Sendai Virus Assembly: M Protein Binds to Viral Glycoproteins in Transit through the Secretory Pathway

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We have examined the relative ability of Sendai virus M (matrix) protein to associate with membranes containing viral glycoproteins at three distinct stages of the exocytic pathway prior to cell surface appearance. By the use of selective low-temperature incubations or the ionophore monensin, the transport of newly synthesized viral glycoproteins was restricted to either the pre-Golgi intermediate compartment (by incubation at 15°C), the medial Golgi (in the presence of monensin), or the trans-Golgi network (by incubation at 20°C). All three of these treatments resulted in a marked accumulation of the M protein on perinuclear Golgi-like membranes which in each case directly reflected the distribution of the viral F protein. Subsequent redistribution of the F protein to the plasma membrane by removal of the low-temperature (20°C) block resulted in a concomitant redistribution of the M protein, thus implying association of the two components during intracellular transit. The extent of M protein-glycoprotein association was further examined by cell fractionation studies performed under each of the three restrictive conditions. Following equilibrium sedimentation of membranes derived from monensin-treated cells, approximately 40% of the recovered M protein was found to cofractionate with membranes containing the viral glycoproteins. Also, by flotation analyses, a comparable subpopulation of M protein was found to be membrane associated whether viral glycoproteins were restricted to the trans-Golgi network, the medial Golgi, or the pre-Golgi intermediate compartment. Additionally, transient expression of M protein alone from cloned cDNA showed that neither membrane association nor Golgi localization occurs in the absence of Sendai virus glycoproteins.

Sendai virus, a member of the paramyxovirus family, produces pleomorphic particles consisting of a lipoprotein envelope surrounding a helical nucleocapsid. The viral envelope is composed of a lipid bilayer, derived from the host plasma membrane, and contains oligomeric forms of both the viral hemagglutinin/neuraminidase protein (HN) and the viral fusion protein (F). Both of these proteins are synthesized as transmembrane glycoproteins in the endoplasmic reticulum, where homologous oligomerization occurs before transport through the exocytic pathway to the plasma membrane (20). In contrast, the helical nucleocapsid of Sendai virus is assembled exclusively in the cytoplasm and is composed of a single unsegmented negative-sense RNA molecule and three additional viral proteins, namely, the nucleocapsid protein (NP), the viral phosphoprotein (P), and the viral polymerase (L). (See reference 25 for a recent review).

During the process of viral assembly, mature nucleocapsids must interact with components of the viral envelope in order to initiate the process of bud formation and subsequent viral release (1, 35). This association is mediated by the matrix (M) protein, which forms an essential bridge between the cytoplasmic domain of the glycoprotein(s) and the NP of the nucleocapsid (6, 29, 34, 35). Although the exact mechanism of paramyxovirus assembly and release remains an enigma, current theory suggests that M protein can bind only to viral glycoproteins which are present within the plasma membrane of the host cell and that this spatial restriction of component interaction facilitates the correct assembly and release of viral particles from the plasma membrane (see

reference 24 for a recent review). Implicit in this model is the concept that glycoproteins in transit through the secretory pathway are in some conformation or subcellular environment which prevents M protein-glycoprotein association.

In this study, we directly questioned this idea as a suitable model for all members of the paramyxovirus family. Specifically, we designed a series of experiments to analyze the sequence of events occurring early in the process of Sendai virus assembly. By use of low-temperature incubations and the drug monensin, we were able to restrict the exocytic transport of viral glycoproteins to either the pre-Golgi intermediate compartment (at 15°C), the medial Golgi (with monensin), or the trans-Golgi network (TGN) (at 20°C). Under each of these conditions, we performed both morphological and biochemical tests to show that M protein can bind to the viral glycoproteins during their transport through the secretory pathway.

MATERIALS AND METHODS

Reagents. Monoclonal antibodies against Sendai virus M, F, and HN proteins were kind gifts from Allan Portner (St. Jude Children's Research Hospital, Memphis, Tenn.). Polyclonal antisera raised against wild-type (WT) Sendai virus and the mannose 6-phosphate receptor (M6PR) were generously donated by J. T. Seto (California State University—Los Angeles) and W. J. Brown (Cornell University, Ithaca, N.Y.), respectively. A cDNA clone encoding the Sendai virus F protein was donated by the late H. Shibuta (Institute of Medical Science, University of Tokyo, Tokyo, Japan). The cDNA clone of the Sendai virus (Z strain) M protein was constructed by Hsiao-Huei Wu. Fluorescein isothiocyanate (FITC)-, rhodamine-, or alkaline phosphatase-conjugated

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FIG. 1. Colocalization of M and F proteins with M6PR in the TGN at 20°C. BHK cells were infected by synchronized adsorption at 4°C and then incubated at 37°C for 5.5 h before being transferred to 20°C for a further 2-h period. Cycloheximide (10 μ g/ml) was added to the cells 1 h after initiation of the 20°C incubation in order to concentrate viral glycoproteins within the TGN. Cells were then fixed and double labeled for fluorescence, using a rabbit polyclonal anti-M6PR antibody and either monoclonal anti-F (A and B) or anti-M (C and D) antibody. All cells were then stained by using a cocktail of rhodamine-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antisera. Panels A and C were viewed by using filters to exclude fluorescein fluorescence; panels B and D were viewed by using filters to exclude rhodamine fluorescence. Arrows indicate an expanded TGN.

secondary antibodies were obtained from Cappel (Organon Teknika Corp., West Chester, Pa.).

Virus and cells. WT Sendai virus (Z strain) obtained from J. T. Seto was grown in 10-day-old embryonated chicken eggs. Baby hamster kidney (BHK) cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum.

Synchronized infection of cells. BHK cells were cultured to approximately 80% confluence in DMEM and then washed twice with phosphate-buffered saline (PBS) (4°C) before infection with Sendai virus at an approximate multiplicity of

infection of 3. The virus inoculum was diluted in cold (4°C) PBS containing 1% bovine serum albumin and adsorbed to cells at 4°C for 90 min. Following adsorption of viral particles, cells were washed twice in PBS (4°C) before the addition of DMEM prewarmed to 37°C. Addition of 37°C medium was taken to represent zero time postinfection in all experiments.

Indirect immunofluorescence. For analyses of internal antigen distribution, cells were thoroughly washed in PBS and fixed by incubation in acetone-methanol (1:1) for 7 min at -20°C. For detection of cell surface antigens, cells were

FIG. 2. Morphological distribution of F and M proteins during a 20°C block and subsequent 37°C chase. BHK cells infected by synchronized adsorption of virus were incubated at 37°C for 5.5 h before being transferred to 20°C for a period of 2 h. Cycloheximide (10 μ g/ml) was added 1 h after initiation of the 20°C incubation. Following accumulation of viral glycoproteins in the TGN at 20°C, cells were shifted back to 37°C for either 15 or 45 min in the presence of cycloheximide. (A and D) Cells at the end of the 20°C incubation (no chase); (B and E) cells fixed following a 20°C block and 15-min chase at 37°C; (C and F) cells incubated at 20°C and then chased for 45 min at 37°C. Panels A to C and D to F were stained with monoclonal antisera to F and M proteins, respectively.

washed twice in PBS and fixed in PBS containing 4% formaldehyde at room temperature for 10 min. Following fixation, cells were washed thoroughly in PBS before being blocked in PBS-0.2% (wt/vol) gelatin (blocking buffer) for 1 h at 37°C. Primary antibodies were diluted to 1/100 with

blocking buffer and incubated with cells for 1 h at 37°C. Cells were then washed by three sequential washes of 15 min each in blocking buffer before the addition of secondary FITC-conjugated antibodies at a dilution of 1/100. After staining, cells were further washed in PBS-0.05% Tween 20 and then

PBS alone before being mounted in a Gelvatol (Monsanto, Indian Orchard, Mass.)-glycerol solution containing 2.5% 1,4-diazabicyclo-[2.2.2]octane (DABCO) (Sigma, St. Louis, Mo.). Transmission fluorescence microscopy was performed with a Nikon Optiphot microscope (Nippon Kogaka, Tokyo, Japan).

Subcellular fractionation. Preparative fractionation of cultured BHK cells was performed as follows. Cells were washed twice in ice-cold PBS before being removed from the flask by scraping. Cells were then harvested by sedimentation, resuspended in hypotonic lysis buffer (10 mM Tris [pH 7.5], 10 mM KCl, 5 mM MgCl₂), and incubated on ice for 10 min before disruption of cells by repeated passage (15 times) through a 26-gauge hypodermic needle. Unbroken cells and nuclei were removed by centrifugation at 100 × *g* for 7 min, and the resulting postnuclear supernatant was subjected to further fractionation either by equilibrium centrifugation through continuous sucrose gradients or by flotation analyses as described below.

Equilibrium gradient centrifugation. Crude postnuclear supernatants were generated as described above before being layered over a 25 to 48% (wt/wt) sucrose gradient containing 50 mM Tris (pH 7.5), 25 mM KCl, and 5 mM MgCl₂. Gradients were centrifuged by using a Beckman SW40 rotor at 4°C for 18 h at 38,000 rpm. Following centrifugation, 1-ml fractions were collected from the top of the gradient by using a Hacki-Buckler Auto Densiflow II gradient remover, and the relative density of each fraction was determined by refractation. Any material that pelleted at the bottom of the gradient was resuspended in the final 1-ml fraction. Membranes and sedimentable material were recovered from each gradient fraction by rate sedimentation as follows. Each 1-ml fraction was diluted approximately fivefold in low-salt buffer (LSB) containing 0.25 M sucrose, 50 mM Tris HCl (pH 7.5), 25 mM KCl, and 5 mM MgCl₂ and recentrifuged in a Beckman SW55 rotor at 45,000 rpm for 90 min at 4°C. Resulting pellets were resuspended in LSB and stored at -20°C. Subsequently, fractions were analyzed for either viral proteins or the M6PR protein by Western immunoblotting. Filters decorated with polyclonal anti-WT Sendai virus or polyclonal anti-M6PR antibody were visualized by using alkaline phosphatase-conjugated secondary antibodies and quantified by densitometric scanning with an LKB 2202 Ultrascan laser densitometer.

Flotation analyses. Flotation analyses were performed according to the method of Sanderson et al. (26), with the following modifications. Crude postnuclear supernatants were prepared as described above. Aliquots (0.5 ml) were then dispersed into 3.5 ml of 70% (wt/wt) sucrose LSB and overlaid with 6.5 ml of 55% (wt/wt) sucrose LSB and approximately 2 ml of 10% (wt/wt) sucrose LSB. Gradients were then centrifuged by using a Beckman SW40 rotor at 4°C for 18 h at 38,000 rpm. Following centrifugation, fractions were collected and analyzed as described above. Flotation analyses of metabolically labeled cells were performed as described above with the exception that gradients were centrifuged in a Beckman SW50 rotor at 4°C for 3 h at 48,000 rpm.

Transient expression of M and F proteins. M and F proteins were transiently expressed by using a T7-vaccinia virus expression system according to the method of Fuerst et al. (11). Briefly, BHK cells were infected with vaccinia virus expressing the T7 RNA polymerase at a multiplicity of infection of 1. Subsequently, cDNA encoding Sendai virus F or M protein was introduced into vaccinia virus-infected cells by lipofection-mediated gene transfer (10). Analyses of

FIG. 3. Membrane association of M protein during a 20°C block of glycoprotein transport. BHK cells infected by synchronized adsorption of Sendai virus at 4°C were incubated at 37°C for 5.5 h before being subjected to a 20°C block of secretory transport for a further 2-h period. Cycloheximide (10 µg/ml) was added 1 h after the shift to 20°C. A crude postnuclear supernatant was then fractionated by flotation as described in Materials and Methods. Following centrifugation, membranes accumulated at the 10/55% (wt/wt) sucrose interface (fraction 3), while non-membrane-associated material such as soluble proteins and viral nucleocapsids remained within the dense 70% (wt/wt) sucrose cushion (fractions 11 to 14). Quantitation of protein distribution was performed by densitometry of Western blots decorated with either a polyclonal anti-M6PR antibody (A) or a polyclonal anti-whole Sendai virus rabbit antibody (B and C) followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. (A) Distribution of the endogenous TGN marker M6PR; (B) distribution of both viral glycoproteins (F and HN) measured independently; (C) distribution of the viral M protein. See Fig. 5 for an example of Western blot analysis.

the expression of Sendai virus genes were performed at approximately 5 h posttransfection, and cells were processed for either immunofluorescence or cell fractionation as described above.

RESULTS

To optimize the concentration of viral glycoproteins within the desired subcellular compartment, it was first essential to determine at what time postinfection viral glycoprotein synthesis was initiated and at what time the proteins reached the host cell plasma membrane. For this

FIG. 4. Accumulation of M protein on Golgi membranes during incubation with monensin. BHK cells infected by synchronized adsorption of virus at 4°C were incubated at 37°C for 5 h; monensin (10 μ M) was then added, and cells were incubated in the presence of monensin for a further 5 h (10 hpi) (A, C, and E). No monensin was added to cells shown in panels B, D, and F. All cells were fixed at 10 hpi and stained for either internal (A to D) or cell surface (E and F) antigens. Panels A, B, E, and F were stained with monoclonal antibodies to the viral F protein; panels C and D were stained with monoclonal antibodies raised against the viral M protein.

purpose, we performed both immunoprecipitation of pulse-labeled proteins and immunofluorescence of virus-infected cells at intervals of 1 h over a 10-h period following synchronized infection at a multiplicity of infection of 3. For immunofluorescence studies, we specifically chose to follow the distribution of the viral F protein, as this protein is

known to have a rate of intracellular transport higher than that of the HN protein (2, 22). Our findings from these experiments showed that expression of M, F, and HN was initiated simultaneously between 5 and 6 h postinfection (hpi) (data not shown), at which time all of the F protein was contained within Golgi and reticular endoplasmic reticulum

FIG. 5. Isopycnic distribution of viral components following incubation of cells with monensin. Sendai virus-infected BHK cells were incubated at 37°C in the absence of monensin for 5 h and subsequently in the presence of monensin (10 μ M) for a further 5 h. A crude postnuclear supernatant was then prepared as described in Materials and Methods and fractionated to equilibrium along a continuous 25 to 48% (wt/wt) sucrose gradient. Proteins and membranes from each fraction were recovered by sedimentation as described in Materials and Methods. Component proteins were separated on sodium dodecyl sulfate–10% polyacrylamide gels before being transferred to a nitrocellulose filter. The filter was then cut into two; the upper part was stained with polyclonal anti-Sendai virus rabbit serum AS 74, which had a relatively high titer for the viral glycoproteins (E), and the lower part of the membrane was stained with rabbit polyclonal anti-Sendai virus serum AS 81, which had a high titer for the viral M protein (F). The relative positions of viral proteins P, HN, F, NP, and M are indicated on the left. (A) Relative density of each fraction; (B) distribution of HN protein; (C) distribution of F protein; (D) distribution of M protein.

membranes, with no detectable F protein being present on the plasma membrane (see Fig. 9A). To obtain a single population of viral glycoproteins within the 20°C compartment, cycloheximide (10 μ g/ml) was added after the transport block was imposed, thereby preventing further protein synthesis but allowing previously synthesized proteins to be transported through the secretory pathway as far as the 20°C compartment.

M protein is associated with membranes of the TGN at

20°C. Incubation of virus-infected cells at 20°C has been extensively used to inhibit vesicular traffic from the TGN (13, 21, 27). To determine whether M protein could associate with viral glycoproteins accumulated within the TGN, a 20°C block of secretory transport was imposed for 2 hours at 5.5 hpi, and double immunofluorescence was performed to show the relative distribution of F or M protein with respect to an endogenous TGN protein, M6PR. Under normal conditions, M6PR is known to cycle between the TGN and the prely-

FIG. 6. Flotation analyses of membranes derived from monensin-treated cells. Sendai virus-infected BHK cells were incubated at 37°C for 5 h without monensin and subsequently for further 5 h after the addition of monensin (10 μ M). A crude postnuclear supernatant was prepared as described in Materials and Methods and fractionated by flotation through dense sucrose. Following centrifugation, membranes were recovered from the 10/55% (wt/wt) sucrose interface (fractions 3 and 4), while soluble material and viral nucleocapsids remained in the dense 70% (wt/wt) sucrose cushion (fractions 11 to 14). (A) Distribution of HN protein; (B) distribution of F protein; (C) distribution of M protein.

sosomal compartment (5, 8); however, when exit from the TGN is impeded, the distribution of M6PR fluorescence is shifted drastically from the prelysosomal compartment to the TGN (33).

Immunofluorescence analyses of M6PR distribution in noninfected BHK cells incubated at 20°C for 2 h showed that the majority of M6PR was localized to perinuclear Golgi-like membranes (data not shown). The results from Sendai virus-infected cells show that inhibition of secretory transport at 20°C resulted in a perinuclear accumulation of both F protein (Fig. 1A) and a population of M protein (Fig. 1C). Furthermore, upon double staining, both F and M proteins were seen to colocalize with the TGN as defined by the postblock distribution of the M6PR protein (Fig. 1B and D). To control for the possibility of antibody cross-reactivity during our colocalization experiments, uninfected cells stained with primary and secondary antibodies as described above were found to show negligible FITC staining of Golgi membranes, while infected cells incubated at 20°C and stained with monoclonal antibodies against M protein showed a clear Golgi-like distribution of the M protein (data not shown). From this colocalization of antigens, we conclude that M protein can associate with the TGN when viral glycoproteins are accumulated within this compartment.

Synchronized transport of F and M proteins from the TGN to the plasma membrane. The observation presented above

suggests that M protein can bind to viral glycoproteins contained within the TGN. To further define the extent of M protein-glycoprotein association, we examined the effect on M protein when the 20°C transport block was removed, allowing redistribution of the viral glycoproteins from the TGN to the plasma membrane. Clearly, if M protein is bound to the viral glycoproteins, we would expect to see a concomitant redistribution of both M and F proteins from the TGN to the plasma membrane. Accordingly, Sendai virus-infected BHK cells were shifted from 37 to 20°C at 5.5 hpi and incubated at 20°C for an additional 2 h. Further biosynthesis of new proteins was prevented by addition of cycloheximide (10 μ g/ml) 1 h after the shift to 20°C. Subsequently, cells were either fixed immediately (Fig. 2A and D) or transferred to 37°C in the presence of cycloheximide for either 15 min (Fig. 2B and E) or 45 min (Fig. 2C and F) before staining for either F (Fig. 2A to C) or M (Fig. 2D to F) protein distribution.

The results exhibited a coordinate redistribution of both F and M proteins from the TGN to the plasma membrane following a temperature shift from 20 to 37°C (Fig. 2). Release of the 20°C block in secretory transport resulted in an initial diffusion of the perinuclear TGN staining, which was accompanied by an increased granular appearance of the cytoplasm (presumably small transport vesicles emanating from the TGN) and the appearance of both antigens at the plasma membrane within 15 min of incubation at 37°C (Fig. 2B and E). After a 45-min incubation at 37°C, the transition from a perinuclear distribution to a plasma membrane location of both M and F proteins was almost complete (Fig. 2C and F). From these data, it appears that a population of M protein associates with Golgi membranes which contain the viral glycoproteins and is then transported along the exocytic pathway together with the viral glycoproteins to the plasma membrane.

A subpopulation of M protein is membrane bound when viral glycoproteins are restricted to the TGN. Flotation analysis is a stringent assay for determining the relative degree of membrane association of a normally soluble or cytoplasmic protein or protein complex. In the context of our experiments, this assay is not designed to resolve different populations of cellular organelles but rather to determine the proportion of M protein which is membrane associated under conditions of restricted secretory transport when viral glycoproteins are concentrated within internal Golgi-like membranes. By dispersing crude cellular extracts in very dense sucrose (70% [wt/wt]) and overlaying with steps of decreasing density (55 and 10% [wt/wt] sucrose), membranes and membrane-associated proteins can be separated from soluble proteins, aggregates, or dense particles, such as viral nucleocapsids, by equilibrium centrifugation. Accordingly, a crude postnuclear supernatant prepared from virus-infected cells incubated at 20°C was analyzed by flotation as described in Materials and Methods (see also Fig. 5 for examples of Western blots and quantification). Following centrifugation, 100% of both viral glycoproteins F and HN as well as M6PR were present in the membrane fraction which migrated to the 55/10% (wt/wt) sucrose interface (Fig. 3A and B, fraction 3). Additionally, approximately 28% of the viral M protein recovered from the gradient was found to cofractionate with the membrane fraction (Fig. 3C), while the remaining 72% of the M protein was present either as soluble protein or as part of a nucleocapsid complex which remained within the dense sucrose cushion (Fig. 3C, fractions 11 to 14). The high concentration of M protein in the dense sucrose cushion was expected, as it had previously

FIG. 7. Subcellular distribution of viral F and M proteins following a 15°C block of secretory transport. BHK cells infected by synchronized adsorption of virus at 4°C were incubated at 37°C for 5.5 h before being subjected to a block of secretory transport at 15°C for 2 h. Panels A and B were stained with monoclonal antibodies against viral F and M proteins, respectively.

been reported that M protein forms a major part of Sendai virus nucleocapsids (16).

Inhibition of secretory transport by monensin. Monensin is known to block glycoprotein transport (32) in the medial-Golgi compartment of virus-infected BHK cells (14). A major advantage of this drug over low-temperature incubations is that protein synthesis can continue in the presence of monensin throughout the incubation period (14). Consequently, the drug can be added early after infection, thereby allowing sufficient concentrations of viral proteins to be synthesized without allowing the viral glycoproteins to reach the plasma membrane. Therefore, to determine whether M protein can associate with viral glycoproteins accumulated within the medial-Golgi complex, BHK cells were infected with Sendai virus by synchronized adsorption at 4°C and then incubated at 37°C, each for a total of 10 h either without monensin (Fig. 4B, D, and F) or with the addition of 10 μ M monensin at 5 hpi (Fig. 4A, C, and E). Cells were then stained to show the relative distribution of both F and M proteins in the presence or absence of monensin. The data in Fig. 4 show that incubation of cells with monensin during infection resulted in a marked redistribution of both F and M proteins. As predicted by previous reports (14, 36), incubation with monensin prevented delivery of viral glycoproteins to the cell surface (compare Fig. 4E and F) and resulted in accumulation of F protein within distended perinuclear membranes (Fig. 4A). We also observed two distinct staining patterns of M protein in the presence of monensin. In addition to showing a diffuse cytoplasmic staining, M protein was present on distended perinuclear Golgi-like membranes similar to those containing the viral F protein (Fig. 4C).

M protein binds to Golgi membranes containing the viral glycoproteins in the presence of monensin. To substantiate the morphological evidence that M protein can bind to viral glycoproteins restricted to the medial Golgi, we performed both equilibrium centrifugation and flotation assays of crude postnuclear supernatants obtained from Sendai virus-infected cells treated with monensin (10 μ M) as described in Materials and Methods. Following equilibrium centrifuga-

tion, each fraction was analyzed by Western blotting with polyclonal rabbit serum against Sendai virus (Fig. 5E and F), and the relative proportion of each viral component was determined by densitometric analysis. Both viral glycoproteins (F and HN) were present within a specific membrane population with an average buoyant density of 1.13 g/ml (Fig. 5A to C), as expected for Golgi-derived membranes. However, we also observed that approximately 40% of the recovered M protein (Fig. 5D) cofractionated with membranes containing the F and HN proteins (Fig. 5B and C). To further show that M protein was indeed membrane associated and not simply cosedimenting with glycoprotein-containing membranes, a separate flotation analysis was performed. The results show that after flotation, 40% of the M protein was again found to be membrane associated (Fig. 6C). This direct correlation between results obtained by two distinct fractionation techniques along with the immunofluorescence data shows that a subpopulation of the M protein is associated with Golgi membranes containing the viral glycoproteins during incubation of cells with monensin.

Inhibition of biosynthetic transport at 15°C. Incubation of cells at 15°C results in the accumulation of viral glycoproteins within an intermediate compartment or pre-Golgi network (27, 28). By imposing a 15°C temperature restriction at 5.5 hpi, we were able to localize the viral F protein to sets of perinuclear stacks (Fig. 7A) which appeared less distended and more clearly defined than membranes seen to contain viral glycoproteins following incubation at 20°C (Fig. 2A) or during incubation with monensin (Fig. 4A). More significantly, the viral M protein was also partially localized to perinuclear stacks following incubation at 15°C (Fig. 7B). Small punctuate cytoplasmic loci thought to be characteristic of the intermediate compartment were less well defined in our preparations than in those used in previous morphological studies (7, 28). However, this could be due to the fact that in the absence of a temperature-sensitive mutant, we were unable to preload the endoplasmic reticulum with a large quantity of viral glycoproteins prior to incubation at 15°C. Consequently, a much lower concentration of viral

FIG. 8. Flotation analyses of M protein membrane association following a 15°C block of secretory transport. BHK cells were infected by synchronized adsorption of virus at 4°C and then incubated at 37°C for 5.5 h before a 15°C block of secretory transport was imposed for 2 h. A crude postnuclear supernatant was then fractionated by flotation through dense sucrose as described in Materials and Methods. Following centrifugation, membranes were recovered from the 10/55% (wt/wt) sucrose interface (fraction 3), while soluble proteins and viral nucleocapsids remained in the dense 70% (wt/wt) sucrose cushion (fractions 11 to 14). (A) Distribution of F protein; (B) distribution of M protein.

antigens was present in our experiments, making detection of smaller structures difficult.

M protein binds to membranes derived from the intermediate compartment following incubation of Sendai virus-infected cells at 15°C. Following flotation of membranes obtained from Sendai virus-infected cells incubated at 15°C for 2 h, approximately 87% of the viral F protein was found to be contained within membranes which floated to the 10/55% (wt/wt) sucrose interface (Fig. 8A). Identical fractions when probed for M protein revealed that 27% of the M protein was membrane bound, while 73% remained non-membrane associated and fractionated with the dense sucrose cushion (Fig. 8B).

Having observed that M protein associates with internal cellular membranes under conditions of restricted transport, we wished to determine whether this association occurred only as a result of the imposed glycoprotein accumulation or whether a similar association occurred during the normal course of Sendai virus infection.

Accordingly, Sendai virus-infected cells were incubated at 37°C for either 5.5 hpi (Fig. 9A and B) or 10 hpi (Fig. 9C and

D), and the relative subcellular distribution of F and M proteins was determined by immunofluorescence. As indicated by the results shown in Fig. 8, a subpopulation of M protein appears to be associated with perinuclear and reticular internal membranes (Fig. 9B and D) similar to those containing the viral F protein (Fig. 9A and C) at both 5.5 and 10 hpi. Consequently, it would appear that the association observed in the presence of transport inhibitors is not simply a deleterious effect but rather the amplification of a normal event occurring during viral replication.

M protein does not accumulate on Golgi membranes in the absence of viral glycoproteins. Finally, to determine whether M protein alone was inherently capable of binding to intracellular Golgi-like membranes in the absence of other Sendai virus components, we transiently expressed either M or F protein alone in BHK cells from cloned cDNA, and the transfected cells were incubated in the presence or absence of monensin at 37°C.

The effect of monensin on the subcellular distribution of each protein was determined by immunofluorescence. The cellular distribution of F protein changed from the plasma membrane in the absence of monensin (Fig. 10A) to the Golgi apparatus in the presence of monensin (Fig. 10B), as expected. However, the relative distribution of M protein was unaffected by the presence of monensin (compare Fig. 10C and D). Interestingly, in the absence of monensin, the cellular distribution of M protein did not reflect that observed during infection (compare Fig. 4D and Fig. 10C). In the absence of viral glycoproteins, M protein was distributed randomly throughout the cytoplasm of transfected cells but did not extend to the peripheral boundary of the cell as seen during Sendai virus infection, suggesting that in the absence of viral glycoproteins, M protein cannot effectively bind to either the Golgi apparatus or the plasma membrane.

To further determine whether M protein alone can bind to intracellular membranes, BHK cells transfected with either the M or F protein cDNA were pulse-labeled for 30 min at 5.5 h posttransfection before being chased for 1 h at 37°C. This chase period was in excess of the reported kinetics of M protein-membrane association observed during Sendai virus infection (4). Cells were then homogenized, and a postnuclear supernatant was analyzed by flotation. The results show that following flotation, F protein predictably migrated along with membranes to the 10/55% (wt/wt) sucrose interface (Fig. 11A) and that 98% of the M protein remained in the dense sucrose cushion (Fig. 11B), indicating that M proteins when expressed alone do not have an inherent capacity to associate with cellular membranes either *in vivo* or during fractionation.

DISCUSSION

The exact mechanism by which M protein interacts with viral glycoproteins during the assembly of paramyxoviruses is poorly understood. However, a working model has been proposed on the basis of observations made during Newcastle disease virus replication (36). Specifically, M protein was found to exhibit strict topographical requirements for glycoprotein binding in that the association between M protein and glycoproteins could occur only after the glycoproteins had reached the plasma membrane of the host cell (36). In this report, we have examined the mechanism of M protein-membrane association during Sendai virus assembly and found it to vary considerably from that reported for Newcastle disease virus (36). The results presented here show that during Sendai virus assembly, a population of M protein

FIG. 9. Association of M protein with internal membranes during normal uninterrupted viral infection. BHK cells were infected by synchronized adsorption of virus at 4°C and then incubated at 37°C for either 5.5 h (A and B) or 10 h (C and D). Cells were then fixed to observe the internal distribution of either F protein (A and C) or M protein (B and D). Panels C and D were photographed at one-fifth the exposure time used for panels A and B in order to reveal the internal distribution of each antigen, which would otherwise be obscured by the prevalent plasma membrane population of each antigen seen at later stages of infection (see Fig. 4B and D).

binds to internal cellular membranes which contain viral glycoproteins before being transported to the plasma membrane. These data further suggest that some M protein-glycoprotein complex can be cotransported along the exocytic pathway. Indeed, there are now several well-documented examples in which M proteins (or their equivalents) of other virus families, e.g., the rhabdovirus (17), alphavirus (19), and retrovirus (3, 18) families, associate with internal membranes containing the respective viral glycoproteins and subsequently travel in a piggyback style along the secretory pathway (3) as proposed here for Sendai virus.

By inhibition of transport at sequential steps along the exocytic pathway, viral glycoproteins were concentrated within the pre-Golgi intermediate compartment (at 15°C), the

medial-Golgi (in the presence of monensin), or the TGN (at 20°C). Under all three of these conditions, we were able to show morphologically by immunofluorescence and biochemically by cell fractionation studies that Sendai M protein associates with internal cellular membranes which contain viral glycoproteins at early stages of secretory transport. Also, by expression of M protein alone in the absence of Sendai virus glycoproteins, we have established that neither membrane association nor perinuclear accumulation of M protein can occur in the absence of viral glycoproteins. Consequently, our results suggest that M protein interacts with membranes of the host cell via a specific interaction with viral glycoproteins and not simply by nonspecific interaction with membrane lipids. This observation is pertinent, as there are published reports of M proteins from both

FIG. 10. Absence of M protein on perinuclear Golgi-like membranes in the absence of viral glycoproteins. BHK cells infected with a recombinant vaccinia virus containing the T7 RNA polymerase were subsequently transfected with cDNA encoding either F protein (A and B) or M protein (C and D). Two hours after transfection, monensin ($10 \mu\text{M}$) was added to cells shown in panels B and D. All cells were incubated for a total of 5.5 h at 37°C before being processed for immunofluorescence.

myxoviruses and paramyxoviruses interacting directly with synthetic liposomes *in vitro* (9, 12). Both our results and the results of Yoshida et al. (36) suggest that intracellular M proteins may show a very different selectivity for membrane association, possibly as a result of an alternative interaction with cellular components such as the cytoskeleton or other cellular or viral proteins which may restrict the ability of the protein to associate with internal membranes.

It is also significant to note that other authors have previously observed a diffuse perinuclear distribution of M protein during the early stages of Sendai virus replication (34). Also, it was previously reported that a higher percentage of M protein is associated with the smooth cellular membranes of Sendai virus-infected cells compared with that observed in Newcastle disease virus-infected cells (36). These observations as well as the data reported here suggest that there may well be mechanistic variation among the assembly processes of different members of the paramyxovirus family.

From the observations presented here, we cannot say whether the binding of M proteins to viral glycoproteins during secretory transport plays any essential role in viral assembly or is simply a passive association. However, recent reports on the bipolar budding of a pantropic Sendai virus mutant (F1-R) raise several interesting points (30, 31). Analyses of the F1-R mutant (31) and several revertants (30) have revealed that one mutation in the F-protein cytoplasmic tail together with two mutations in the M protein were sufficient and responsible for polarized missorting of both the mutated F protein and the unmutated HN protein. Interestingly, the authors proposed that formation of an aberrant complex between M protein and both glycoproteins

FIG. 11. Drastic reduction in M protein membrane association in the absence of viral glycoproteins. BHK cells were infected with a recombinant vaccinia virus containing the T7 RNA polymerase gene before being transfected with cDNA encoding either the Sendai virus F or M protein. Following transfection, cells were incubated at 37°C for 5.5 h before being pulse-labeled for 30 min and chased for a further hour at 37°C . Following metabolic labeling, cells were homogenized and fractionated by flotation as described in Materials and Methods. After centrifugation, membranes were recovered from the 10/55% (wt/wt) sucrose interface (fractions 1 and 2), while non-membrane-associated material was recovered from the dense 70% (wt/wt) sucrose cushion (fractions 4 to 6). (A) Distribution of F protein; (B) distribution of M protein.

could result in the incorrect polarized targeting of complexed proteins. Obviously, if this were true, in order to affect the polarized sorting of transmembrane proteins, the M protein-glycoprotein complex would have to be formed prior to the exit of glycoproteins from the TGN, as this compartment facilitates the normal segregation of proteins destined for transport to either the apical or the basolateral plasma membranes (15, 23). In fact, we have also analyzed the distribution of the M protein in mutant F1-R-infected cells and found that like the WT M protein, the mutated F1-R M protein was associated with viral glycoproteins before exiting from the TGN (data not shown). To conclusively determine whether M protein can either play an active role in or interfere with correct protein sorting, it will be necessary to isolate viral mutants or revertants that possess mutations only within the M gene but still exhibit the bipolar missorting phenotype. Alternatively, we need to show that a mutated form of the M protein, when expressed with WT forms of the viral glycoproteins, can affect their normal polarized distribution. Such experiments are in progress.

Our data strongly suggest that a population of M protein

can and does interact with the viral glycoproteins before they reach the host cell plasma membrane. However, although the cellular location of membrane-bound M protein appears to be determined by the location of the viral glycoproteins, we have not yet demonstrated a definitive physical interaction between M and either F or HN. To identify exactly which proteins are interacting with each other and which domains of each are involved in the interaction, we are currently expressing both the WT and mutated forms of all three components (F, HN, and M). We conclude from the data presented here that the plasma membrane-specific model of M protein-glycoprotein interaction cannot be applied to Sendai virus assembly. Instead, we propose that Sendai virus M protein and possibly also mature nucleocapsids can bind to membranes which contain a sufficient concentration of viral glycoproteins either as a result of enforced accumulation or as a result of the natural concentration of proteins that occur during transit through the secretory pathway. We propose that virus budding and release may occur predominantly from the plasma membrane not because of a strict topographic requirement for M protein-glycoprotein binding but possibly because the physical process of viral bud formation occurs selectively or more readily at the plasma membrane. It will be interesting, therefore, to determine whether nucleocapsid binding and even budding can occur in the Golgi apparatus following incubation with monensin as was previously reported for Semliki forest virus (14).

Finally, interaction between M protein and viral glycoproteins has been rather difficult to pursue because of the lability of the bonds between M protein and glycoproteins. Coimmunoprecipitation often disrupts the M protein-glycoprotein complex or nonspecifically precipitates M protein. However, separation of membrane-bound and soluble proteins by flotation provides a clean quantitative assay for determining the extent of M protein-glycoprotein association in vivo and can now be used to examine the specificity of the interaction and to determine the exact domains of either the M protein or glycoproteins involved in binding. Such studies are in progress.

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