Sendai Virus M Protein Binds Independently to either the F or the HN Glycoprotein In Vivo

C. M. SANDERSON, H.-H. WU, AND D. P. NAYAK*

Department of Microbiology and Immunology, Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, California 90024-1747

Received 11 August 1993/Accepted 21 September 1993

We have analyzed the mechanism by which M protein interacts with components of the viral envelope during Sendai virus assembly. Using recombinant vaccinia viruses to selectively express combinations of Sendai virus F, HN, and M proteins, we have successfully reconstituted M protein-glycoprotein interaction in vivo and determined the molecular interactions which are necessary and sufficient to promote M protein-membrane binding. Our results showed that M protein accumulates on cellular membranes via a direct interaction with both F and HN proteins. Specifically, our data demonstrated that a small fraction (8 to 16%) of M protein becomes membrane associated in the absence of Sendai virus glycoproteins, while >75% becomes membrane bound in the presence of both F and HN proteins. Selective expression of M protein together with either F or HN protein showed that each viral glycoprotein is individually sufficient to promote efficient (56 to 73%) M protein-membrane binding. Finally, we observed that M protein associates with cellular membranes in a time-dependent manner, implying a need for either maturation or transport before binding to glycoproteins.

The structural simplicity of paramyxoviruses makes them ideal for analyzing the complex process of enveloped virus assembly. Sendai virus particles are pleomorphic structures composed of a lipoprotein envelope surrounding a single unsegmented viral nucleocapsid. The viral envelope is composed of a lipid bilayer containing the viral fusion (F) protein, the hemagglutinin/neuraminidase (HN) protein, and the matrix (M) protein which forms an electron-dense shell immediately below the membrane. Coiled around the inside surface of the viral envelope is the nucleocapsid, a composite structure composed of a single negative-sense viral RNA genome, the nucleoprotein (NP), the phosphoprotein (P), and the polymerase protein (L).

During the final stages of infection, assembly of virus particles is triggered by the binding of nucleocapsids to cellular membranes which contain the two viral spike proteins (F and HN). Mechanistically, very little is known about the molecular interactions which occur during viral morphogenesis. However, there is strong evidence to suggest that the process is mediated by the viral M protein (18, 21). On the basis of data obtained from the analyses of temperature-sensitive mutants (13, 14, 20) and chemical cross-linking experiments (11), a model which predicts that M protein forms a bridge between the NP of the viral nucleocapsid and the cytoplasmic tails of both F and HN proteins has been proposed (12). However, as yet, there is no direct evidence to support the proposed interaction of M protein with one or both of the viral glycoproteins, nor do we understand the sequential order in which components interact during virus budding.

In this study, we have addressed some of these questions by directly analyzing the molecular interactions which are both necessary and sufficient to promote M protein-membrane binding in vivo. Specifically, we have constructed three recombinant vaccinia viruses which encode Sendai virus F (RVVF), HN (RVVHN), or M (RVVM) protein. By selective infection with these viruses, we have successfully reconstituted M protein-glycoprotein interaction in vivo and determined the mechanism by which M protein interacts with cellular membranes.

Two independent assays were used to determine the relative degree of M protein-membrane binding. First, we have used a membrane rotation assay (16) to quantify the efficiency of M protein-membrane binding in the presence or absence of Sendai virus glycoproteins. Second, a morphological assay was used to qualitatively demonstrate M protein-glycoprotein binding. Essentially, in this assay, Sendai virus glycoproteins were accumulated within the medial Golgi compartment by incubation with monensin (8). Under these conditions, a subpopulation of M protein was found to concentrate around perinuclear Golgi-like membranes (16), thereby providing a convenient morphological assay for M protein-glycoprotein interaction. We discuss the implications of these observations on the accuracy of the proposed model of paramyxovirus assembly.

MATERIALS AND METHODS

Reagents. Monoclonal antibodies against Sendai virus proteins M, F, and HN were kind gifts from A. Portner (St. Jude Children’s Research Hospital, Memphis, Tenn.) A polyclonal antiserum raised against wild-type Sendai virus (AS 74), a monospecific polyclonal antiserum against Sendai HN protein (AS 61), and a bispecific polyclonal antiserum against Sendai virus HN and F proteins (AS 59) were generously donated by J. T. Seto (California State University, Los Angeles). Two cDNA clones encoding the Sendai virus F and HN proteins were 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 H.-H. Wu. Fluorescein isothiocyanate-, rhodamine-, or alkaline phosphatase-conjugated secondary antibodies were obtained from Cappel Laboratories, West Chester, Pa.

Virus and cells. Wild-type Sendai virus (Z strain) obtained from J. T. Seto (California State University, Los Angeles) was grown in 10-day-old embryonated chicken eggs. Baby hamster kidney (BHK) cells were maintained in Dulbecco modified Eagle’s medium containing 10% fetal calf serum. Wild-type
vaccinia virus was obtained from Bernard Moss, National Institutes of Health, Bethesda, Md.

Construction of recombinant vaccinia viruses. Recombinant vaccinia viruses were constructed by the method of Chakrabarti et al. (4). Sendai virus Z strain cDNAs encompassing the open reading frames of the F, HN, and M proteins were inserted into the multiple cloning site of the vaccinia virus expression vector pSC11, which contains the promoter sequence of the 7.5 protein upstream of the multiple cloning site and the thymidine kinase gene. All vaccinia viruses were propagated and plaque titered in HeLa cells.

Indirect immunofluorescence. For analyses of internal antigen distribution, cells were thoroughly washed in phosphate-buffered saline (PBS) and fixed by incubation in acetone-methanol (1:1) for 7 min at −20°C. Following fixation, cells were blocked in PBS containing 0.2% (wt/vol) gelatin (blocking buffer) for 1 h at 37°C. Primary antibodies were diluted to 1/100 for monoclonal antiseras or 1/1500 for polyclonal antiseras (using 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 the blocking buffer before the addition of secondary fluorescein-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.)-gelsol solution containing 2.5% 1,4-diazobicyclo-[2.2.2.] octane (DABCO; Sigma, St. Louis, Mo.). Transmission fluorescence microscopy was performed with a Nikon Optiphot microscope (Nippon Kogaka K. K., 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 dishes by scraping. Cells were then harvested by centrifugation, 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 1,000 × g for 5 min, and the resulting postnuclear supernatant was subjected to further fractionation by flotation as described below.

Flotation analyses. Flotation analyses were performed as described by Sanderson et al. (16), with the following modifications. Crude postnuclear supernatants were prepared as described above. Aliquots (0.5 ml) were then dispersed into 2 ml of 72% (wt/vt) sucrose in low-salt buffer (LSB) containing 50 mM Tris HCl (pH 7.5), 25 mM KCl, and 5 mM MgCl₂ and overlaid with 2.5 ml of 55% (wt/vt) sucrose LSB and approximately 0.6 ml of 10% (wt/vt) sucrose LSB. Gradients were then centrifuged by using a Beckman SW 55 Ti rotor at 4°C for approximately 8 h at 38,000 rpm. Following centrifugation, 0.8-ml fractions were collected from the top of the gradient, using a Hacki-Buckler Auto Densiflow II gradient remover. Any material which pelleted during centrifugation was resuspended within the final 0.8-ml fraction of each gradient. Prior to immunoprecipitation, all fractions were diluted with 3 ml of LSB before addition of 1 ml of 5×-concentrated radiolabeled immunoprecipitation assay buffer. Samples were shaken at 4°C for 2 h before addition of antiseras. Each fraction was immunoprecipitated with a cocktail of 1 μl of a polyclonal rabbit anti-Sendai virus serum (AS 74) and 0.2 μl of a monoclonal antiserum against the Sendai virus M protein. In the context of our experiments, this assay was not meant to resolve different populations of cellular membranes (all of which should empirically float) but rather was designed to simply determine the relative proportion of M protein which binds to membranes in the presence or absence of selected Sendai virus glycoproteins.

Transient expression of M and F proteins from recombinant vaccinia viruses. BHK cells were infected with recombinant vaccinia viruses at the indicated multiplicity of infection (MOI) for 1 h at 37°C. After infection, virus was removed and cells were incubated in minimal essential medium at 37°C until being processed as described in the text.

RESULTS

Expression of Sendai virus F, HN, and M proteins. To facilitate the selective expression of Sendai virus proteins, three recombinant vaccinia viruses which encoded either Sendai virus F protein (RVVF), HN protein (RVVHN), or M protein (RVVM) were constructed. BHK cells infected with either recombinant vaccinia viruses or Sendai virus were labeled for 20 min at 7 h postinfection (hpi) and processed for immunoprecipitation with or without a chase period. As shown in Fig. 1A, Sendai virus proteins expressed from recombinant vaccinia viruses were specifically precipitated by a polyclonal rabbit antiserum (AS 74) raised against wild-type Sendai virus and were shown to comigrate with authentic Sendai virus proteins in sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. The comparatively low level of M protein immunoprecipitated directly after pulse-labeling is thought to be due to a time-dependent antigenic maturation of the M protein, as more M protein was immunoprecipitated after a 1-h chase in the absence of radioisotopes (Fig. 1A; compare lanes 2 and 6). Similar results were also reported by other workers (15, 17). Specificities of polyclonal sera (AS 61 and AS 59) and monoclonal antibodies are shown in Fig. 1B and C, respectively. Although not shown here, these polyclonal and monoclonal antibodies also immunoprecipitated HN, F, or M protein after expression from recombinant vaccinia viruses.

The morphological distribution of individually expressed Sendai virus proteins was analyzed by immunofluorescence. As can be seen in Fig. 1D and E, Sendai virus glycoproteins expressed from recombinant vaccinia viruses show a typical plasma membrane-type distribution in which peripheral cellular extensions are well defined, as are the internal Golgi-reticular membranes which contain viral glycoproteins en route to the plasma membrane. In contrast, M protein exhibited a diffuse cytoplasmic distribution (Fig. 1F) which did not define either peripheral cellular extensions or internal membranes. Significantly, none of the antibodies used in these experiments recognized cells infected with wild-type vaccinia virus (Fig. 1G to I).

M protein does not bind efficiently to membranes in the absence of Sendai virus glycoproteins. To determine the extent to which M protein binds to cellular membranes in the absence of Sendai virus glycoproteins, BHK cells were infected with RVVM alone and then analyzed by membrane flotation. Flotation analysis is a stringent assay for determining the relative degree of membrane association of a soluble cytoplasmic protein or complex. In the context of our experiments, this assay is not designed to resolve different populations of cellular organelles but rather is designed to determine the proportion of M protein which binds to any cellular membrane. Cells were pulse-labeled for 20 min at 7 hpi and chased for 2.5 h before being processed for membrane flotation as described in Materials and Methods. Following equilibrium centrifugation, membranes which corresponded to the 10%/55% (wt/wt) sucrose interface (Fig. 2C) were recovered in fraction 1, while non-membrane-associated proteins remained within the dense sucrose cushion (fractions 4 to 6). The results presented in Fig. 2D show that >90% of the total M protein remained within the dense sucrose cushion following equilibrium centrifugation,
indicating that in the absence of Sendai virus glycoproteins, M protein exists predominantly as a non-membrane-bound cytoplasmic protein. It should also be noted that after infection with RVVM, two forms of Sendai virus M protein, corresponding to the phosphorylated and nonphosphorylated forms of M protein observed during Sendai virus infection (9), were detected. To further test our flotation data, we used Western blots (immunoblots) to analyze the equilibrium subcellular distribution of M protein after 9 h of infection. The results from these experiments showed that even under steady-state conditions, M protein was again predominantly (>80%) contained within the dense sucrose cushion following flotation (data not shown). These results confirm our previous observation that M protein does not efficiently bind to cellular
membranes in the absence of Sendai virus glycoproteins (16) and also demonstrate that the observed low levels of M protein-membrane binding were not simply due to the time limitations of the chase period used throughout our experiments.

**Binding of M protein to membranes containing both F and HN proteins.** To determine whether M protein could bind to Sendai virus glycoproteins in the absence of other Sendai virus components, BHK cells were coinfected with a combination of RVVF, RVVHN, and RVVM in a ratio of 3:3:1. Cells were pulse-labeled for 20 min at 7 hpi and then fractionated either immediately or after a 2.5-h chase period. The results presented in Fig. 3 show the relative distribution of all three proteins (F, HN, and M) following flotation centrifugation. As expected, both integral membrane proteins (F and HN) were recovered from fraction 1 regardless of whether cells were chased (Fig. 3A and C). In contrast, M protein was initially synthesized as a non-membrane-bound cytoplasmic protein (Fig. 3B) which subsequently bound to membranes containing Sendai virus glycoproteins during the 2.5-h chase period (Fig. 3D).

These data indicate that M protein binds efficiently to membranes which contain F and HN proteins. To morphologically analyze this effect, BHK cells were infected either with a combination of RVVF, RVVHN, and RVVM or with RVVM alone. Monensin (20 μM) was added at 5 hpi, and cells were subsequently incubated in the presence of monensin for a further 3-h period before being processed for immunofluorescence as described in Materials and Methods. In the presence of monensin (20 μM), F and HN proteins were predominantly restricted to distended perinuclear membranes (Fig. 4A), whereas M protein expressed alone maintained a diffuse cytoplasmic distribution (Fig. 4C). In contrast, coexpression of F, HN, and M proteins in the presence of monensin resulted in a perinuclear accumulation of M protein which was clearly not seen in the absence of Sendai virus glycoproteins (compare Fig. 4B and C). Consequently, taken together with the cell fractionation data, these results demonstrated that M protein binds efficiently to cellular membranes which contain both F and HN proteins.

**Double-component coexpression experiments.** Having established that M protein binds efficiently to cellular membranes containing the two Sendai virus glycoproteins, we were interested in determining whether each viral glycoprotein was individually sufficient to promote M protein-membrane binding. For this reason, either F or HN protein was selectively coexpressed with M protein, and the degree of M protein-membrane binding was determined by flotation and immunofluorescence assays. In these experiments, the MOI of recombinant vaccinia viruses encoding the relevant glycoprotein (F or HN) was increased twofold (i.e., to an MOI of 6) in order to maintain both the same total MOI per cell and the same ratio of ligand (M protein) to receptor (F or HN protein), thereby
facilitating direct comparison of the relative efficiency of M protein-glycoprotein binding between experiments. In all flotation experiments using mixed components, a $T = 0$ time point was included in order to define the basal level of M protein binding for that particular experiment and to show that membrane binding was not simply due to nonspecific association which occurred as a result of cell fractionation.

F protein alone is sufficient to promote efficient M protein-membrane association. BHK cells, infected with a combination of RVVF and RVVM at MOIs of 6 and 1, respectively, were pulse-labeled for 20 min at 7 hpi and then either processed immediately or chased for a further 2.5 h before fractionation. The results show the relative distribution of both F and M proteins following flotation (Fig. 5A to F) and the morphological distribution of each protein during coexpression in the presence of monensin (Fig. 5G and H). From the cell fractionation data presented in Fig. 5A and B, it is apparent that the majority of F protein was converted to a higher-molecular-weight form during the chase period. This observation is consistent with posttranslational modifications which occur during exocytic transport. It is important to note, however, that the relative distribution of F protein within the flotation gradient did not change significantly during the chase period (Fig. 5E). Again, this result was expected since the F protein is a transmembrane protein (10) and therefore should float irrespective of its subcellular location or glycosylation pattern. The slight tailing off of the F protein signal toward the bottom of the gradient observed when cells were fractionated immediately after pulse-labeling was probably due to a less efficient flotation of dense rough endoplasmic reticulum-derived membranes in this experiment. In contrast, M protein exhibited a clear time-dependent redistribution between the cytoplasmic and membrane fractions of the flotation gradients (Fig. 5F). Immediately after pulse-labeling, M protein was predominantly detected as a soluble cytoplasmic protein or complex which remained within the dense sucrose cushion (fractions 4 to 6) following flotation. However, after the 2.5-h chase period, the majority of M protein cofractionated with membranes containing F protein (fraction 1).

To further investigate the interaction between F and M proteins, BHK cells were coinfectcd with RVVF and RVVM as described above. Five hours after infection, monensin was added to a final concentration of 20 μM, and cells were incubated for a further 3-h period before being processed for immunofluorescence. The addition of monensin resulted in a massive accumulation of F protein within distended perinuclear Golgi-like membranes (Fig. 5G). In addition, M protein was seen to accumulate in a perinuclear region of the cell, thus demonstrating a glycoprotein-dependent accumulation of M protein. Since M protein alone did not show any Golgi-like redistribution in the presence of monensin (Fig. 4C), we conclude that F protein is individually sufficient to promote M protein-membrane binding.

HN protein is independently sufficient to promote M protein-membrane association. To determine whether HN protein also facilitates M protein-membrane interaction, BHK cells were coinfectcd with a combination of RVVHN and RVVM and pulse-labeled for 20 min at 7 hpi. Cells were then either processed immediately or chased for a further 2.5 h before fractionation. Like F protein, HN protein was predominantly localized within the floated membrane fraction (fraction 1) irrespective of the time of processing (Fig. 6E). In contrast, the M protein was seen to redistribute from a cytoplasmic to a membrane distribution within the 2.5-h chase period (Fig. 6F).

To further investigate the interaction of HN and M proteins, we coinfectcd BHK cells with RVVHN and RVVM in the presence of monensin as described above and analyzed the relative subcellular distribution of each protein by immunofluorescence. The results (Fig. 6G and H) show a glycoprotein-dependent accumulation of M protein in a perinuclear region of the cell which was not seen when M protein was expressed alone in the absence of either Sendai virus glycoprotein (Fig. 4C). These data are directly comparable to the results obtained when M protein was coexpressed with F and HN proteins or with F protein alone. Consequently, we conclude that HN protein is also independently sufficient to promote membrane-association of M protein.

Kinetics of M protein-membrane interaction. To investigate the sequential mechanism of M protein-membrane interaction, we analyzed the relative kinetics of M protein-membrane binding either in the presence or in the absence of Sendai virus glycoproteins. We were particularly interested in determining whether the kinetics of M protein-membrane association was comparable to that reported for vesicular stomatitis virus (5). Accordingly, BHK cells were infected with either a combina-
FIG. 5. F protein alone is sufficient to promote M protein-membrane association. (A to F) Results of membrane flotation experiments. BHK cells were infected with a combination of recombinant vaccinia viruses encoding Sendai virus F and M proteins at MOIs of 6 and 1, respectively. Cells were pulse-labeled for 20 min at 7 hpi and then either fractionated immediately (A and C) or chased for a further 2.5 h before being processed for membrane flotation (B and D). The relative distributions of F protein and M protein following flotation are shown in panels A and B and panels C and D, respectively. Quantification of autoradiographs was performed with an LKB laser scanner, and the results are shown in panels E and F. Values shown on graphs represent percentage of total detected protein. Morphological analyses of F protein-M protein interaction were performed to confirm data obtained by cell fractionation; these results are shown in panels G and H. In these experiments, BHK cells were infected with a combination of recombinant vaccinia viruses encoding F and M proteins, and monensin was added to a final concentration of 20 μM at 5 hpi. Cells were then incubated in the presence of monensin for a further 3 h before being processed for immunofluorescence. Cells shown in panel G were stained with a monospecific rabbit polyclonal antiserum against Sendai virus F protein (AS 59); cells shown in panel H were stained with a monoclonal antiserum against Sendai virus M protein.

FIG. 6. HN protein alone is sufficient to promote M protein-membrane association. (A to F) Results of membrane flotation experiments. BHK cells were infected with a combination of recombinant vaccinia viruses encoding Sendai virus HN and M proteins. Cells were pulse-labeled 7 hpi and then either fractionated immediately (A and C) or chased for a further 2.5 h before being processed for membrane flotation (B and D). The relative distributions of HN protein and M protein following flotation are shown in panels A and B and panels C and D, respectively. Quantification of autoradiographs was performed with an LKB laser scanner, and the results are shown in panels E and F. Values shown on graphs represent percentage of total recovered protein. Morphological analyses of HN protein-M protein interaction were performed to confirm data obtained by cell fractionation; these results are shown in panels G and H. In these experiments, BHK cells were infected with a combination of recombinant vaccinia viruses encoding HN and M proteins, and monensin was added to a final concentration of 20 μM at 5 hpi. Cells were then incubated in the presence of monensin for a further 3 h before being processed for immunofluorescence. Cells shown in panel G were stained with a monospecific rabbit polyclonal antiserum against Sendai virus HN protein (AS 61); cells shown in panel H were stained with a monoclonal antiserum against Sendai virus M protein.

The M protein detected by immunoprecipitation was initially synthesized as a non-membrane-bound cytoplasmic protein which, in the presence of F and HN proteins, started to bind to membranes within the first 45 min of chase (Fig. 7B). Also, we observed that the relative level of membrane-bound M protein increased gradually throughout the chase period, reaching a maximal value of >75% after 3 h of chase, while the cytoplasmic population showed a concomitant decrease with time (Fig. 7A to D). Interestingly, M protein expressed alone did demonstrate some time-dependent membrane binding (Fig. 7E to H), but the efficiency was clearly much reduced (<20%) compared with that observed in the presence of both F and HN proteins (>75%) (Fig. 7I). These experiments were replicated three times, and in each case the relative kinetics of M protein-membrane binding in the presence and absence of viral glycoproteins were essentially similar (data not shown). From these data, we conclude that M protein requires a lag period before binding to cellular membranes. Also, M protein may follow the same pattern of maturation or transport irrespective of the presence or absence of Sendai virus glyco-
proteins. However, concentration of M protein within cellular membranes is greatly enhanced by Sendai virus F and HN proteins.

DISCUSSION

The data presented in this report demonstrate three important aspects of Sendai virus M protein-membrane interactions in vivo. First, Sendai virus glycoproteins are required for efficient membrane association of M protein in vivo. Second, each viral glycoprotein (F or HN) is individually sufficient to promote M protein-membrane binding. Finally, M protein requires a period of maturation or transport before binding to cellular membranes. These observations are important because they identify specific interactions which may occur during the initial stages of viral morphogenesis. In addition, our results substantiate several indirect observations on which the current hypothesis of paramyxovirus assembly is based.

For example, ultrastructural analyses of Sendai virus-infected cells led to the idea that M protein binds to regions of the plasma membrane which contain viral glycoproteins (1, 3, 12) and in doing so induces a site for localized clustering of envelope components, to which viral nucleocapsids subsequently bind (12). Implicit in this idea is the fact that a population of M protein can bind to viral glycoproteins before binding to viral nucleocapsids. Our results confirm that this model would be mechanistically feasible in vivo, as we demonstrate that M protein can bind to viral glycoprotein in the absence of other Sendai virus components.

In addition, identification of temperature-sensitive mutants which produced viral particles that selectively lacked either F (13) or HN (19) protein led to the assumption that M protein must possess the ability to interact with either of the viral glycoproteins individually. Clearly, our data provide direct physical and morphological evidence to confirm this assumption.

Nonmyristylated M proteins are found in several different virus families (6), including orthomyxo-, paramyxo-, and rhabdoviruses. In each case, the M protein is presumed to provide an essential link between viral nucleocapsids and the envelope. Given the common function of M proteins, it is tempting to assume that there may be a single unifying mechanism of M protein-envelope interaction. However, recent reports concerning the mechanism of M protein-membrane interaction in rhabdovirus (5) showed that M protein binds directly to cellular membranes independent of any interaction with the viral spike or G protein. Also, the authors suggested that M protein-membrane binding must occur rapidly after synthesis while the M protein is still in a nascent or plastic conformation. However, it is evident from our data that the mechanism of membrane interaction for Sendai virus M protein is clearly different. Whether these differences represent mechanistic variations which exist between viral families or simply reflect technical limitations of different transient expression systems remains to be determined. Consequently, although M proteins appear to serve the same function in different viruses, it remains to be determined whether they do so by directly comparable mechanisms.

It would appear from our kinetic data that the rate of M protein-membrane association is slow compared with that observed during Sendai virus infection (2). However, it is important to note that a direct comparison between the two systems cannot be made for the following reasons. First, the rate of M protein-membrane association observed during Sendai virus infection may be faster simply because viral assembly is a coordinated cooperative process in which multiple interactions serve to promote membrane incorporation of all viral components. Second, during Sendai virus infection, M protein may associate with membranes directly by binding to viral glycoproteins or indirectly as part of a mature viral nucleocapsid. Consequently, it is difficult to analyze the rate of association of either population individually during Sendai virus infection. In contrast, our assay specifically analyzes direct M protein-glycoprotein interactions. As such, the two sets of data should not be directly compared.

In the experiments reported here, we have shown that M protein does not bind efficiently to cellular membranes in the absence of viral glycoproteins. This conclusion is in contrast to the in vitro results which showed that the M protein of Newcastle disease virus binds to lipoprotein membranes in the absence of any other proteins (7). We believe that this apparent discrepancy arises from the fact that in vivo, M
protein may interact with other cellular or viral components which restrict or mask its ability to bind directly to the lipid bilayer. It is also possible that hydrophobic domains are artificially exposed as a result of the stringent extraction procedures used to obtain the M protein used in in vitro binding assays (7). It should also be noted that the efficiency of M protein-liposome binding in vitro was very low, and it is possible that these results correspond to the low levels of glycoprotein-independent M protein-membrane interaction which we also observe in vivo. Whether this small percentage of glycoprotein-independent M protein-membrane binding is mechanistically relevant to Sendai virus assembly remains to be seen.

During Sendai virus infection, M protein exists in both phosphorylated and nonphosphorylated forms (9); however, after cell fractionation, the phosphorylated form could not be easily detected (9). In contrast to these observations, our data show that both forms of the M protein were readily detectable after cell fractionation. Interestingly, our results show that there is no difference between the relative distribution of phosphorylated and nonphosphorylated M protein following membrane flotation, as both forms of the M protein were detected in the cytoplasm and on membranes. Therefore, it would appear that within the context of our reconstitution assay, the phosphorylation state of the M protein does not absolutely dictate its subcellular localization.

In conclusion, we believe that this assay faithfully replicates the early stages of Sendai virus assembly and provides a simple in vivo system which may be used to define the precise molecular interactions which occur between M protein and each viral glycoprotein during viral assembly. Molecular dissection of each protein is being performed to determine the exact epitopes which mediate the component interactions during Sendai virus assembly.

ACKNOWLEDGMENTS

Work reported here was supported by NIAID research grants RO1AI16748 and RO1AI12749.

REFERENCES