Virology 276, 289–303 (2000)
doi:10.1006/viro.2000.0556, available online at http://www.idealibrary.com on IDEAL®

Assembly of Sendai Virus: M Protein Interacts with F and HN Proteins and with the Cytoplasmic Tail and Transmembrane Domain of F Protein

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Received May 26, 2000; returned to author for revision June 27, 2000; accepted July 26, 2000

Sendai virus matrix protein (M protein) is critically important for virus assembly and budding and is presumed to interact with viral glycoproteins on the outer side and viral nucleocapsid on the inner side. However, since M protein alone binds to lipid membranes, it has been difficult to demonstrate the specific interaction of M protein with HN or F protein, the Sendai viral glycoproteins. Using Triton X-100 (TX-100) detergent treatment of membrane fractions and flotation in sucrose gradients, we report that the membrane-bound M protein expressed alone or coexpressed with heterologous glycoprotein (influenza virus HA) was totally TX-100 soluble but the membrane-bound M protein coexpressed with HN or F protein either individually or together was predominantly detergent-resistant and floated to the top of the density gradient. Furthermore, both the cytoplasmic tail and the transmembrane domain of F protein facilitated binding of M protein to detergent-resistant membranes. Analysis of the membrane association of M protein in the early and late phases of the Sendai virus infectious cycle revealed that the interaction of M protein with mature glycoproteins that associated with the detergent-resistant lipid rafts was responsible for the detergent resistance of the membrane-bound M protein. Immunofluorescence analysis by confocal microscopy also demonstrated that in Sendai virus-infected cells, a fraction of M protein colocalized with F and HN proteins and that some M protein also became associated with the F and HN proteins while they were in transit to the plasma membrane via the exocytic pathway. These studies indicate that F and HN interact with M protein in the absence of any other viral proteins and that F associates with M protein via its cytoplasmic tail and transmembrane domain.

INTRODUCTION

Sendai viruses, a member of the *Paramyxoviridae* family of viruses that includes many human and animal pathogens, are enveloped viruses containing a single RNA genome of negative polarity (Lamb and Kolakofsky, 1995). Members of this group of viruses bud from the plasma membrane of infected cells producing pleomorphic spheroidal particles containing a long helical nucleocapsid. The viral envelope is composed of lipid bilayers derived from the host plasma membrane and contains oligomeric forms of two transmembrane glycoproteins, F (type I) and HN (type II). F and HN proteins are synthesized in the endoplasmic reticulum and transported via the exocytic pathway to the plasma membrane, the assembly and budding site of Sendai viruses. Consequently, complete virus particles are not usually observed inside the cell.

Morphogenesis of enveloped viruses in general and Sendai viruses in particular is not well understood. The assembly and budding processes of enveloped viruses are complex and involve multiple steps. They involve not only the formation of nucleocapsid complex but also the envelopment of the viral nucleocapsid at the plasma membrane and budding of virions. Two steps are obligatory in the assembly and budding processes of virions: First, all subviral components, either individually or as a complex, must be transported to the assembly and budding site, and second, these components must interact with one another in an orderly way during the transport or at the assembly site or both in order to initiate the budding process leading to the production of infectious virions (Nayak, 1996, 2000).

From the standpoint of understanding the viral assembly and budding processes, the Sendai viral structure can be divided into three major subviral components, each of which must be brought to the assembly site for morphogenesis to occur. These subviral components are as follows: (i) the helical viral nucleocapsid containing the vRNA, NP (nucleoprotein), and transcriptase complex consisting of L and P proteins, which together form the inner core of virus particles; (ii) the matrix protein (M protein), which forms an outer protein shell around the nucleocapsid and constitutes the bridge between the viral envelope and the nucleocapsid; and (iii) the viral envelope (or membrane), the outermost barrier of virus particles, consisting of the virally encoded F and HN transmembrane glycoproteins and host cell lipids (see Dubois-Dalcq *et al.,* 1984).

During the process of viral assembly, mature nucleocapsids must interact with the components of the viral

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0042-6822/00 $35.00

envelope to initiate the process of bud formation. This association is presumed to be mediated by M protein, which is a key component in virus assembly and morphogenesis. It is the most abundant protein in virus particles and is the rate-limiting component in particle formation since virus particle formation is greatly inhibited when M protein synthesis is either defective or reduced (see Peeples, 1991). However, particles with reduced amounts of glycoproteins can be formed efficiently although such virus particles may be less infectious or noninfectious (Portner *et al.,* 1975; Leyrer *et al.,* 1998; Mebatsion *et al.,* 1996). Freeze-fracture electron microscopy has shown that during bud formation, fine crystalline arrays of 5-nm spikes possibly composed of viral glycoproteins are present on the outer side, whereas on the inner side of virus-infected cells and in virus particles, M protein appears to be present as a sheet between the lipid bilayer and the viral nucleocapsid. This intramembranous array of M protein is presumed to form the landing site of the nucleocapsid (Büechi and Bächi, 1982). Viral glycoproteins are initially inserted randomly on the plasma membrane but the presence of M protein produces a clustering effect, increasing the density and concentration of viral glycoproteins, and visible spikes appear only in the regions of membrane where M protein is present (Nagai *et al.,* 1976; Scheid *et al.,* 1978; Markwell and Fox, 1980). Furthermore, cocapping experiments using antibodies against viral glycoproteins demonstrated a possible interaction of M protein with viral glycoproteins and nucleocapsids (Yoshida *et al.,* 1979; Tyrrell and Ehrnst, 1979). *In vitro* experiments using isolated viral components also demonstrated that the attachment of glycoproteins to the nucleocapsid occurred only if M protein was present (Yoshida *et al.,* 1976, 1979). These and other studies indicate that the M protein is likely to interact both with the lipid bilayer and the associated viral glycoproteins of the membrane on the outer side and with the viral nucleocapsid on the inner side of the virus particles.

However, although various studies have provided evidence of the complex formation of M protein with the viral nucleocapsids (Markwell and Fox, 1980; Nagai *et al.,* 1978; Stricker *et al.,* 1994), attempts to demonstrate the interaction of matrix proteins and glycoproteins have yielded varying results with both orthomyxo- and paramyxoviruses (Enami and Enami, 1996; Kretzschmar *et al.,* 1996; Zhang and Lamb, 1996; Sanderson *et al.,* 1994; Stricker *et al.,* 1994). Using coexpression of M protein with glycoproteins and flotation gradient analysis, some studies showed a significant increase in membrane association of the M protein when coexpressed with F and HN glycoproteins while others did not observe such an increase in membrane association of M protein in the presence of homologous glycoproteins (Sanderson *et al.,* 1993; Stricker *et al.,* 1994). The major problem in all of these studies has been the inherent membrane association of matrix proteins due to the presence of amphiphilic helix in both orthomyxovirus (Kretzschmar *et al.,* 1996) and paramyxovirus matrix proteins (Motett *et al.,* 1996). Furthermore, variations ranging from 10 to 49% have been observed at the level of membrane association of M protein expressed alone, which can be attributed to a number of factors including different expression systems used (e.g., recombinant vaccinia virus infection versus T7 vaccinia virus transfection expression system), labeling conditions, the procedures used for preparing the cytoplasmic fraction for flotation analyses and variation from experiment to experiment.

To avoid these problems in the present report, we have used Triton X-100 (TX-100) detergent treatment, which completely eliminates the membrane binding of Sendai virus M protein when expressed alone, thus making it possible to assay the specific membrane binding of M protein in the presence of Sendai viral glycoproteins. Using this procedure, we have demonstrated that Sendai viral M protein interacts with homologous F and HN but not with heterologous influenza viral HA. We further show that M protein interacts with both the cytoplasmic tail and the transmembrane domain of F protein.

RESULTS

Membrane association of Sendai virus M protein in Sendai virus-infected cells and in cells infected with recombinant vaccinia viruses expressing M protein

To determine the membrane association of M protein in Sendai virus-infected baby hamster kidney (BHK) cells, virus-infected cells were pulse-labeled at 6.5 h postinfection (hpi) for 15 min and chased for 90 min. The 4K supernatants were prepared by disrupting the cells in hypotonic buffer and analyzed by flotation gradient centrifugation as described under Materials and Methods. Fractions were collected, immunoprecipitated, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Results show that the majority of F and HN proteins (>60%) were membrane-associated during both pulse and chase (Figs. 1A and 1B, fractions 1 and 2). On the other hand, the majority of NP protein remained soluble and nonmembrane-associated (fractions 3, 4, and 5) during both pulse and chase, as expected. About 15% of M protein was present in the membrane fraction of the 4K supernatant immediately after pulse and the membrane-associated fraction of M protein increased to 35% after a 90-min chase in Sendai virus-infected cells (Figs. 1A and 1B, fractions 1 and 2).

To determine whether the M protein associated with membranes in the absence of other viral proteins, BHK cells were infected with recombinant vaccinia viruses expressing M protein (RVVM) at 10 m.o.i. and the 4K supernatants were prepared and analyzed by flotation

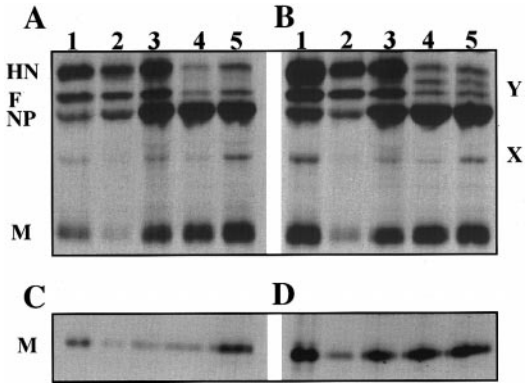


FIG. 1. Membrane association of M protein in cells infected with Sendai virus or RVVM expressing M protein. Pulse (A) and chase (B) analysis of the 4K supernatant of Sendai virus-infected BHK cells. At 6.5 hpi, Sendai virus-infected BHK cells (5×10^6) were pulse-labeled with 200 μCi of ^{35}S Easy Tag for 15 min and chased for 1.5 h. Labeled cells were fractionated as described under Materials and Methods and the 4K supernatant was analyzed by flotation gradients (Sanderson *et al.,* 1994). Each fraction was immunoprecipitated with anti-Sendai virus polyclonal antibodies and analyzed by SDS–PAGE. Fractions 1 (top) and 5 (bottom). X and Y are nonspecific proteins possibly of host origin. Pulse (C) and chase (D) analysis of the 4K supernatant of RVVM-infected BHK cells. BHK cells were infected with RVVM at 10 m.o.i. At 6 hpi, cells were pulse-labeled with 200 μCi of ^{35}S Easy Tag and chased as above, 4K supernatant was analyzed by flotation gradients, and fractions were immunoprecipitated and analyzed by SDS–PAGE.

gradient as above. Results show that in the 4K supernatant, about 20% of the Sendai virus M protein expressed alone was present in the membrane fraction immediately after pulse and after a 90-min chase, the membrane-associated fraction increased to 33% (Figs. 1C and 1D). These results show that a significant fraction of M protein expressed alone was membrane-associated as has been reported previously (Mottet *et al.,* 1996; Sanderson *et al.,* 1993; Stricker *et al.,* 1994) and that the percentage of membrane-associated M was variable due to a number of factors as mentioned earlier.

TX-100 detergent treatment of the membrane-bound M protein in RVVM-infected BHK cells

Since a significant amount of M protein expressed alone in the absence of other Sendai virus proteins became membrane-associated, it was difficult to assay the specific membrane association of M protein due to its interaction with F and HN proteins. We therefore decided to eliminate the membrane-associated M protein by treating the membrane with a nonionic detergent such as TX-100 and analyzing the detergent-treated M protein/membrane complex in a flotation gradient. We reasoned that TX-100 treatment would render the randomly membrane-bound M protein soluble. However, orthomyxo- and paramyxoviruses are known to bud apically and the apical glycoproteins of paramyxo- and orthomyxoviruses have been shown to bind to lipid rafts in both polarized and nonpolarized cells. These lipid rafts are enriched in cholesterol and glycosphingolipids and are relatively resistant to TX-100 extraction (Kundu *et al.,* 1996; Lin *et al.,* 1998; Keller and Simons, 1998; Manié *et al.,* 2000; Scheiffele *et al.,* 1997). Therefore, if M protein interacts with F or HN, it will become TX-100 insoluble, remain membrane-associated, and float to the top of the gradient. We therefore wanted to determine the minimum concentration of TX-100 that will solubilize the membrane-associated M protein in the absence of Sendai virus glycoproteins. Since there was variation in the ratio of the membrane-bound versus non-membrane-bound M protein in the 4K supernatant from one experiment to another as mentioned earlier, we used the pure membrane fraction isolated from the 4K supernatant. Accordingly, the membrane-bound fraction of M protein was first isolated from the 4K supernatants of BHK cells infected with RVVM alone by flotation gradient (see Materials and Methods) and only the membrane-bound M protein fraction was then treated with various concentrations of TX-100 and analyzed again by a second flotation gradient. Results show that, as expected, without any detergent treatment the M protein/membrane complex floated to the top of the gradient (Fig. 2A, fractions 1 and 2) and none was present in the bottom fractions. However, treatment with varying concentrations of TX-100 reduced the level of the membrane-associated M protein, and finally, TX-100 at 0.03% or higher concentration completely solubilized the membrane-bound M protein and M protein did not float to the top but remained in the bottom of the gradient (Fig. 2A, fractions 3, 4, and 5).

To determine the effect of TX-100 on the membrane-bound proteins in Sendai virus-infected cells, infected BHK cells were pulse-labeled for 30 min and chased for 90 min and the membrane fractions were isolated from the 4K supernatant. Aliquots of the pure membrane fractions were treated with or without TX-100 (0.03%) and analyzed by flotation gradient. As shown in Fig. 2B, after TX-100 treatment, over 50% of Sendai viral glycoproteins (F and HN) and M protein remained membrane-bound (Fig. 2b, fractions 1 and 2) compared to 0% of the membrane-bound M protein expressed alone (Fig. 2A, 0.03%). A higher concentration of TX-100 treatment further reduced the membrane-bound fraction of both glycoproteins and M protein in Sendai virus-infected cells (data not shown). Since 0.03% TX-100 completely solubilized the membrane-bound M protein expressed alone, we used 0.03% TX-100 in all subsequent experiments unless otherwise mentioned, to eliminate the nonspecific membrane-bound M protein alone from the specific membrane-bound M protein due to its interaction with the Sendai viral transmembrane glycoproteins, F and HN. It should be also noted that in addition to the non-membrane-bound soluble proteins, some F and HN were present in the bottom fractions before detergent treatment (see Fig. 1, fractions 3, 4, and 5), possibly due to

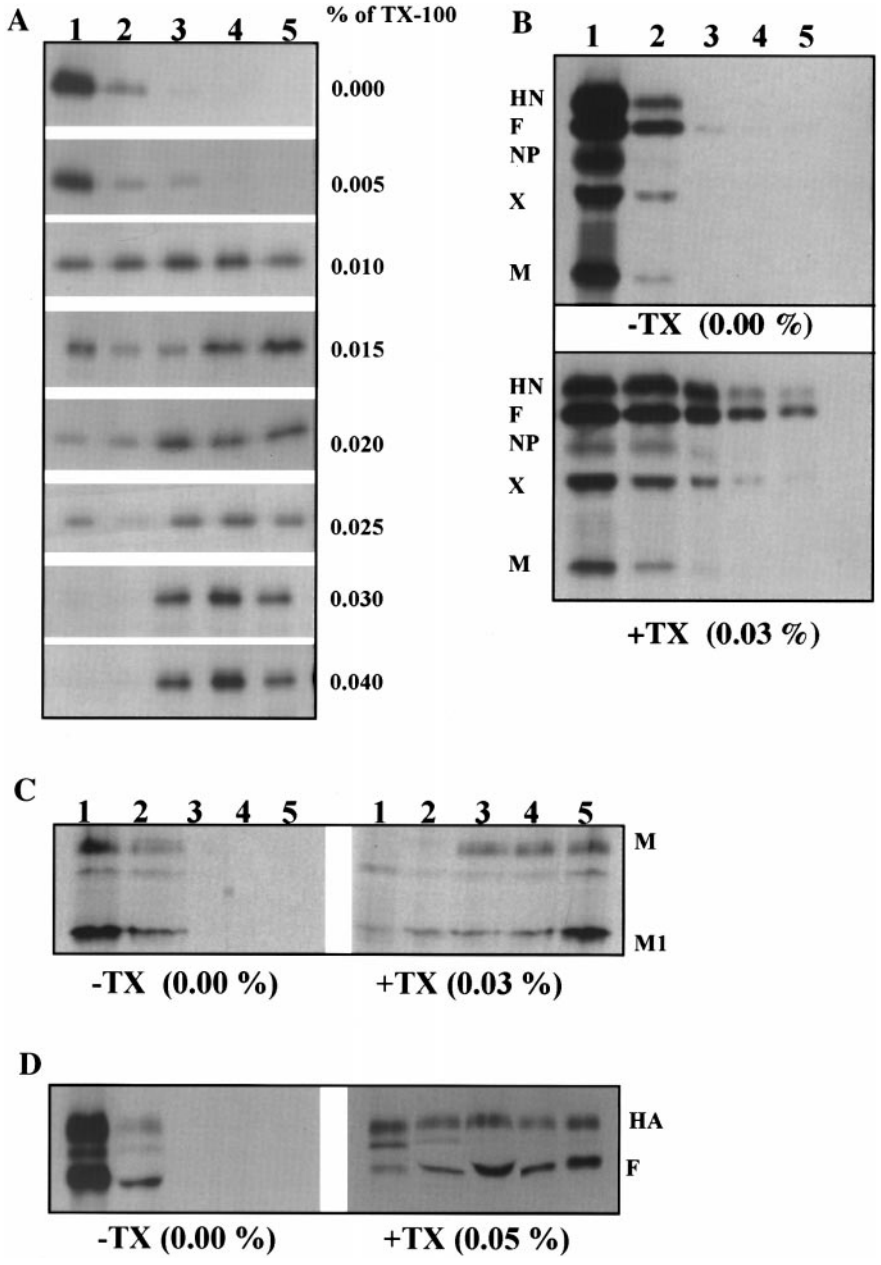


FIG. 2. TX-100 detergent treatment of membrane-associated viral proteins expressed in recombinant vaccinia virus-infected and Sendai virus-infected cells. (A) Detergent resistance of the membrane-associated M protein expressed alone. BHK cells (5×10^6) were infected with 10 m.o.i. of RVVM and labeled at 6 hpi for 30 min with 300 μCi of ^{35}S Easy Tag and chased for 1.5 h. The 4K supernatants were isolated and analyzed in flotation gradients. The pure membrane fractions were isolated from the top of the gradient and aliquots were either mock-treated or treated with varying concentrations (0.005, 0.010, 0.015, 0.020, 0.025, 0.030, and 0.040%) of TX-100 detergent for 15 min on ice. Each sample was then analyzed by a second flotation gradient as described under Materials and Methods. (B) Detergent resistance of the membrane-associated viral proteins in Sendai virus-infected cells. BHK cells were infected with Sendai virus (10 m.o.i.), pulse-labeled (30 min) at 6.5 hpi, and chased (90 min). Pure membrane fractions were prepared from the 4K supernatant, treated without (0%) or with (0.03%) TX-100, and analyzed by a flotation gradient as above. (C) Comparative detergent resistance of Sendai virus M and influenza virus M1 expressed by recombinant vaccinia viruses. The membrane-bound M and M1 were isolated, TX-100 treated ((0.03%), and analyzed by flotation gradient as above. (D) Comparative detergent resistance of HA and F expressed by recombinant vaccinia viruses. The membrane fractions containing F and HA proteins were isolated, treated with TX-100 (0.05%), and analyzed by flotation gradients as above. All fractions were immunoprecipitated using rabbit anti-Sendai virus antibodies (AS74) and influenza virus antibodies and analyzed by SDS–PAGE as described under Materials and Methods. Fractions 1 (top) and 5 (bottom).

their interaction with high-density membranes, e.g., endoplasmic reticulum (ER) membrane. However, since we used only the pure membrane fractions, which always floated to the top of the gradient before detergent treatment, our assay for detergent resistance was not influenced by the presence of such proteins.

Our assay for determining the detergent resistance of the membrane-bound M protein was different from the

standard assay for lipid raft association of apical proteins using a higher concentration of TX-100 (usually 1%). We used 0.03% TX-100 treatment for a number of reasons. Raft association of these transmembrane apical proteins as measured by 1% TX-100 resistance was not 100%. Furthermore, TX-100 resistance among the different transmembrane apical proteins varied considerably, i.e., the fraction of apical transport of a protein does not often correlate with TX-100 insolubility (Lin *et al.,* 1998; Barman and Nayak, 2000; Manié *et al.,* 2000). Sendai virus glycoproteins appear to be less TX-100 resistant than glycoproteins of other paramyxoviruses such as measles virus (Manié *et al.,* 2000). This was also seen for Sendai virus F and influenza virus HA proteins after treatment with 0.05% TX-100 (Fig. 2D). The top fractions contained only 15% of F compared to 55% of HA after 0.05% TX-100 detergent treatment (Fig. 2C, fractions 1 and 2 vs fractions 3, 4, and 5). In fact, under our conditions, higher concentrations, such as 0.3% TX-100 or more, rendered both glycoproteins and M protein of Sendai virus completely detergent-soluble by flotation assay (data not shown). The reason for the variation in TX-100 resistance among the different transmembrane apical proteins remains unclear. It could be due to variation in the affinity of different apical proteins for lipid rafts or heterogeneity among the lipid rafts. Even the TX-100 sensitivity of non-lipid-raft-associated membrane-bound proteins such as influenza virus M1 and Sendai virus M protein also varied. Membrane-bound Sendai virus M protein was less TX-100 resistant than influenza virus M1 (Fig. 2C). After treatment with 0.03% TX-100, Sendai virus M protein was completely soluble, whereas 20 to 30% of influenza virus M1 remained membrane-associated when both were expressed from recombinant vaccinia viruses (RVV). Therefore we used 0.03% TX-100 to eliminate nonspecific membrane-bound M protein.

F and HN proteins affect the detergent resistance of the membrane-bound M protein

To investigate the effect of Sendai virus F and HN proteins on the detergent resistance of the membrane-bound M protein, BHK cells were infected with RVVM alone, RVVM and RVVF, RVVM and RVVHN, or all three recombinant vaccinia viruses together as described under Materials and Methods. At 6.0 hpi, cells were pulse-labeled and chased, and the membrane fractions were isolated, detergent-treated, and analyzed by flotation gradient. Results show that the membrane-bound M protein expressed alone from RVVM was completely detergent-soluble, as expected (Fig. 3A, Table 1). However, in cells coexpressing F and M, 70% of the membrane-bound F and 80% of the membrane-bound M protein were detergent-resistant and floated to the top of the gradient (Fig. 3B, Table 1). Similarly, in cells coexpressing HN and M, 75% of HN and 70% of the membrane-bound M were detergent-resistant (Fig. 3C, Table 1). When all three proteins (F, HN, and M) were coexpressed, 95% of the membrane-bound M protein was detergent-resistant and floated to the top (Fig. 3D, fractions 1 and 2) of the gradient. These results demonstrate that a major fraction of the membrane-bound M protein became detergent-

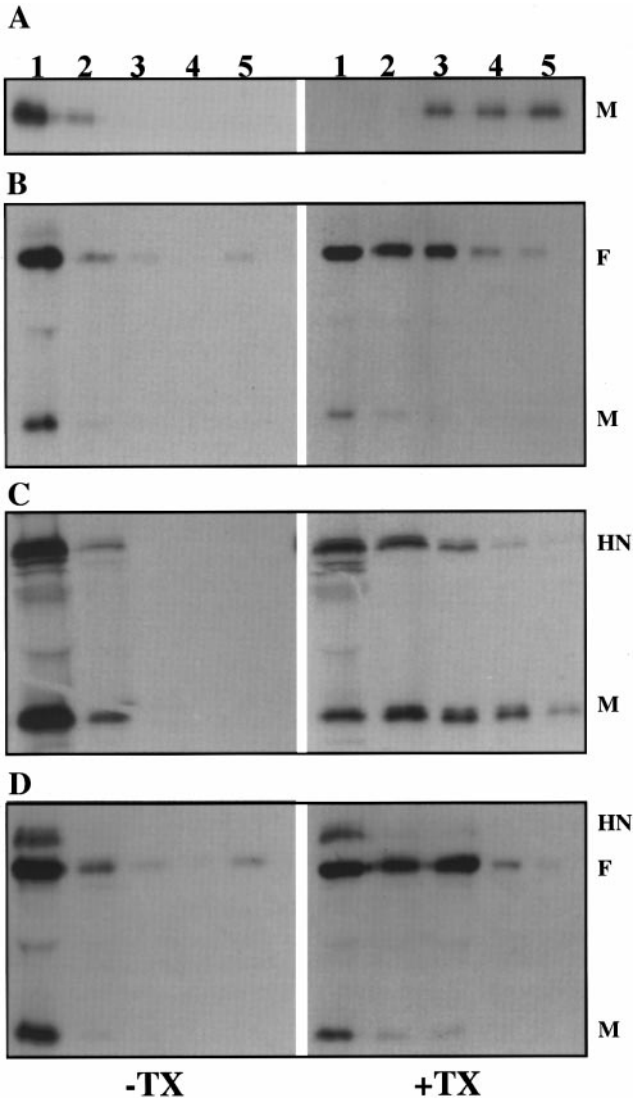


FIG. 3. Detergent resistance of the membrane-bound M protein when coexpressed with Sendai virus F and HN. BHK cells (5×10^6) were infected with recombinant vaccinia viruses expressing M alone or M in combination with F or HN or with both F and HN proteins together as stated under Materials and Methods. At 6 hpi, cells were labeled with 300 μCi of ^{35}S Easy Tag for 30 min and chased for 90 min. Cells were fractionated to prepare the 4K supernatant. Pure membrane fractions were isolated from the 4K supernatant by flotation gradient and either untreated (−) or treated (+) with TX-100 (0.03%) and analyzed by a second flotation gradient. (A) Sendai virus M expressed alone. (B) Sendai virus M coexpressed with Sendai virus F protein. (C) Sendai virus M coexpressed with HN protein. (D) M coexpressed with both Sendai virus HN and F. Left-hand panels are without (−) TX-100 treatment and right-hand panels are after TX-100 treatment (+). (Fractions were collected from the top (1), immunoprecipitated, and analyzed by SDS–PAGE.)

TABLE 1

Detergent Resistance of the Membrane-Bound Sendai Virus M Protein and Glycoproteins in Coexpressing BHK Cells

Coexpressing proteins	% of detergent-resistant membrane-bound M protein	% of detergent resistant membrane-bound glycoproteins
M only	0	—
Influenza HA + M	0	60 ± 5
F + M	80 ± 5	70 ± 5
HN + M	70 ± 6	75 ± 8
F + HN + M	95 ± 5	62 ± 5 (F), 80 ± 8 (HN)
FHF + M	80 ± 7	75 ± 8
FFH + M	50 ± 5	60 ± 5

Note. These results are calculated by densitometric analysis of autoradiographs shown in Figs. 3 and 4 from three or more independent experiments. All proteins were expressed using recombinant vaccinia virus.

resistant when coexpressed with HN and F (Table 1). Variations in the percentage of detergent resistance of M protein when expressed with different glycoproteins may be due to varying levels of expressed proteins as well as to the affinity of interaction and stability of the complex and therefore may not reflect the relative contribution of F and HN proteins in membrane binding of M protein in Sendai virus-infected cells.

Domains of F protein involved in rendering the membrane-bound M protein detergent-resistant

Since Sendai virus F protein affected the detergent resistance of the membrane-bound M protein when both F and M proteins were coexpressed, we wanted to investigate the domains of F protein involved in rendering the membrane-bound M protein detergent-resistant. Accordingly, chimeras between Sendai virus F and heterologous influenza virus HA protein, both type I transmembrane proteins, were constructed by switching either the transmembrane domain (FHF) or the cytoplasmic tail (FFH) of F with that of HA (Fig. 4A). and recombinant vaccinia viruses were made with these chimeric constructs. The junction sequences in the chimeras FHF and FFH are shown in Fig. 4B. Both chimeras were expressed efficiently and transported to the plasma membrane. Migration behavior and endo H resistance demonstrating the maturation of the chimeric (FFH and FHF) and wild-type proteins (F and HA) are shown in Fig. 4C. Since the ectodomain of F protein cannot interact with M protein, FHF and FFH will have the cytoplasmic tail and transmembrane domain of F, respectively, for interaction with M protein. BHK cells were infected with RVVM alone or coinfected with recombinant vaccinia viruses coexpressing M and HA, M and FHF, or M and FFH. At 6.0 hpi, recombinant vaccinia virus-infected cells were pulse-labeled and chased; pure membrane fractions were isolated from the 4K supernatant, treated with (+) or without (−) detergent, and analyzed by flotation gradients as stated under Materials and Methods. Results show that the membrane-bound M protein expressed alone or in the presence of a heterologous influenza virus HA protein was completely detergent soluble (Figs. 5A and 5B, + TX) although 60% of the membrane-bound HA was detergent-resistant (Fig. 5B, +TX). However, upon coexpression of M protein with the chimera containing the transmembrane domain of F (FFH), 50% of the membrane-bound M protein became detergent-resistant (Fig. 5D, Table 1). Similarly, coexpression of the chimeric protein containing the cytoplasmic tail of F (FHF) rendered 80% of the membrane-bound M protein detergent-resistant (Fig. 5C, Table 1). These results demonstrate that both the cytoplasmic tail and the transmembrane domain of F can render M protein resistant to detergent treatment and therefore support the interaction of M protein with both the cytoplasmic tail and the transmembrane domain of F and HN proteins. However, as mentioned earlier, the relative percentage of detergent resistance of the membrane-bound M protein may not indicate the relative contribution of these domains in interacting with M protein. The transmembrane domain and cytoplasmic tail of HA were also shown to interact with influenza virus M1 (Ali *et al.,* 2000).

Membrane-bound M protein became detergent-resistant in Sendai virus-infected BHK cells

To determine whether the membrane-bound M protein became detergent-resistant in early and late phases of the Sendai virus infectious cycle, BHK cells were infected with Sendai virus (10 m.o.i.), pulse-labeled for 20 min at 2.5 hpi (early) and 6.5 hpi (late), and chased in the presence of cycloheximide for 3 h (early) or 1 h (late). Membrane fractions were isolated from cells immediately after pulse-labeling or after chase, treated with (+) or without (−) TX-100, and analyzed by flotation gradients. Results show that both early and late in the infectious cycle, the membrane-bound glycoproteins (F and HN) immediately after pulse were predominantly detergent-soluble [only 5–8% detergent-resistant for both F and HN (Figs. 6A and 6C, +TX; and Table 2)], as expected. After chase, however, the majority of the labeled F and HN proteins became detergent-resistant as expected because of their association with the lipid raft in the *trans*-Golgi and plasma membrane of virus-infected cells (Figs. 6B and 6D, +TX; and Table 2). The membrane-bound M protein, immediately after pulse at 2.5 hpi, was completely detergent-soluble (Fig. 6A, +TX; and Table 2). However, late in the infectious cycle, a fraction (25%, Table 2) of the membrane-bound M protein became detergent-resistant (Fig. 6C, +TX). This would be expected since at 6.5 hpi, mature glycoproteins will be present in the *trans*-Golgi network (TGN) and plasma

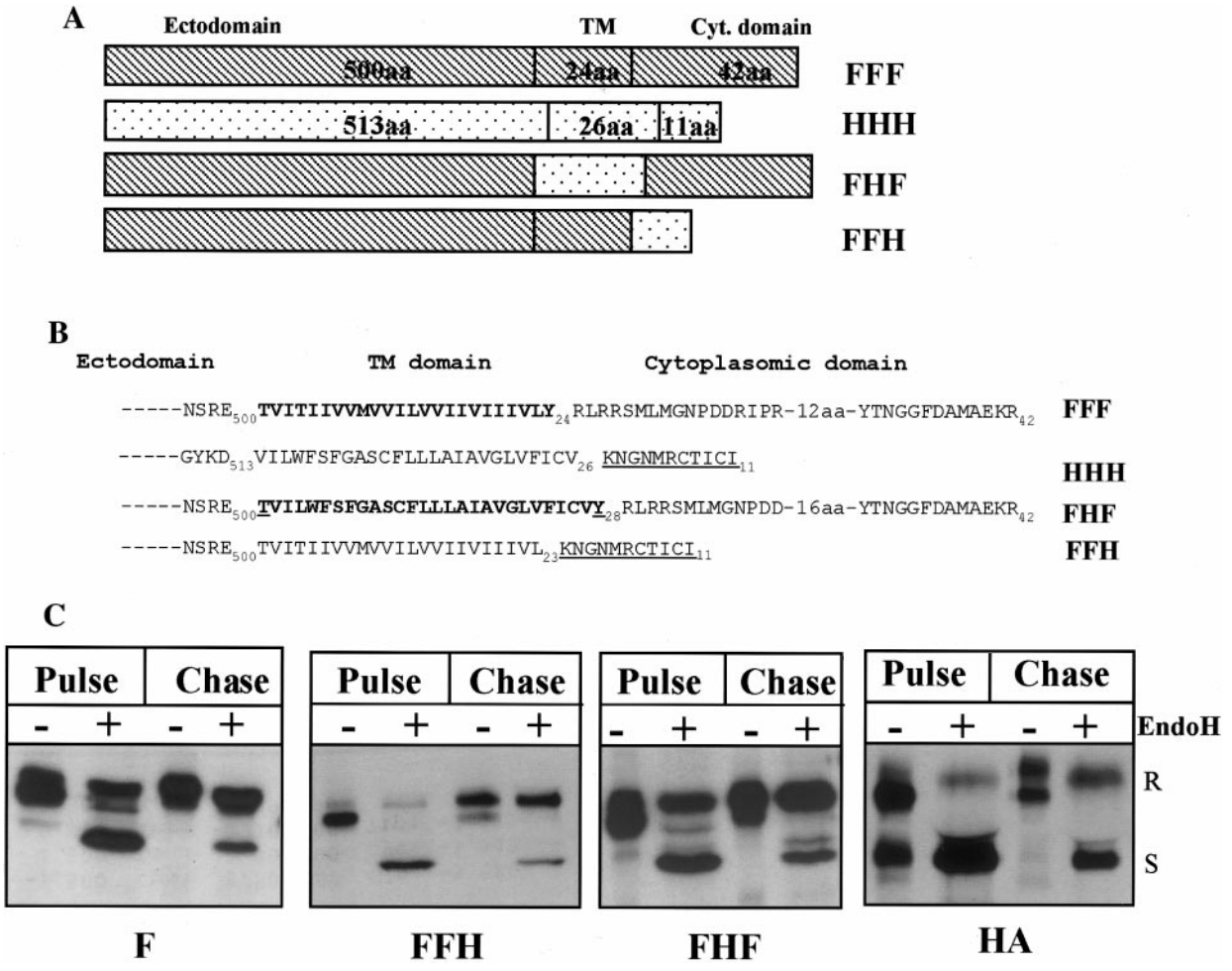


FIG. 4. Construction and expression of chimeras (FHF, FFH) containing the transmembrane domain or cytoplasmic tail of influenza virus hemagglutinin. (A) Schematic representation of chimeric constructs between Sendai virus F and influenza virus HA. (B) The sequence and junction sites of the transmembrane domain and cytoplasmic tail in the chimeric and wild-type proteins. (C) The endo H resistance (+) of wild-type and chimeric proteins after pulse (30 min) and chase (1 h) in BHK cells infected with recombinant vaccinia viruses expressing the wild-type and chimeric proteins. FFF and HHH represent the ectodomain, transmembrane domain, and cytoplasmic tail of the Sendai virus F and influenza virus HA, respectively. Two underlined amino acids (T and Y) in FHF are from the transmembrane domain of F protein. The other underlined sequence is cytoplasmic tail of HA. R, resistant; S, sensitive.

membrane containing detergent-resistant lipid rafts and some of the newly synthesized M proteins would bind to these preexisting mature glycoproteins and thereby become detergent-resistant.

However, after chase, approximately 90% of the membrane-bound M protein became detergent-resistant both early and late in the infectious cycle (Figs. 6B and 6D, Table 2). This could be explained due to maturation of glycoproteins during the chase and the interactions of M protein with mature F and HN proteins. Mature F and HN would acquire detergent resistance due to their association with lipid rafts at the TGN and plasma membrane. Early in the infectious cycle, the chase was extended to 3 h to ensure that most glycoproteins became mature and associated with lipid rafts. These results support the finding that in Sendai virus-infected cells, the membrane-bound Sendai virus M protein acquired a detergent-resistant form due to its interaction with mature glycoproteins present in the lipid rafts. In addition, a significant fraction of NP was also membrane-bound both before and after detergent treatment (Figs. 6A–6D, fractions 1 and 2). Membrane binding of NP before and after detergent treatment was likely due to the interaction of nucleocapsid with the membrane via M protein and glycoproteins as has been observed previously (Mottet *et al.,* 1996; Sanderson *et al.,* 1993; Stricker *et al.,* 1994). It is interesting to note that the membrane-bound NP (nucleocapsid) became more detergent-resistant with chase (compare TX+ and TX−, Fig. 6), suggesting the possible maturation of nucleocapsids and their interaction with the detergent-resistant membrane. It should be noted that in Fig. 6 only the pure membrane fractions were used, whereas in Fig. 1 the total 4K supernatant was used in flotation analysis. Therefore, more non-mem-

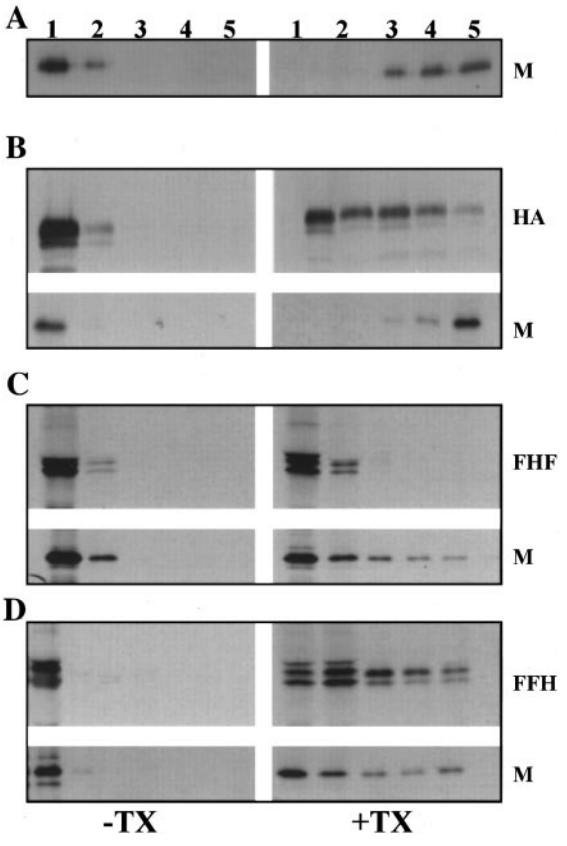


FIG. 5. Detergent resistance of the membrane-associated M protein when coexpressed with chimeric constructs of influenza HA and Sendai virus F proteins. (A–D) Detergent resistance of membrane-associated M protein when coexpressed in the presence of heterologous or chimeric proteins. BHK cells (5×10^6) were infected with RVV expressing M alone (A), M with influenza virus HA (B), M with FHF (C), or M with FFH (D) as described under Materials and Methods. At 6 hpi, cells were pulse-labeled with 300 μCi of ^{35}S Easy tag for 30 min and chased for 90 min. Pure membrane fractions we isolated from 4K supernatants, treated without (−) or with (+) TX-100 (0.03%), and analyzed by flotation gradients. Fractions were collected from the top (1), immunoprecipitated, and analyzed by SDS–PAGE.

brane-bound cytoplasmic NP and M proteins were present in the 4K supernatant (Figs. 1A and 1B, fractions 3, 4, and 5) than in membrane fractions before TX-100 treatment (Figs. 6A and 6C, fractions 3, 4, and 5).

Colocalization of M protein with F and HN in cells infected with Sendai viruses or recombinant vaccinia viruses

To determine whether M protein colocalizes with F and HN, two sets of experiments were done. BHK cells were infected synchronously at 4°C with 10 m.o.i. of Sendai virus. Subsequently, cells were washed and incubated at 37°C for 2 h when monensin (1 mM, final concentration) was added to one set of virus-infected cells and the other set was incubated without monensin. Following further incubation for 5 h at 37°C with or without monensin, cells were fixed, permeabilized, and stained for M and F or for M and HN using polyclonal anti-M and monoclonal anti-F or anti-HN antibodies and a mixture of fluorescein-tagged anti-rabbit IgG and rhodamine-tagged anti-mouse IgG. The stained cells were examined by confocal microscopy. Results show that in the absence of monensin both F and HN (red) and M protein (green) were present throughout the cell including the cell periphery although compared to M protein, both F and HN proteins were more concentrated in the perinuclear region (compare Figs. 7A and 7B versus Figs. 7J and 7K). Superimposition of staining clearly showed orange and yellow staining, indicating colocalization of M with F and HN particularly in the perinuclear region (Figs. 7C and 7L).

Colocalization was further demonstrated in Sendai virus-infected cells by monensin treatment. Monensin blocks the exocytic transport of glycoproteins (F and HN) in the mid-Golgi region and this can be seen from the one-sided, perinuclear concentration of F and HN proteins (Figs. 7E and 7N) in cells after monensin treatment. However, it was also observed that the distribution of M protein that is not transported via the exocytic pathway was also distinctly different in these monensin-treated cells (Figs. 7D and 7M) when compared to that in cells without monensin treatment (Figs. 7A and 7J). It can be seen that in general in monensin-treated cells M protein was also more concentrated in the perinuclear region and present much less in the cell periphery. Superimposition of staining further showed a distinct colocalization of M with F and HN in specific orange or yellow spots (Figs. 7F and 7O). Three-dimensional rotation of images after superimposition as well as analysis of total cell sections supported the colocalization of these proteins inside the cells. These results show that M protein colocalized with both F and HN proteins in Sendai virus-infected cells either in the presence or in the absence of monensin treatment.

We also examined the colocalization of M with F and HN in the absence of other Sendai viral proteins by coexpressing M with either F or HN using a recombinant vaccinia virus expression system. Essentially, a pattern similar to that observed in Sendai virus-infected cells was also observed in coexpressing cells. Both M protein and glycoproteins were more diffuse in the cytoplasm in the absence of monensin and became distinctly perinuclear in the presence of monensin, supporting colocalization of M with F and HN (data not shown). These results indicate that colocalization of M protein with F or HN did not depend on other viral proteins, supporting a direct interaction of M protein with F or HN.

However, it should be noted that colocalization of these proteins throughout the cells was neither uniform nor complete. Such variation was expected because of the different levels of each protein present in different subcellular components at a given stage of the viral life cycle and because of the different functions of M and

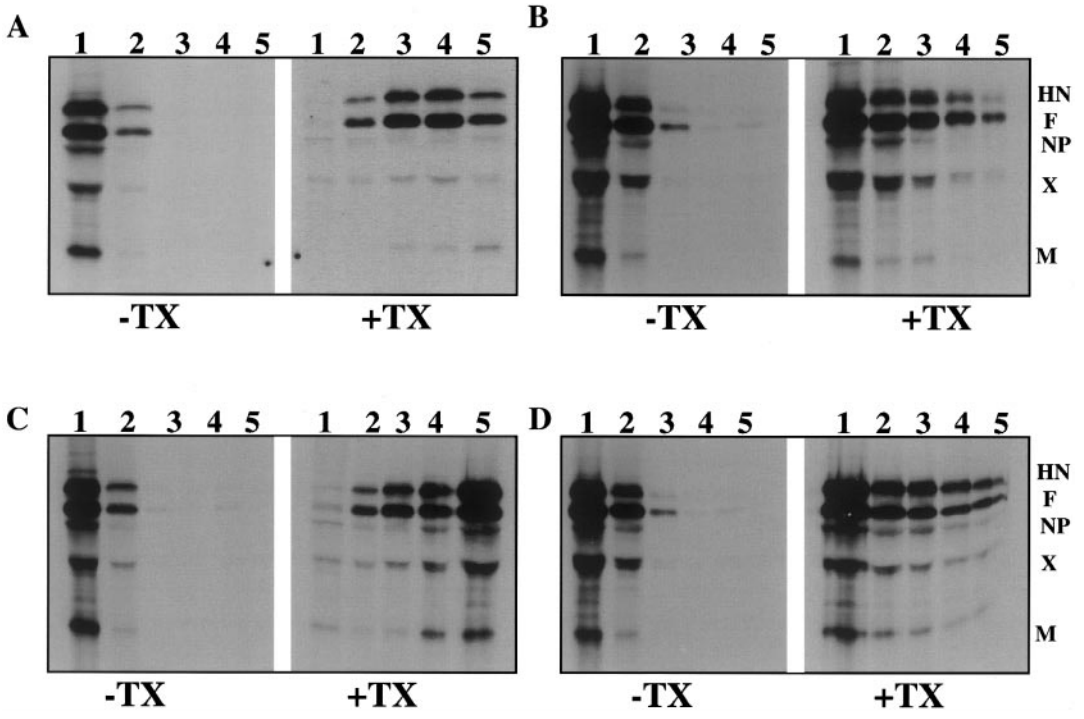


FIG. 6. Detergent resistance of the membrane-associated M protein in Sendai virus-infected BHK cells. (A and B) TX-100 treatment of the membrane-associated M protein synthesized early (2.5 hpi) in the virus replication cycle. BHK cells (5×10^6) were infected with Sendai virus (10 m.o.i.), pulse-labeled with 300 μCi at 2.5 hpi for 20 min (A), and chased for 3 h (B) in the presence of 1.0 mM cycloheximide. Cells were then harvested and fractionated, and pure membranes were isolated from the 4K supernatant by flotation gradient. The pure membranes were then treated without (−) or with (+) 0.03% TX-100 and analyzed by a second flotation gradient. Fractions were immunoprecipitated and analyzed by SDS–PAGE. (C and D) Analysis of M protein synthesized late (6.5 hpi) in the virus replication cycle. Sendai virus-infected BHK cells were labeled with 300 μCi at 6.5 hpi for 20 min (C) and chased for 1 h (D) in the presence of cycloheximide as described above. Membrane fractions were isolated from virus-infected cells as above, treated without (−) or with (+) 0.03% TX-100, and analyzed by flotation gradient. Fractions were immunoprecipitated and analyzed by SDS–PAGE. X, nonspecific protein band.

glycoproteins in the virus life cycle. Furthermore, M protein interacts not only with glycoproteins but also with viral nucleocapsid containing NP (Peeples, 1991). Therefore, distribution of M protein will be different from that of glycoproteins. However, it is not known whether M protein can directly interact with NP protein when coexpressed in the absence of nucleocapsid. With influenza viruses, it has been shown that although M1 interacts with RNP, M1 and NP can form homodimers and homooligomers but they do not form M1/NP heterodimers when coexpressed (Avalos *et al.,* 1997; Zhang and Lamb, 1996; Ye *et al.,* 1999).

TABLE 2

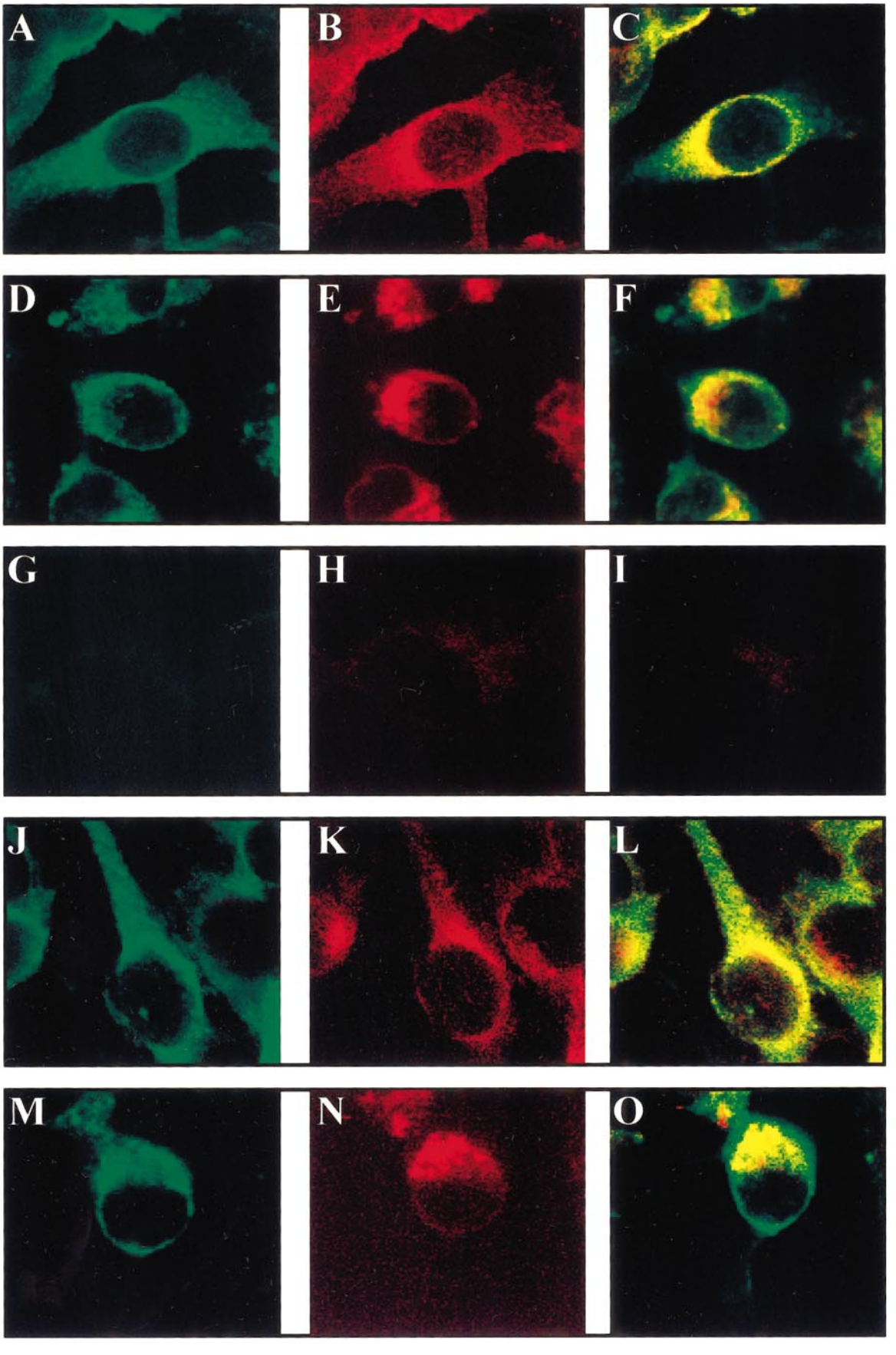
TX-100 Resistance of Membrane-Bound Sendai Virus Proteins in Sendai Virus-Infected Cells

	Early		Late	
	Pulse	Chase	Pulse	Chase
HN	5 ± 5	78 ± 6	5 ± 5	75 ± 5
F	8 ± 5	68 ± 8	8 ± 5	66 ± 6
M	0	90 ± 10	25 ± 5	88 ± 10
NP	5 ± 5	80 ± 5	25 ± 6	75 ± 5

Note. Sendai virus-infected cells were pulse-labeled and chased at 2.5 hpi (early) and 6.5 hpi (late) as described in the legend to Fig. 5. Standard deviations were determined from three or more independent experiments.

DISCUSSION

M protein plays a critical role in the assembly and budding of paramyxoviruses (Peeples, 1991; Dubois-Dalcq *et al.,* 1984). This was demonstrated by numerous studies showing a correlation of reduced Sendai virus particle formation with the reduced production of M protein or synthesis of defective M protein (Machamer *et al.,* 1981; Roux *et al.,* 1984; Yoshida *et al.,* 1979). Ultrastructural, biochemical, and immunological studies of virus-infected cells and virus particles indicate that M protein is likely to interact with the components of nucleocapsid on the inner side and the viral envelope on the outer side. Association of M protein with viral nucleocapsid may involve its interaction with RNA (Heggeness *et al.,* 1981), NP (Markwell and Fox, 1980; Nagai *et al.,* 1978), L protein (Hamaguchi *et al.,* 1983), and possibly other components including P protein. Crosslinking, electron mi-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croscopic, and biophysical analyses have also shown that M protein interacts with M protein forming dimers or homo-oligomers (Markwell and Fox, 1980; Nagai *et al.,* 1978; Büechi and Bächi, 1982; Hewitt and Nermut, 1977; Heggeness *et al.,* 1981).

Numerous studies have supported the interaction of M protein with viral glycoproteins in Sendai virus-infected cells. Monensin treatment prevented both viral glycoproteins and M protein from reaching the plasma membrane (Yoshida *et al.,* 1986; Sanderson *et al.,* 1993). Using antibody-induced cocapping, it has been reported that antibodies against viral glycoproteins resulted in capping of M and NP and that cytochalasin B treatment reduced cocapping of NP, but not of M protein, with glycoproteins in cells chronically infected with measles virus (Tyrrell and Ehrnst, 1979). Yoshida *et al.* (1979) also observed that antibody-inducing capping of Sendai viral glycoproteins was faster and more complete at the nonpermissive temperature than at the permissive temperature in cells infected with a Sendai virus ts M mutant, suggesting greater mobility of glycoproteins in the absence of M protein. Furthermore, it was also reported that *in vitro* interaction of glycoproteins with nucleocapsid depended on the presence of M protein (Yoshida *et al.,* 1976). However, despite this indirect evidence supporting the interaction of M protein with Sendai virus glycoproteins, evidence for direct interaction between Sendai or any paramyxoviral glycoproteins and M protein is lacking or conflicting. Coimmunoprecipitation of the M/glycoprotein complex using anti-M, anti-F, or anti-HN antibodies or crosslinking experiments have failed to demonstrate interaction of M with F or HN (Markwell and Fox, 1980). Similarly, *in vitro* experiments using cotranslation of M protein and glycoproteins have failed to demonstrate an interaction of M with glycoproteins (unpublished data). Finally, as mentioned earlier, attempts to demonstrate interaction of M and glycoproteins by coexpression and flotation analysis have yielded conflicting results due to the inherent membrane-binding property of M protein (Mottet *et al.,* 1996; Sanderson *et al.,* 1993; Stricker *et al.,* 1994). Therefore, the goal of this report was twofold: (i) to demonstrate the interaction of M protein with F and HN and (ii) to define the domains of F protein involved in the interaction with M protein.

In the experiments reported here, we used a protocol treating the membrane fraction with TX-100 detergent, which eliminates the membrane binding of M protein alone in the absence of F and HN. The procedure used in this report permits assaying the specific membrane binding of M protein due to its interaction with Sendai viral glycoproteins. As mentioned earlier, in this protocol we have taken advantage of apical glycoproteins acquiring higher TX-100 resistance due to their interaction with lipid rafts in the *trans*-Golgi region. However, as mentioned earlier, the specific concentration of TX-100 needed to demonstrate the M protein/glycoprotein interaction may vary for different transmembrane viral proteins. Using this procedure, we have demonstrated that M protein can interact with F and HN in the absence of any other viral proteins. This was further confirmed by confocal microscopy demonstrating the colocalization of M with F and HN in Sendai virus-infected cells both in the presence and in the absence of monensin. Similar results on colocalization were also observed by Stricker *et al.* (1994) from the analysis of cells infected with defective interfering viruses except that they concluded that a third factor, namely, the viral nucleocapsid, was also involved in the formation of glycoprotein/M complex. The data presented in this paper demonstrate that F and HN can interact with M protein in the absence of other Sendai viral proteins and that the viral nucleocapsid is not an obligatory factor in the M/glycoprotein interaction. Earlier studies by other workers also support our observation that NP (or nucleocapsid) is not essential for interaction of M with glycoproteins. Tyrrell and Ehrnst (1979) observed that cytochalasin B treatment, which dissociates actin filaments, reduced cocapping of NP but not cocapping of M and viral glycoproteins. Interaction of M protein with HN protein is also supported from the studies of Roux *et al.* (1984), who concluded that in cells persistently infected with standard and DI viruses, M protein was responsible for the higher instability of HN protein. Recent studies also showed that in influenza virus-infected cells and in cells coexpressing M1, HA, and NA, M1 protein interacts with both HA and NA but not with heterologous Sendai virus F protein (Ali *et al.,* 2000). This is not to suggest that the viral nucleocapsid (or NP) does not affect or facilitate M/glycoprotein interaction. Flotation analysis data of Sendai virus-infected cells presented here (Fig. 6) and previously reported (Sanderson *et al.,* 1993; Stricker *et al.,* 1994) clearly showed that nucleocapsids became membrane-associ-

FIG. 7. Distribution of M and glycoproteins (HN and F) in Sendai virus-infected BHK cells by confocal microscopy. BHK cells (4×10^5) were grown on coverslips and synchronously infected with 10 m.o.i. of Sendai virus at 4°C or mock-infected and incubated at 37°C. Monensin at 10 μM final concentration was added to a set of some cells at 2 hpi (D–F, G–I, and M–O) and incubated for another 5 h at 37°C. At 7 hpi, all virus-infected cells were fixed with ice-cold acetone:methanol (1:1) incubated with a mixture of anti-M rabbit polyclonal and anti-F mouse monoclonal antibodies (A–F) with a mixture of anti-M rabbit polyclonal and anti-HN mouse monoclonal antibodies (J–O), or with a mixture of anti-M rabbit polyclonal antibodies, anti-HN mouse monoclonal antibodies, and anti-F mouse monoclonal antibodies (G–I) and stained with anti-rabbit IgG (green) and anti-mouse IgG (red). The stained cells were examined by confocal microscopy as described under Materials and Methods. (G–I) Mock-infected with monensin. (A–C and J–L) Virus-infected cells without monensin treatment; (D–F and M–O), virus-infected cells treated with monensin. Image analysis was done as follows: (A, D, G, J, and M) green; (B, E, H, K, and N) red; (C, F, I, L, and O) red and green (original magnification, 600×).

ated in Sendai virus-infected cells, possibly due to their interaction with viral glycoproteins via M protein. Similarly, reconstitution experiments *in vitro* also demonstrated that the interaction of viral glycoproteins with nucleocapsid required the presence of M protein (Yoshida *et al.,* 1976).

Our studies with chimeric constructs demonstrated that both the cytoplasmic tail and the transmembrane domain of F protein were involved in interactions with M protein (Fig. 5). Earlier studies with SSPE (subacute sclerosing panencephalitis) measles virus (Cattaneo *et al.,* 1988) indicated that mutations in the cytoplasmic tail of F protein could affect interactions with M protein and consequently virion formation. The cytoplasmic tails of HN and F proteins were found to process critical determinants for their incorporation into Sendai virus particles (Fouillot-Coriou and Roux, 2000; Takimoto *et al.,* 1998) and in the assembly of paramyxovirus simian virus 5 (Schmitt *et al.,* 1999) and measles virus (Cathomen *et al.,* 1998). Similarly, the cytoplasmic tails of influenza virus HA and NA proteins have been shown to affect the lipid raft association influenza virus glycoproteins as well as morphogenesis of influenza viruses (Zhang *et al.,* 2000). Although a majority of studies have indicated a possible interaction of M protein with the cytoplasmic tail of glycoprotein (see reviews by Dubois-Dalcq *et al.,* 1984; Peeples, 1991), our data also show that M protein, in addition to the cytoplasmic tail of glycoproteins, also interacts with the transmembrane domain of F (Fig. 4D). However, this was not unexpected since M protein expressed alone has been shown to be membrane-bound (Mottet *et al.,* 1996). Furthermore, freeze-fracture analysis showed that clusters of M protein were associated with the inner leaflet of the lipid bilayer of the membrane (Büechi and Bächi, 1982), thereby making it possible for M protein to be in contact with the transmembrane domain of glycoproteins. With influenza virus, M1 protein has been shown to interact with both the transmembrane domain and the cytoplasmic tail of HA (Ali *et al.,* 2000), supporting a similar interaction of matrix proteins and glycoproteins among both orthomyxo- and paramyxoviruses.

Finally, confocal microscopy analysis of both Sendai virus-infected cells and cells coexpressing M, F, and HN shows colocalization of M protein with F and HN (Fig. 7). These data also show that colocalization can occur at the plasma membrane as well as during the transit of glycoproteins through the exocytic pathway. The data presented here confirm and extend the earlier finding that M protein that does not use the exocytic pathway can be blocked to the perinuclear region in Sendai virus-infected cells either using defective M protein (Mottet *et al.,* 1999) or lower temperature block (Sanderson *et al.,* 1993). In addition, the biochemical and morphological data presented here demonstrate that interaction of M protein with F and HN protein can occur in the absence of other viral proteins including NP. Although our data show the interaction of M protein with glycoproteins in the mid-Golgi region blocked by monensin, we cannot rule out a possible interaction of M with glycoproteins in the earlier extocytic transport compartments including the ER. However, neither our data nor the data presented earlier show that interaction of M protein with glycoproteins occurs only during the exocytic pathway but not at the plasma membrane. More likely, the concentration of glycoproteins in a specific membrane compartment will affect M protein colocalization.

In conclusion, the data presented here demonstrate that using TX-100 detergent, it is possible to eliminate the nonspecific membrane binding of M protein and thereby assay the specific membrane binding of M protein in the presence of F and HN. Furthermore, our data show that specific interaction occurs only with homologous glycoproteins and not with heterologous glycoproteins such as influenza virus HA. We also show that M protein interacts with both the transmembrane domain and the cytoplasmic tail of F protein. Now it will be possible to define the specific sequences of interaction between M protein and glycoproteins. Finally, immunofluorescence analysis using confocal microscopy shows specific colocalization of M with F and HN proteins both at the plasma membrane and during exocytic transport. These results clearly support the specific interaction of M protein with F and HN proteins in Sendai virus-infected cells.

MATERIALS AND METHODS

Cells, virus, and antibodies

Wild-type Sendai virus (Z strain) was grown in 10-day-old embryonated chicken eggs (Sanderson *et al.,* 1993). BHK, CV-1 and HeLa cells were grown in minimal essential medium (MEM, Gibco BRL, Richmond, CA) supplemented with 10% fetal bovine serum (FBS), 250 U of penicillin/ml, and 250 μg of streptomycin/ml. Monoclonal antibodies against Sendai virus F and HN proteins were gifts from Dr. Allen Portner, (St. Jude Research Hospital, Memphis, TN). Polyclonal anti-M antibodies were made in rabbits. Polyclonal antibodies against whole Sendai virus and Sendai F protein were obtained from J. Seto (California State University, Los Angeles, CA). Anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) and anti-mouse IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of recombinant vaccinia viruses

Recombinant vaccine viruses expressing Sendai virus F, HN, M, influenza virus HA genes, or chimeric constructs were made using the procedure described previously (Chakrabarti *et al.,* 1985). cDNAs of WSN

(A/WSN/33) influenza virus HA, Sendai virus Z strain F, and chimeric constructs were inserted into the multiple cloning site of the vaccinia virus expression vector pSC11 containing the 7.5 promoter sequence upstream of the multiple cloning site and the thymidine kinase gene. Chimeric constructions were done by swapping domains between WSN HA and Sendai virus F (Fig. 4A). Chimeric constructs were designated FHF or FFH, indicating the ectodomain, transmembrane domain, and cytoplasmic tail, respectively, of either Sendai virus F protein (F) or influenza virus HA protein (H). Each construct was sequenced to ensure that additional PCR mutations were not created. All vaccinia viruses were propagated in HeLa cells and plaque-titered in CV-1 as previously described (Sanderson *et al.,* 1994). For expression, BHK or HeLa cells were infected with RVV expressing specific proteins at 10 m.o.i.

Radiolabeling

BHK cells (5×10^6) were infected with Sendai virus or with recombinant vaccinia viruses at 10 m.o.i. The infected cells were then incubated at 37°C in MEM plus 2.5% FBS. At the indicated times postinfection, cells were starved with MEM without methionine and cysteine for 30 min and pulse-labeled with ^{35}S Easy Tag Express Protein labeling mix (NEN Life Science Products Inc., Boston, MA). The labeling medium was then replaced with the chase medium (MEM plus 2.5% FBS supplemented with a 10 mM concentration of unlabeled cysteine and methionine) and chased for various times as indicated. The pulse and chase times and the amount of ^{35}S-labeled amino acids varied with different experiments and are stated in the figure legends.

Subcellular fractionation and flotation analysis

For subcellular fractionation, BHK cells infected with Sendai viruses or recombinant vaccinia viruses were washed twice in ice-cold phosphate-buffered saline containing Ca^{2+} and Mg^{2+}, scraped from dishes, and pelleted by centrifugation. The cell pellet was resuspended in ice-cold hypotonic lysis buffer [10 mM Tris HCl (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$] and incubated on ice for 30 min. Cells were then disrupted by repeated passage (25 times) through a 26-gauge hypodermic needle, and unbroken cells and nuclei were removed by centrifugation at 1000*g* for 5 min (SW 50 rotor at 4000 rpm) at 4°C. The postnuclear supernatant (4K supernatant) was then subjected to flotation analysis as described (Sanderson *et al.,* 1994). Briefly, aliquots of the 4K postnuclear supernatants (0.4 ml) were dispersed into 2 ml of 75% (wt/wt) sucrose in low-salt buffer (LSB) containing 50 mM Tris–HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$ and layered on 0.5 ml of 80% (wt/wt) sucrose, overlaid with 2 ml of 55% (wt/wt) sucrose in LSB and approximately 0.6 ml of 5% (wt/wt) sucrose in LSB. Gradients were then centrifuged for 18 h at 38,000 rpm using a SW 55 Ti rotor at 4°C, and a 500-μl fraction containing the visible membrane fraction (called the pure membrane fraction) was collected from the top and pooled. Four-hundred-microliter aliquots of this membrane fraction were treated with or without TX-100 (Boehringer Mannheim, Mannheim, Germany) on ice for 15 min and used for a second flotation gradient as above. Five 1-ml fractions were collected from the top by using a Hacki-Buchler Auto Densiflow II gradient remover (Buchler Instruments, Lenexa, KS) and used for immunoprecipitation. Therefore, in all gradients the top fraction is 1 and bottom fraction is 5. In these flotation gradients, fractions 1 and 2 contain the membrane fraction and fractions 3, 4, and 5 contain the nonmembranous, soluble proteins. To avoid any variation in detergent and membrane concentration, the same number of cells was used in each experiment, the protein concentration in the pure membrane fraction was determined, and the same amounts of membrane fractions were used for detergent treatment and flotation gradient analysis.

Immunoprecipitation

All gradient fractions were diluted with 3 ml of LSB before addition of 1 ml of 5× concentrated radioimmunoprecipitation assay (RIPA) buffer [1× RIPA buffer contains 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1.0% TX-100, 0.5% sodium deoxycholate, 0.1% SDS, 1.0 mM phenylmethylsulfonyl fluoride (Sigma), 2% aprotinin (Sigma). Samples were then shaken in RIPA buffer at 4°C for 2 h before the addition of antibodies. Each fraction was immunoprecipitated with polyclonal anti-Sendai virus rabbit antibodies (AS 74) or polyclonal anti-WSN antibodies. Subsequently, 7 mg of protein A–Sepharose beads (Pharmacia, Uppsala, Sweden) was added to each sample and the mixture was incubated for 1.5 h at 4°C. Immunoprecipitates bound to Sepharose beads were pelleted by centrifugation and washed three times in RIPA buffer containing 5 mg of bovine serum albumin (BSA) per milliliter, followed by another wash with RIPA buffer. Immunoprecipitates were then dissolved in SDS–sample buffer [50 mM Tris–HCl (pH 6.8), 5% 2-β mercaptoethanol, 2% SDS, 10% (wt/vol) glycerol, and 0.1% (wt/vol) bromophenol blue] at 95°C for 5 min and analyzed by SDS (0.1%)–10% PAGE and autoradiography. Quantifications were done by densitometric scanning of autoradiographs with a LKB 2222-020 Ultrascan-XL Laser densitometer (Pharmacia-LKB, Uppsala, Sweden) using QuanTN software (Molecular Dynamics, Sunnyvale, CA). Data from three or more independent experiments were used for quantification analysis.

Immunofluorescence by confocal microscopy

For immunofluorescence analysis, BHK cells (4×10^5) were grown overnight in tissue culture coverslips (Nunc,

Naperville, IL) and synchronously infected with Sendai virus or RVV for 1.0 h at 4°C. Following adsorption, 1.5 ml of prewarmed (37°C) MEM containing 2.5% FBS was added to the cell monolayers for the indicated times. For monensin (Sigma) treatment of the virus-infected cells, prewarmed MEM containing 2.5% FBS and monensin (10 μM final concentration) was added at 2 hpi and incubated for a further 5 h at 37°C. At 7 hpi, infected BHK cells were fixed with a methanol:acetone mixture (1:1) at −20°C for 20 min. RVV-infected HeLa cells were fixed with 4% formaldehyde for 20 min at room temperature and permeabilized with 1% NP-40 for 30 min at room temperature. To block the nonspecific antibody binding, the cells were incubated in 3% BSA (Sigma) for 30 min. Primary antibodies, anti-M rabbit polyclonal antibodies, and anti-F and anti-HN mouse monoclonal antibodies were diluted in 3% BSA and incubated with cells for 1 h at room temperature as described previously (Avalos *et al.,* 1997). Cells were then stained with FITC-tagged anti-rabbit IgG and TRITC-tagged anti-mouse IgG (Sigma). Cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Specimens were imaged on a Leica TCS-SP inverted confocal microscope (Leica Microsystems, Heidelberg GmbH, Germany) equipped with an argon laser for 488-nm blue excitation for FITC and a krypton laser for 568-nm red excitation for TRITC. The thickness of each digital section obtained by the microscope was 0.6 μm and at least 30 serial sections throughout the cells were analyzed. Image analysis was performed using the Leica TCS-NT software provided with the microscope. Images were superimposed digitally to allow fine comparison. Colocalization by superimposition of green (FITC) and red (TRITC) signals in a single pixel produces yellow or orange, while separated signals remain green and red.

ACKNOWLEDGMENTS

This work was supported by USPHS grants (AI-16348, AI-41681) from the NIH, NIAID. We are grateful to Jose Orozco for constructing the recombinant vaccinia viruses and Eleanor Berlin for typing the manuscript. Confocal microscopy and image analysis were done using the UCLA Brain Research Institute Core Imaging Facility. We thank Dr. Matthew J. Schibler for his kind help in confocal microscopy.

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