and Biken (pTZ-BMW) M cDNAs (Fig. 4A) (1). The proteins synthesized from these constructs were tested by the in vitro nucleocapsid-binding assay.

Only the Edmonston M protein was able to bind efficiently to the viral nucleocapsids (Fig. 4B, EMW). Chimeric M proteins carrying mutations in the amino-terminal, carboxy-proximal, or carboxy-terminal region all failed to bind to the MV nucleocapsids (Fig. 4B, Ba, Bb, and Bc, respectively).

These results indicate that the mutations in all three regions of the Biken M protein can abrogate the binding of M protein to the viral nucleocapsids.

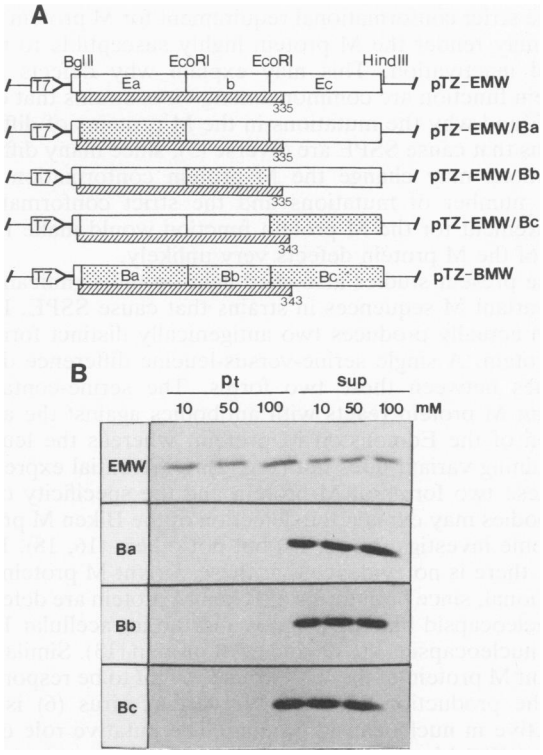

FIG. 4. Nucleocapsid-binding function of chimeric M proteins carrying different Biken mutations. (A) Chimeric M genes containing the mutations affecting the amino-terminal (*Bgl*II-*Eco*RI), carboxy-proximal (*Eco*RI-*Eco*RI), or carboxy-terminal (*Eco*RI-*Hin*dIII) regions of the Biken M protein were constructed by swapping different regions between pTZ-EMW and pTZ-BMW (1). Open areas represent Edmonston cDNA sequences; stippled areas represent Biken cDNA sequences; Hatched areas represent M protein-coding regions. (B) ^{35}S-labeled M protein of Edmonston strain (EMW) and chimeric M proteins carrying the amino-terminal, carboxy-proximal, and carboxy-terminal mutations of the Biken strain (Ba, Bb, and Bc, respectively) were incubated with extracts from Nagahata strain-infected CV-1 cells at 10 to 100 mM NaCl. The nucleocapsid-associated (pt) and free (sup) M proteins were separated and analyzed as described in the legend to Fig. 1B.

DISCUSSION

The M protein of MV is believed to serve a role in virus maturation by interacting with the viral nucleocapsids, and defects in the expression or function of the M protein are thought to play a role in the nonproductive infection by MV strains that cause SSPE. Because of the lack of a functional assay, it was difficult to substantiate the presumed role of the M protein of MV and the postulated defects in the M protein of strains that cause SSPE. Recently, we developed a simple in vitro assay to demonstrate that the M proteins produced by an MV strain that causes acute infection and its descendant strain that causes SSPE are functionally different (13). In the present study, we extended this observation to show that the M proteins of three MV strains that cause acute infection interact efficiently with the viral nucleocapsids, whereas the M proteins and cryptic M proteins produced by four strains that cause SSPE are defective in this interaction.

Functional analysis of chimeric M proteins suggests a possible mechanism for the defect in the M protein of Biken strain. Chimeric M proteins carrying the amino-terminal, carboxy-proximal, or carboxy-terminal region of the Biken M protein all fail to interact with the viral nucleocapsids (Fig. 4). This strongly suggests that the overall M protein conformation is important for nucleocapsid binding. The M protein-nucleocapsid association is dependent on the salt concentration, suggesting that it involves electrostatic interactions (13). The mutations in the Biken M protein do not affect the many positively charged arginine and lysine residues thought to be important for protein-protein interactions (4). Rather, the mutations in the amino- and carboxy-terminal regions of the Biken M protein have been shown to alter the antigenicity of a distant protein region (1). Therefore, we believe that these mutations alter the tertiary structure of the M protein important for positioning the crucial amino acids for interaction with the viral nucleocapsid. We have not ruled out the possibility that the Biken sequences are simply incompatible with the Edmonston sequences in a chimeric context.

The strict conformational requirement for M protein function may render the M protein highly susceptible to mutational inactivation. This may explain why defects in M protein function are common among virus strains that cause SSPE and why the mutations in the M proteins of different strains that cause SSPE are diverse (5), since many different mutations may change the M protein conformation. The large number of mutations and the strict conformational requirement for the M protein function would make reversion of the M protein defects very unlikely.

The present studies also shed light on the significance of the variant M sequences in strains that cause SSPE. Biken strain actually produces two antigenically distinct forms of M protein. A single serine-versus-leucine difference distinguishes between these two forms. The serine-containing variant M protein reacts with antibodies against the amino region of the Edmonston M protein, whereas the leucine-containing variant does not (Fig. 2). Differential expression of these two forms of M protein and the specificity of the antibodies may explain the detection of the Biken M protein by some investigators (1, 14) but not others (16, 18). However, there is no evidence that these variant M proteins are functional, since both forms of Biken M protein are defective in nucleocapsid binding (Fig. 3) and the intracellular Biken viral nucleocapsids are devoid of M protein (13). Similarly, a variant M protein of the IP-3 strain thought to be responsible for the production of the 3-1 revertant virus (6) is also defective in nucleocapsid binding. The putative role of the variant IP-3 M sequences in virus production awaits confirmation.

The defective interaction between the M protein and the viral nucleocapsids probably explains the nonproductivity of MV strains that cause SSPE. In MV Biken-infected cells, the M protein is not incorporated into the viral nucleocapsids and is not assembled into virions (13). It is not known whether the nonproductive mode of infection is a cause or effect of SSPE. It is now possible to assess the biological function of the M proteins encoded by cDNAs obtained directly from brain tissues of patients with SSPE and to answer the question of whether the defect in M protein function is a universal feature in SSPE. The present system will also facilitate detailed analysis of the molecular interaction between the M protein of MV and the viral nucleocapsid.

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