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The matrix proteins of neurovirulent subacute sclerosing panencephalitis virus and its acute measles virus progenitor are functionally different

(virus maturation/persistent infection)

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ABSTRACT **Persistence of measles virus in the brains of patients with subacute sclerosing panencephalitis (SSPE) is accompanied by changes in the viral matrix (M) protein. To understand the significance of these changes, cell culture and cell-free assays were developed to compare the functions of the M proteins of an SSPE virus Biken strain and its acute measles virus progenitor Nagahata strain. The Nagahata viral M protein is associated with the intracellular viral nucleocapsids and the plasma membrane, whereas the Biken viral M protein is localized mainly in the cytosol. The lack of M protein in the Biken viral nucleocapsids is due to a failure of the Biken M protein to bind to the viral nucleocapsids. The Biken M protein also fails to bind to the Nagahata viral nucleocapsids. Conversely, the Nagahata M protein can bind to the Biken viral nucleocapsids, although this association is not as stable at physiological salt concentration. These results offer concrete evidence that the M protein of an SSPE virus is functionally different from that of its progenitor acute measles virus.**

Subacute sclerosing panencephalitis (SSPE), a fatal degenerative neurological disorder, is due to chronic infection by measles virus (MV) in the central nervous system (1). Unlike the MV strains that initiate the original acute disease, the virus strains isolated from SSPE brain tissues typically do not produce extracellular virions (2, 3). The nucleotide sequences of acute MV and SSPE virus strains differ at numerous positions (4). The genetic differences are often manifested as abnormalities in the expression, structure, or stability of the matrix (M), fusion (F), or hemagglutinin (H) protein (5–15). These abnormalities are thought to be responsible for the nonproductive mode of SSPE virus infection. However, there has been no experimental evidence linking these abnormalities to a defect in the protein function.

Two major obstacles have made it difficult to obtain such evidence. First, the direct progenitors of most SSPE virus strains have not been isolated. It is difficult to determine whether the sequence differences between the SSPE virus strains and the reference MV strains represent biologically relevant mutations or natural genetic variation. Second, functional assays for most MV proteins have not yet been devised, hindering the use of reverse genetics approaches to evaluate the significance of specific mutations.

We recently made a major step toward solving the first problem by identifying the Nagahata strain of acute MV as the likely progenitor of the Biken strain, a defective SSPE virus (16, 17). The M protein of the Biken SSPE virus differs from that of the Nagahata MV by 15 amino acid substitutions and a carboxyl-terminal extension. In the present work, we developed cell culture and cell-free assays to study the functions of the M proteins of Biken SSPE virus and Nagahata MV.

MATERIALS AND METHODS

Cells and Viruses. The African green monkey kidney cell line CV-1 and Biken-CV-1 cells, persistently infected with the Biken strain of SSPE virus (16, 18), were maintained in Eagle's minimum essential medium supplemented with 10% newborn calf serum. The Edmonston strain (19) and Nagahata strain (17) of MV were propagated in CV-1 cells.

Antisera. Polyclonal GM antiserum was made against the total proteins of the Nagahata MV (20). The BC and BBC antisera were made, respectively, against the Edmonston and Biken M protein expressed in bacteria (6, 21). The F-specific antiserum was made against the Edmonston F_1 peptide expressed in bacteria (18). The mouse monoclonal antibodies (mAbs) have been described (5).

Analysis of Viral Proteins in the Nucleocapsids. CV-1 cells were infected with Nagahata MV at a multiplicity of infection of 1–5. When 100% of the cells developed cytopathic effects, the cells were labeled with [^{35}S]methionine for 3 hr. The cells were washed and scraped into phosphate-buffered saline, pelleted at 300 × *g* for 10 min, suspended in 10 mM Tris·HCl, pH 7.4/10 mM NaCl/1.5 mM $MgCl_2$, and incubated on ice for 15 min. Nonidet P-40 was added to 0.65% (wt/vol) and the cells were homogenized with 20 strokes in a Dounce homogenizer (B pestle). Nuclei were pelleted by centrifugation at 1000 × *g* for 5 min. EDTA (2 mM) and Nonidet P-40 (2%) were added to the supernatant. The cytoplasmic fractions were centrifuged in discontinuous sucrose gradients (20%/30%/40%, wt/wt) at 26,500 rpm for 2.5 hr at 4°C in a Beckman SW50.1 rotor. Fractions (200–300 μl) were collected and the viral proteins were analyzed by immunoprecipitation and SDS/10% PAGE.

Preparation of Membrane-Associated or Cytosolic Proteins. ^{35}S-labeled cells were washed, scraped into phosphate-buffered saline, and pelleted at 300 × *g* for 10 min. Cell pellets were suspended in 3 volumes (relative to the packed cell volume) of a hypotonic buffer (10 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/1 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride), incubated on ice for 10 min, and homogenized with 10 strokes in a Dounce homogenizer. The nuclei were removed by centrifugation at 3300 × *g* for 15 min. Half of the supernatant was treated with Triton X-100 (1%, wt/vol), and the detergent-treated and untreated cell homogenates were centrifuged at 100,000 × *g* for 10 min. The supernatant (S100) and pellet (P100), containing cytosolic and plasma membrane-associated proteins, respectively, were collected. The viral proteins in each fraction were dissolved in RIPA buffer (10 mM Tris·HCl, pH 7.5/150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% SDS) and analyzed by immunoprecipitation and SDS/PAGE.

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Abbreviations: MV, measles virus; SSPE, subacute sclerosing panencephalitis; mAb, monoclonal antibody.

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Identity of the plasma membrane fraction was confirmed by measuring the hydrolysis of [^{3}H]AMP by 5′-nucleotidase activity, a cellular enzyme marker specific for the plasma membrane (22).

***In Vitro* Transcription and Translation from Cloned Genes.** The cloned M genes of the Nagahata strain (pTZ-NagM) (17) or the Biken strain (pTZ-BM1) (5) driven by a T7 promoter were linearized at the *Hin*dIII site downstream of the cDNA inserts and transcribed *in vitro*. Equal amounts of RNA were translated in rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine (DuPont/NEN). Protein synthesis was monitored by measuring the trichloroacetic acid-precipitable ^{35}S-labeled proteins.

Preparation of Cell Extracts for Nucleocapsid Binding Assay. Uninfected or Nagahata MV-infected CV-1 cells and Biken-CV-1 cells grown in 850-cm^2 roller bottles were washed and scraped into phosphate-buffered saline, pelleted at 300 × *g* for 10 min, and suspended in 10 mM Tris·HCl, pH 7.4/10 mM NaCl/1.5 mM $MgCl_2$ (four times the packed cell volume). After 30 min of incubation on ice, cells were homogenized with 30 strokes in a Dounce homogenizer. Nuclei and debris were removed by a 10-min centrifugation at 3000 × *g*. Aliquots of the supernatant (100–200 μl) were frozen at −80°C.

Nucleocapsid Binding Assay. The assay was performed essentially as described (23). Equal amounts of trichloroacetic acid-precipitable ^{35}S-labeled M protein translated in reticulocyte lysate were added to samples (100 or 200 μl) of cell lysate containing 2 mM EDTA and 10–250 mM NaCl. The mixtures were incubated on ice for 2 hr, then layered onto 3 ml of 50% (vol/vol) glycerol in a buffer containing 10 mM Tris·HCl (pH 7.6), 1 mM EDTA, and the same amount of NaCl as used in the binding reaction. The samples were centrifuged at 35,000 rpm for 150 min at 11°C in a Beckman SW50.1 rotor. After centrifugation, 200-μl samples were taken from the top of the gradient and the pellet was dissolved in 200-μl of RIPA buffer. An 80-μl portion of each sample was mixed with 20 μl of 5× protein loading buffer (1× is 70 mM Tris·HCl, pH 6.8/11.2% glycerol/3% SDS/0.01% bromophenol blue/5% 2-mercaptoethanol), boiled for 2 min, and analyzed by SDS/PAGE.

RESULTS

Biken Viral M Protein Is Not in the Intracellular Viral Nucleocapsids. The intracellular nucleocapsids of the Nagahata strain MV contained four major proteins (Fig. 1*A*, lane b) that were immunoprecipitated by the GM antiserum against total MV proteins (Fig. 1*B*, lane b). Monospecific antibodies identified three of these as the phosphoprotein (P), nucleoprotein (N), and M protein (Fig. 1*B*, lanes d–f, respectively). The N protein in the viral nucleocapsids is antigenically distinguishable from the free N protein. The GM antiserum recognized both the nucleocapsid-associated and the free form of N protein (Fig. 1*B*, lanes b and g), but the N46 mAb preferentially recognized the nucleocapsid-associated form (Fig. 1*B*, lanes e and j). The N46-specific form of N protein thus served as a useful marker of the nucleocapsid fraction. The high molecular weight MV-specific protein in this fraction was presumed to be the polymerase (L) (Fig. 1*B*, lane b). The majority of H and F proteins were in the free protein fraction but not in the viral nucleocapsids (Fig. 1*B*, lanes b, c, g, and h). The Nagahata viral M protein was found in both the nucleocapsid and free protein fractions (Fig. 1*B*, lanes f and k).

The Biken viral nucleocapsids contained at least five major proteins (Fig. 1*A*, lane d) that reacted with the GM antiserum (Fig. 1*C*, lane b). Two of these were confirmed as P and N proteins by the monospecific antibodies (Fig. 1*C*, lanes d and e, respectively). The majority of the Biken viral H protein was also found in the free protein fraction (Fig. 1*C*, lanes c and h). A protein that migrated near the expected position for M protein (Fig. 1*A*, lane d) was not M protein, because it reacted with antibodies against the P protein (Fig. 1*C*, lane d, asterisk) but not with antibodies against the Biken M protein (Fig. 1*C*, lane f, arrowhead). The 45-kDa protein (ac) in the Biken nucleocapsid fraction (Fig. 1*A*, lane d) was identified as cellular actin by immunoprecipitation with anti-actin antibodies (data not shown). Most importantly, unlike the Nagahata virus M protein, the Biken viral M protein was found exclusively in the free protein fraction (Fig. 1*C*, lane k) and not in the nucleocapsids (Fig. 1*C*, lane f).

These results show that the Biken viral nucleocapsids do not contain M protein and are associated with cellular actin and an aberrant P-related protein. It is not clear whether actin

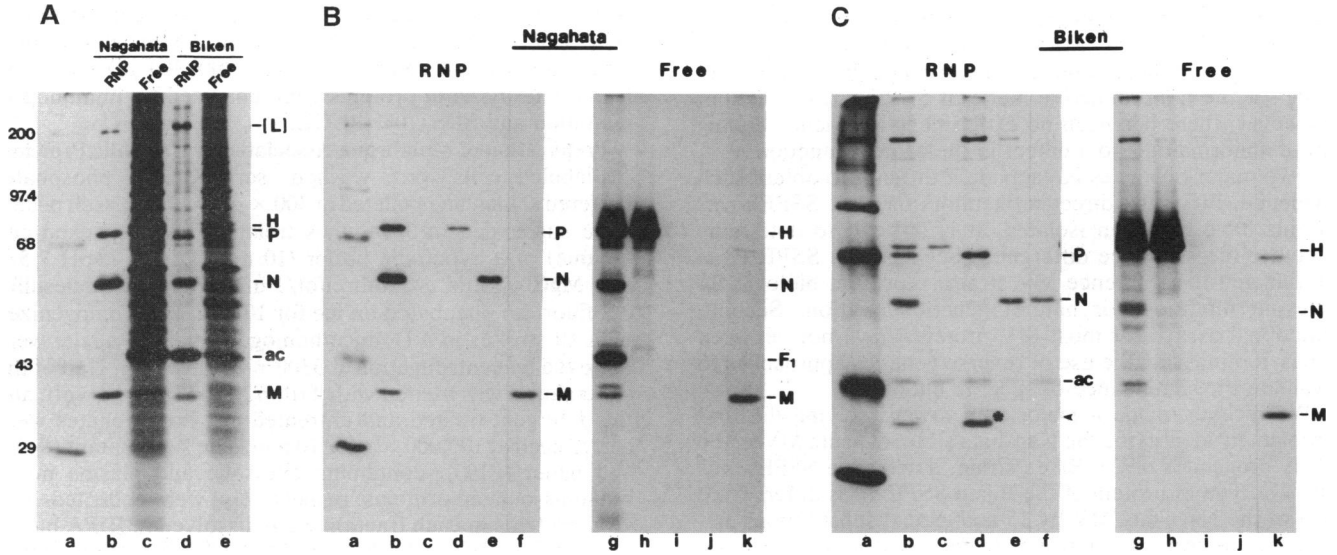

FIG. 1. Proteins in the intracellular viral nucleocapsids. ^{35}S-labeled viral proteins from Nagahata MV-infected CV-1 or Biken-CV-1 cells were fractionated into nucleocapsid-associated (ribonucleoprotein, RNP) and free protein fractions and analyzed by SDS/PAGE directly (*A*, lanes b–e) or by immunoprecipitation (*B* and *C*) with the GM antiserum against total MV proteins (lanes b and g), H mAb (lanes c and h), P mAb (lanes d and i), N mAb (lanes e and j), or the BC or BBC antiserum for the Nagahata or Biken M protein, respectively (lanes f and k). Lanes a, molecular size markers (sizes at left in kilodaltons). Arrowhead indicates the expected position for M protein, and the aberrant P-related protein is marked by an asterisk.

interacts or simply cosediments with the Biken viral nucleocapsids. The P-related protein is phosphorylated (data not shown) and may be a cleavage product of the P protein.

Biken Viral M Protein Is Not Associated with the Plasma Membrane. We next examined the association of the M protein with the plasma membrane by cell fractionation. The P100 fraction from Biken-CV-1 cells contained 78% of the total 5′-nucleotidase activity, a marker for the cellular plasma membrane. The nuclear and S100 fractions contained 5% and 17% of the activity, respectively. Therefore, the P100 fraction contained most of the plasma membrane, and the S100 fraction contained mostly soluble cytosolic proteins.

In the Nagahata MV-infected cells, all the viral proteins were immunoprecipitated from the P100 fraction (Fig. 2*A*, lane b). The Nagahata M protein was distributed roughly equally between the P100 and S100 fractions (Fig. 2*A*, lanes c and f, respectively). On the other hand, the H and F glycoproteins were found mainly in the P100 rather than the S100 fraction (Fig. 2*A*, lanes b, d, e, and g). When the cell homogenate was treated with Triton X-100 before fractionation, all the membrane-bound Nagahata viral proteins were released into the S100 fraction (Fig. 2*B*, lanes b–g).

The P100 fraction from the Biken-CV-1 cells contained the H, F, and three unidentified proteins of 55–65 kDa (Fig. 2*C*, lanes b and d). Detergent treatment released only the H and F proteins from the P100 into the S100 fraction (Fig. 2*D*, lanes b, d, e, and g), but the 55- to 65-kDa proteins continued to sediment in the P100 pellet (Fig. 2*D*, lanes b–d). The latter proteins were most likely from aggregates that contaminated the P100 fraction, since they were precipitated nonspecifically by all the antisera tested (Fig. 2*C*, lanes b–d). Most importantly, the Biken M protein was localized mainly in the S100 but not the P100 fraction (Fig. 2*C*, lanes f and c, respectively). The differential localization of the Nagahata and Biken M proteins was observed in different plasma membrane preparations that contained up to 84% of the cellular 5′-nucleotidase activity (data not shown).

Therefore, all the viral proteins in the Nagahata MV-infected cells, including F, H, and a fraction of the M and nucleocapsid-associated proteins, were associated with the plasma membrane. In Biken-CV-1 cells, only the F and H proteins were localized at the plasma membrane. The Biken M protein was found only in the cytosol.

Biken M Protein Does Not Bind to the Nucleocapsids of Wild-Type MV. The lack of M protein in the nucleocapsids of the Biken virus could be due to a defect in the M protein or the nucleocapsid. To distinguish between these possibilities, we studied the binding of the M protein to the viral nucleocapsids *in vitro*. ^{35}S-labeled M protein translated *in vitro* from the Nagahata or Biken M RNA was incubated with cell extracts from uninfected or Nagahata MV-infected CV-1 cells under different ionic conditions. The M protein bound to the viral nucleocapsids was separated from the unbound M protein by centrifugation through 50% glycerol and analyzed by SDS/PAGE.

The Nagahata or Biken M protein alone could not be pelleted through the glycerol cushion (data not shown). Also, when the Nagahata or Biken M protein was incubated with

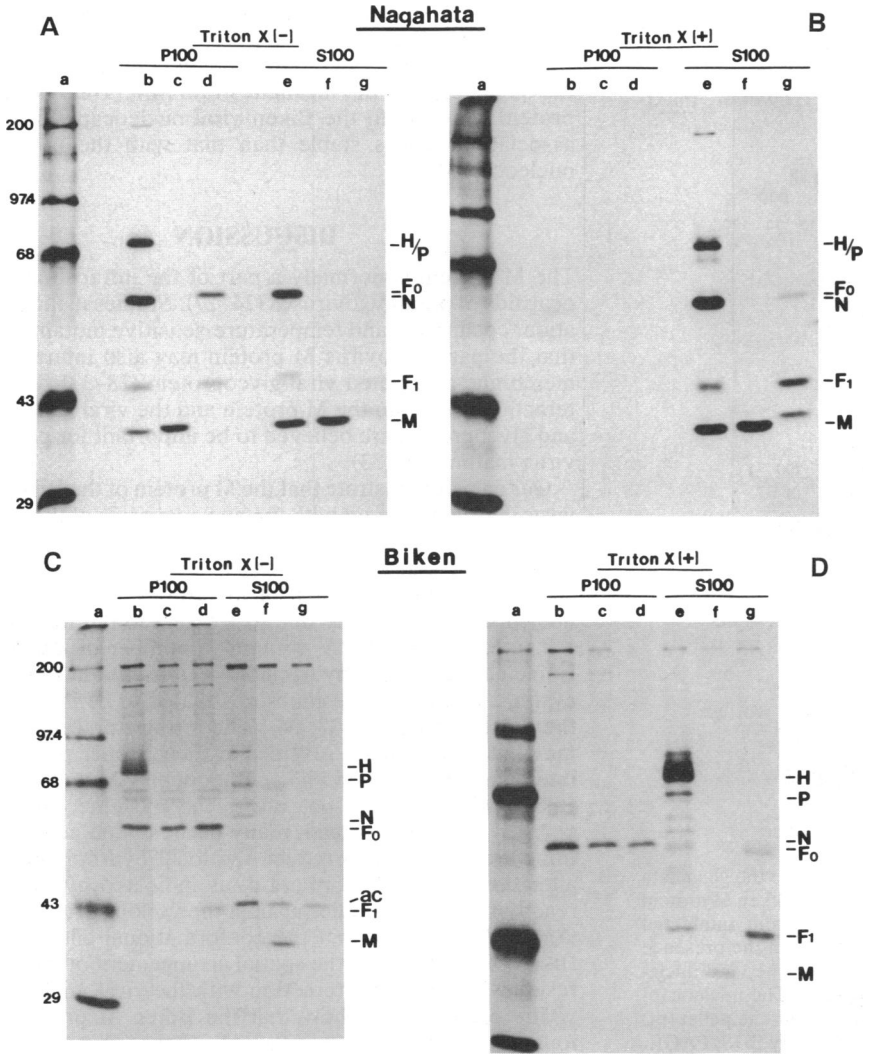

FIG. 2. Membrane-associated viral proteins. ^{35}S-labeled proteins in the postnuclear supernatant from Nagahata MV-infected CV-1 or Biken-CV-1 cells were fractionated into the plasma membrane (P100) and cytosolic (S100) fractions without (*A* and *C*) or with (*B* and *D*) Triton X-100 treatment. Viral proteins were immunoprecipitated with the GM antiserum (lanes b and e), the BC or BBC antiserum for the Nagahata or Biken M protein, respectively (lanes c and f), or the F-specific antiserum (lanes d and g), and analyzed by SDS/PAGE. Lanes a, molecular size markers.

uninfected CV-1 cell extracts, all the M protein stayed near the top of the centrifuge tube, regardless of the salt concentration (Fig. 3*A*). This suggests that the Nagahata and Biken M proteins do not form pelletable aggregates.

When the Nagahata M protein was incubated with the Nagahata MV-infected cell extracts, about 50% of the M protein appeared in the pellet (Fig. 3*B*). This pellet contained MV nucleocapsids, as shown by the presence of genomic-length MV-specific RNA and N46-specific form of N protein (data not shown). The association between the Nagahata M protein and the nucleocapsids was most efficient under physiological ionic conditions and was stable in a solution containing up to 200 mM NaCl (Fig. 3*B*). The Nagahata M protein also bound to sucrose gradient-purified MV nucleocapsids. The binding was specific, since up to 500 μg of a nonspecific competitor (bovine serum albumin) failed to compete with the Nagahata M protein for binding to the nucleocapsids (data not shown).

In sharp contrast, the Biken M protein did not associate with the viral nucleocapsids in the Nagahata MV-infected cell extract (Fig. 3*C*). A low but detectable level of Biken M protein was recovered from the pellet containing the Nagahata viral nucleocapsids. However, this background level of binding was observed only at the lower salt concentrations (10–50 mM) (Fig. 3*C*), suggesting that it was due to nonspecific ionic interactions.

These results show that the wild-type M protein associates with the viral nucleocapsids and that the Biken M protein is defective in this function.

Nagahata M Protein Binds with Low Affinity to the Nucleocapsids of Biken Virus. We further tested whether the Biken viral nucleocapsids were recognized by the M protein of the Nagahata or Biken virus. The Nagahata M protein could bind to the Biken viral nucleocapsids (Fig. 4*A*). However, maximal binding was observed at 50 mM NaCl. At physiological or higher salt concentrations, binding of the Nagahata M protein to the Biken viral nucleocapsids was less stable than binding to the Nagahata viral nucleocapsids (compare Figs. 3*B* and 4*A*).

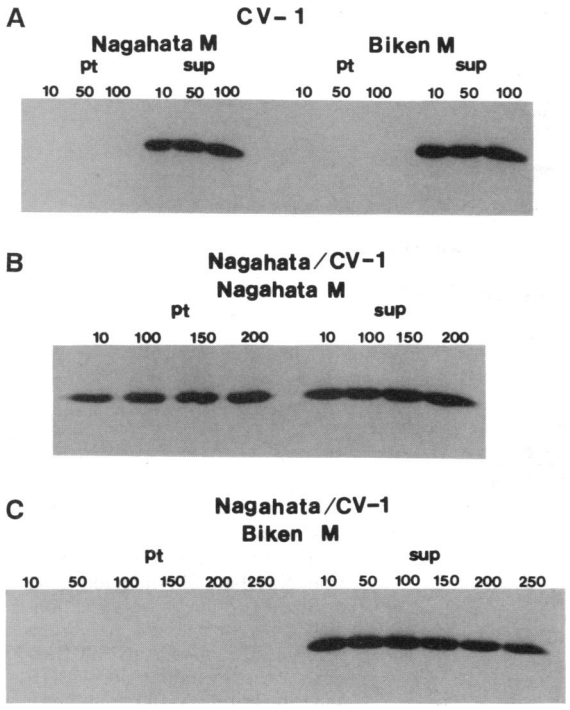

FIG. 3. Binding of Nagahata or Biken M protein to the Nagahata viral nucleocapsids *in vitro*. ^{35}S-labeled Nagahata or Biken M protein translated *in vitro* was incubated with cell extracts from uninfected (*A*) or Nagahata MV-infected (*B* and *C*) CV-1 cells in the presence of 10–250 mM NaCl as indicated above each lane. The viral nucleocapsids were pelleted through a glycerol cushion. The nucleocapsid-associated and free M protein was recovered from the pellet (pt) and top (sup) fractions, respectively, and analyzed by SDS/PAGE.

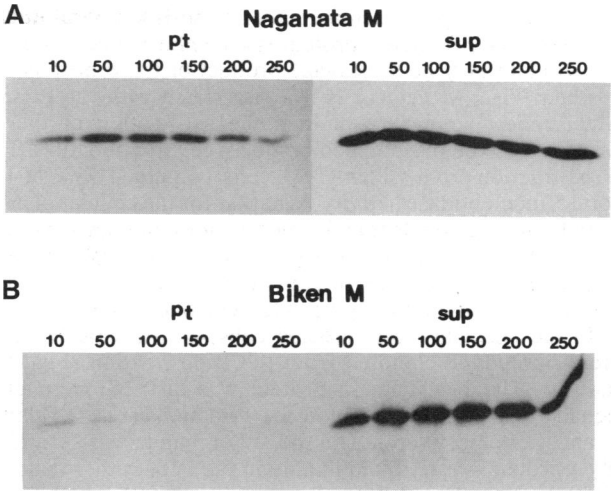

FIG. 4. Binding of Nagahata or Biken M protein to the Biken viral nucleocapsids *in vitro*. ^{35}S-labeled Nagahata or Biken M protein translated *in vitro* was incubated with Biken-CV-1 cell extract in the presence of 10–250 mM NaCl. The nucleocapsid-associated (pt) and free (sup) M protein was analyzed as described in the legend to Fig. 3.

The Biken M protein did not bind to the Biken viral nucleocapsids (Fig. 4*B*). Therefore, the M protein of the Biken virus cannot bind to the homologous nucleocapsids or the nucleocapsids of Nagahata strain MV. The Nagahata M protein can bind to the Biken viral nucleocapsids, but this association is less stable than that with the homologous nucleocapsids.

DISCUSSION

The M protein is normally a part of the intracellular nucleocapsids of paramyxoviruses (24–27). Studies using fractionation, cocapping, and temperature-sensitive mutants suggest that the paramyxovirus M protein may also interact with a membrane-associated viral glycoprotein (28–32). Proper interactions between the M protein and the viral nucleocapsid and glycoprotein are believed to be important for paramyxovirus maturation (33).

Our data demonstrate that the M protein of the Biken SSPE virus does not interact with the nucleocapsids of the Biken or Nagahata virus. On the other hand, the Biken viral nucleocapsids can associate with the M protein of the Nagahata strain. Therefore, the lack of M protein in the Biken viral nucleocapsids is mainly due to a defect in the M protein.

The M protein of MV contains a number of arginine and lysine clusters in the hydrophilic regions and has conserved cysteine and proline residues postulated to be important for the protein structure (33, 34). The tertiary protein structure may hold the charged residues in their proper positions for interaction with other viral components. Mutations in the Biken M protein do not affect the cysteine, proline, or charged residues. Instead, many of the amino acid substitutions are predicted to reduce the local hydrophobicity and alter the protein conformation, as indicated by the loss of reactivity with antibodies against the various protein regions (5, 17). We suggest that the conformational changes in the Biken M protein alter the spatial arrangement of the charged residues crucial for interaction with the viral nucleocapsid.

The present data show that the Biken M protein is not found at the plasma membrane. The carboxyl region of the M

protein of MV contains a predicted β-sheet structure associated with nonpolar amino acids (34). This postulated structure is hypothesized to allow the M protein to interact with the membrane without spanning the lipid bilayer (34). None of the mutations in the Biken M protein directly affects this predicted β-sheet structure or alters the nonpolar residues in this carboxyl region (5). Interestingly, the Nagahata and Biken M proteins expressed from the cloned genes by DNA transfection exhibit little difference in membrane association. Only a small fraction of the M proteins of both strains thus expressed becomes membrane-associated (unpublished data). Therefore, the nonpolar residues in the carboxyl region of the M protein are insufficient for a strong association with the plasma membrane. Membrane localization of the MV M protein may require interaction with other viral components, possibly one of the viral glycoproteins.

Therefore, the defect resulting in the failure of the M protein to localize at the plasma membrane in Biken-CV-1 cells can reside in the M protein or a viral glycoprotein. The F genes of SSPE virus strains are frequently mutated such that the predicted cytoplasmic domains of the gene products are mutated or truncated (11). Notably, the Biken virus F gene also carries such a mutation (unpublished data). Even though the Biken F protein is transported to the plasma membrane (Fig. 2), whether the F protein retains its normal biological functions remains to be tested.

The M protein is also thought to interact with the N protein in the viral nucleocapsid. The N proteins of Sendai virus and Newcastle disease virus can be crosslinked to M protein in newly released virions (32, 35). The amino-terminal region of N protein is basic (36) and is associated with the genomic RNA (37). The carboxyl region of N protein is acidic (38) and is exposed on the viral nucleocapsid (37, 39). The N proteins of SSPE virus strains accumulate more mutations in the carboxyl-terminal region (11). It is tempting to speculate that the negatively charged carboxyl region of the N protein may interact with the positively charged residues in the M protein. In this context, the unstable binding of the Nagahata M protein to the Biken viral nucleocapsids at high salt concentrations (Fig. 4) may reflect subtle changes in the Biken N protein.

Since the Nagahata M protein can bind to the Biken viral nucleocapsids *in vitro*, the Biken viral genomes may be rescuable from the Biken-CV-1 cells by the wild-type MV proteins. While exploring that possibility, Hirano (18) discovered that the Biken virus dominantly suppressed the replication of the Nagahata strain of MV. This suggests that some of the Biken viral gene products may interfere with the functions of the wild-type counterparts. This phenomenon may have allowed the Biken SSPE virus to displace the parental virus during the course of the central nervous system infection.

In summary, the present study provides direct evidence that a major defect in the Biken virus lies in the lack of interaction between the M protein and the viral nucleocapsid and that this defect resides in the M protein. The assays developed in this study open avenues for understanding the significance of the mutations in the MV proteins by reverse genetics approaches.

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