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Inhibition of the assembly of Newcastle disease virus by monensin

Tetsuya Yoshida [1,*], Yasuhiro Nakayama [2], Hiroshi Nagura [2], Tetsuya Toyoda [2], Kazuo Nishikawa [2], Michinari Hamaguchi [2] and Yoshiyuki Nagai [2]

[1] *Nagoya University College of Medical Technology, Nagoya 461, and* [2] *Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya 466, Japan*

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Summary

Monensin inhibits the intracellular transport of the glycoproteins of Newcastle disease virus between *cis* and *trans* Golgi stacks of infected BHK cells, as evidenced by its effect upon their post-translational modifications such as fatty acid acylation, glycosylation and proteolytic cleavage. Thus the drug has markedly altered the subcellular distribution of the glycoproteins so that they accumulate in the internal smooth membranes but are virtually absent in the plasma membrane. These glycoproteins that accumulated in intracellular membranes have a cytoplasmic domain susceptible to protease digestion and thus are transmembranous. Under such conditions, the behavior of M protein, which plays a crucial role in virus assembly (Y. Nagai et al., 1976, Virology 69, 523–538), has been analyzed. It has been found that the M protein can neither associate with the internal membranes nor bind to the plasma membrane. Thus no virus budding has been observed, either at the plasma membranes or at internal membranes. These results substantiate the view that the interaction between M and glycoproteins is of great importance for virus assembly and suggest further that this interaction is possible only when the glycoproteins have been incorporated into the plasma membrane.

Newcastle disease virus, monensin, viral glycoproteins, M protein

* To whom reprint requests should be addressed.

Introduction

The membrane or matrix (M) protein of paramyxoviruses has been shown to play a crucial role in virus assembly (see for reviews Compans and Klenk, 1979; Matsumoto, 1982; Dubois-Dalcq et al., 1984). Soon after synthesis, the M proteins associate with the areas of host-cell plasma membrane which already contain the viral glycoproteins. This is immediately followed by the formation of a patch of virus-specific membrane, the immediate precursor for the envelope, at restricted areas of the plasma membrane. The association of the nucleocapsid with the envelope is also mediated by M proteins, by its binding capacity to the nucleocapsid. The intracellular membranes such as the Golgi apparatus or smooth endoplasmic reticulum also contain the viral glycoproteins, because they pass through these membranes during transport from the site of synthesis, the rough endoplasmic reticulum (RER), to the plasma membrane (Nagai et al., 1976a; Lamb and Choppin, 1977). However, paramyxoviruses as well as certain other enveloped RNA viruses such as orthomyxoviruses do not bud from intracellular membranes but do so exclusively from the plasma membrane. There could be a number of explanations for this selectivity of the virus assembly site, and, in view of the above role of M protein in virus assembly, it is important to clarify whether or not M protein can recognize intracellular membranes containing viral glycoproteins. Previous cell fractionation studies have shown that the M protein is indeed present in the intracellular smooth membrane fraction (Nagai et al., 1976a; Lamb and Choppin, 1977). However, the amount present there relative to the glycoproteins seemed to vary significantly depending on the virus–cell system used; it is usually low in NDV-infected cells (Nagai et al., 1976a) but relatively high in Sendai virus-infected cells (Lamb and Choppin, 1977). In addition, low levels of contamination with membrane vesicles derived from plasma membrane seem to be inevitable in that type of study (Nagai et al., 1976a). Therefore, the specificity of M protein for association with intracellular smooth membranes has not yet been firmly established. It also appears important to define whether or not such a direct interaction of M and the lipid bilayer is involved in the virus assembly, as suggested for the assembly of influenza virus (Gregoriades, 1980; Gregoriades and Frangione, 1981).

Monensin, a linear polyether with a high affinity for Na^+ ions (Pressman, 1976), is known to block the transport to the cell surface of various viral and secretory proteins (Tartakoff, 1983). Therefore, we have been able to use monensin to change greatly the quantitative distribution of viral glycoproteins between plasma membranes and internal membranes, so that the latter are greatly enriched while the former contain low levels. Under such conditions, we examined the intracellular behavior of M protein of NDV and show that it can neither interact with those intracellular membranes even if large amounts of viral glycoproteins accumulate there, nor bind to the plasma membrane devoid of the glycoproteins.

Materials and Methods

Cells and viruses

BHK-21 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum and 10% tryptose phosphate broth (TPB). MDCK cells were grown in MEM containing 5% calf serum and 10% TPB. Stock viruses of NDV, avirulent strain D26 (Nagai et al., 1980) and virulent strain Miyadera, were prepared in embryonated eggs as described previously (Nagai et al., 1972). Stock vesicular stomatitis virus (VSV), strain New Jersey, was prepared in BHK cells. The cells were infected with VSV at a multiplicity of approximately 0.01 pfu per cell and incubated in MEM at 37°C for 24 h. The infectivity of NDV was assayed by determining the 50% egg infectious dose (EID_{50}) for D26 (Yoshida et al., 1976) or by the plaque method on BHK cell monolayers for Miyadera (Nagai et al., 1972). The infectivity of VSV was also assayed by the plaque method on BHK cell monolayers.

Virus infections and labeling of infected cells with radioisotopes

Confluent monolayers of BHK or MDCK cells were washed once with Hank's solution and infected with NDV or VSV at an input multiplicity of approximately 20 EID_{50} (or pfu) per cell. After an adsorption period of 1 h at 37°C, the monolayers were washed three times with Hank's solution and incubated in MEM at 37°C. For the labeling of infected cells with [^{35}S]methionine, the medium was removed at various times after infection, and then MEM deficient in methionine and containing 10 μCi/ml of [^{35}S]methionine (> 800 Ci/mmol, Amersham International, Amersham, U.K.) was added for appropriate periods. For chases, the radioactive medium was removed and the cells were incubated in MEM containing a tenfold excess of methionine. For the labeling of infected cells with [^{3}H]sugars, 10 μCi/ml of D-[1,6-^{3}H]glucosamine HCl (30–60 Ci/mmol) or D-[1-^{3}H]galactose (10–25 Ci/mmol) purchased from New England Nuclear, Boston, MA, was added to the medium for appropriate periods. The labeling of infected cells with [9,10-^{3}H]palmitic acid (23.5 Ci/mmol, New England Nuclear) was done as described by Johnson and Schlesinger (1980).

Antisera and antibody fluorescent staining

Antiserum against NDV was prepared by immunizing rabbits several times with a mixture of purified D26 virions solubilized by 2% Triton X-100 and Freund's adjuvant. Monospecific antisera against HN, F, NP or M polypeptides of NDV described by Nagai et al. (1983) were used in the present study. These antisera were prepared by immunization of rabbits with each of HN, F, NP and M polypeptides purified by SDS–polyacrylamide gel electrophoresis. The specificity of these antisera was determined by immunoprecipitation of lysates of NDV-infected BHK cells followed by polyacrylamide gel electrophoresis, and the sera specifically precipitated the respective viral antigens (Nagai et al., 1983). NDV-infected BHK cells on coverslips were processed for fluorescent antibody staining as described previously (Yoshida et al., 1979), using various anti-NDV antisera and FITC-conjugated anti-rabbit IgG. Microscopy was carried out with an Olympus fluorescence microscope BH.

Isolation of plasma membranes

Plasma membranes were isolated from BHK cells as cell ghosts by a modification of the fluorescein mercuric acetate method of Warren et al. (1966) as described previously (Nagai et al., 1976a).

Cell fractionation

The procedure of Caliguiri and Tamm (1970) was used. BHK cells were disrupted by a tight-fitting Dounce homogenizer and the cytoplasmic extracts were fractionated by centrifugation on a discontinuous sucrose gradient as described previously (Nagai et al., 1976a). Usually 6 bands could be discriminated. The second band in the 25% sucrose layer and the third band in the upper half of the 30% layer were used for smooth membrane fractions and the fifth band at the interface between the 40% and 45% layer for the RER fraction. The fourth band at the interface between the 30% and 40% layer containing both smooth and rough membranes was also used.

Immunoprecipitation and SDS–polyacrylamide gel electrophoresis

Immunoprecipitation of NDV-infected BHK cells labeled with [^{35}S]methionine, [^{3}H]sugar or [^{3}H]palmitic acid was carried out as described previously (Naruse et al., 1981) using anti-NDV and fixed *Staphylococcus aureus*. Electrophoresis on 10% SDS–polyacrylamide gels and fluorography were done as described previously (Yoshida et al., 1982).

Electron microscopy

Monolayers of infected cells on 10-cm dishes were washed with phosphate-buffered saline (PBS), fixed in 2% glutaraldehyde in phosphate buffer (pH 7.4) and scraped off by rubber policemen. The cells were pelleted by centrifugation, fixed further for 2 h at 4°C and washed in PBS. The pellets were then post-fixed in 2% osmium tetroxide in phosphate buffer (pH 7.4) for 2 h, dehydrated in ethanol and embedded in Epon. Ultrathin sections, stained sequentially with uranyl acetate and lead citrate, were examined with an electron microscope (Hitachi HU-300).

Results

Effect of monensin on the maturation of NDV in BHK cells

Monolayers of BHK cells were infected with the virus, and various amounts of monensin were added 1 h after infection. The yield of progeny virus at 9 h after infection was reduced to less than 1% of the control at concentrations higher than 0.1 μM monensin (Table 1). Fig. 1 shows further that the drug does not allow production of either infectious progeny or physical particles. Viral and cellular protein synthesis in monensin-treated cells was slightly reduced at these concentrations (to approximately 80% of the control). When the infected cells were examined by electron microscopy, many dilated smooth membrane vesicles were observed in the cytoplasm of monensin-treated cells. No virus budding was observed either at the plasma membrane or at intracellular vesicle membranes (Fig. 2), confirming that

TABLE 1

EFFECT OF MONENSIN ON THE GROWTH OF NDV IN BHK CELLS

Monolayers of BHK cells were infected with NDV, strain D26. After an adsorption period of 1 h, the monolayers were incubated at 37°C in MEM containing various amounts of monensin. The infectivity titers were assayed 9 h after infection as described in Materials and Methods.

Monensin (μM)	Infectivity (EID_{50}/ml)
0	$10^{7.7}$
0.01	$10^{7.3}$
0.05	$10^{6.3}$
0.1	$10^{5.5}$
0.5	$10^{5.7}$

the drug inhibits the production of infectious progeny as well as physical particles. To localize each viral protein in monensin-treated BHK cells, the infected cells were examined by fluorescent antibody staining using anti-NDV or monospecific antisera against HN, F, NP or M. When the cells were stained with anti-NDV antiserum without acetone fixation, specific fluorescence was almost entirely absent on the surface of monensin-treated cells (Fig. 3B), whereas untreated cells showed remarkable membrane fluorescence (Fig. 3A). When the cells were fixed with acetone before staining, the monensin-treated cells showed distinct fluorescence of anti-F antibody in the cytoplasm (Fig. 3D). However, the staining pattern was quite unusual when

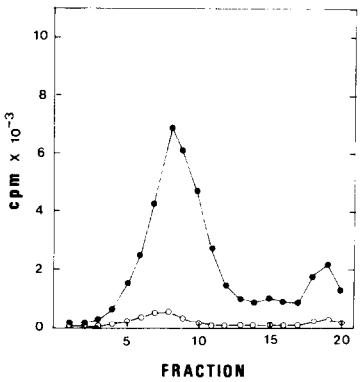


Fig. 1. Effect of monensin on release of virus particles from BHK cells. NDV-infected BHK cells in the absence (●) or presence (○) of 0.2 μM monensin were labeled with 10 μCi/ml [^{35}S]methionine in MEM containing 1/100 unlabeled methionine 4 h after infection. The media were harvested 5 h later and centrifuged at low speed, and the supernatants were incubated for 2 h at 4°C with 7% polyethylene glycol 6000 and 2.3% NaCl. Virions were sedimented by centrifugation at 2000 rpm for 20 min, and the pellet resuspended in MEM was loaded on top of a linear 20–50% sucrose gradient. After centrifugation at 24000 rpm for 2 h in a Hitachi RPS 40, samples of 0.6 ml were collected from the bottom, precipitated with 10% TCA, and counted on a Whatman glass-fiber filter GF/B. It has been confirmed by polyacrylamide gel electrophoresis that the radioactive peak represents the sedimenting virus particles.

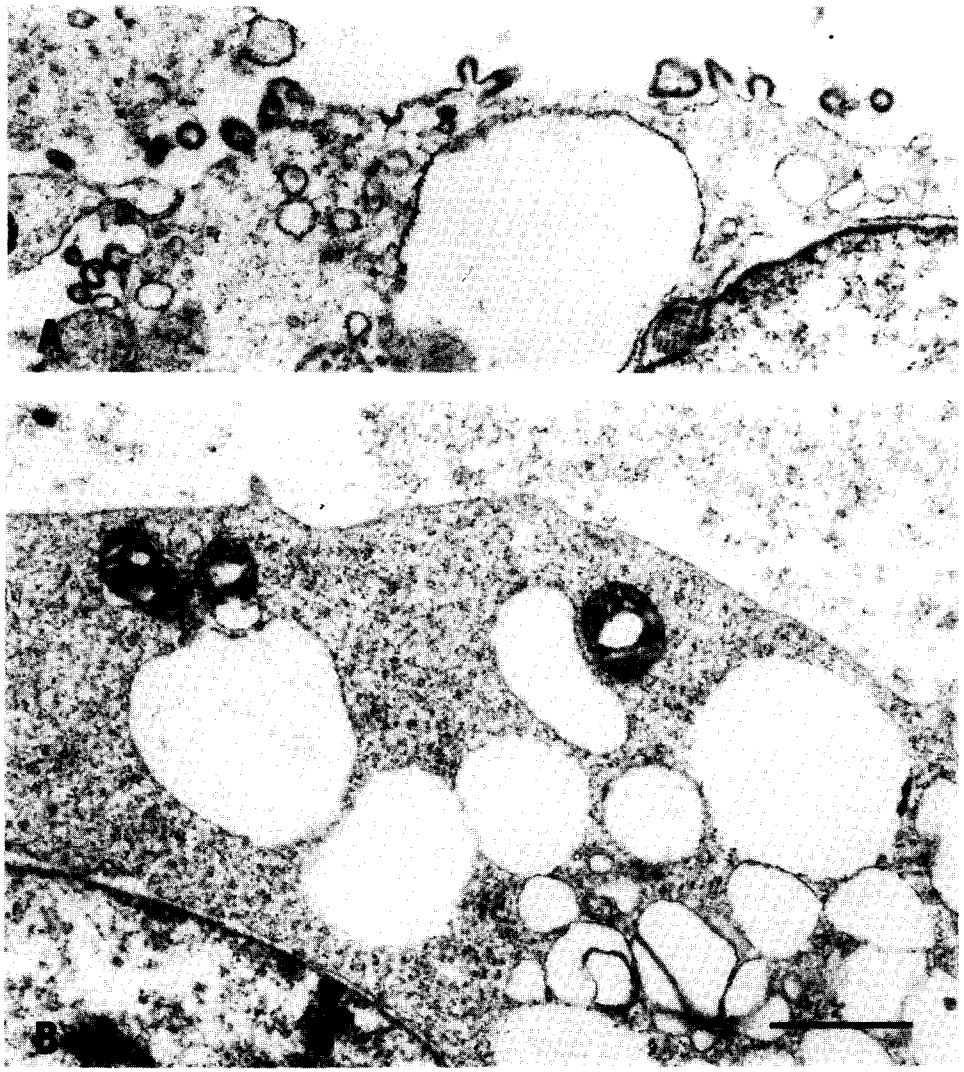


Fig. 2. Electron microscopy of NDV-infected BHK cells. Monolayers of BHK cells were infected with NDV, strain D26, and incubated in the absence (A) or presence (B) of 0.2 μM monensin. The cells were examined 9 h after infection by electron microscopy as described in Materials and Methods. Bar represents 1 μm, and A has the same magnification as B.

compared with that of untreated cells. In addition to the diffuse fluorescence in the cytoplasm, the specific fluorescence seems to be associated with dilated vesicular membranes. Untreated control cells revealed massive fluorescence in the perinuclear region and diffuse or finely granular fluorescence in the cytoplasm (Fig. 3C). Their plasma membranes seem to be also heavily stained in contrast to monensin-treated cells, which showed almost no fluorescence. Similar differences in staining pattern

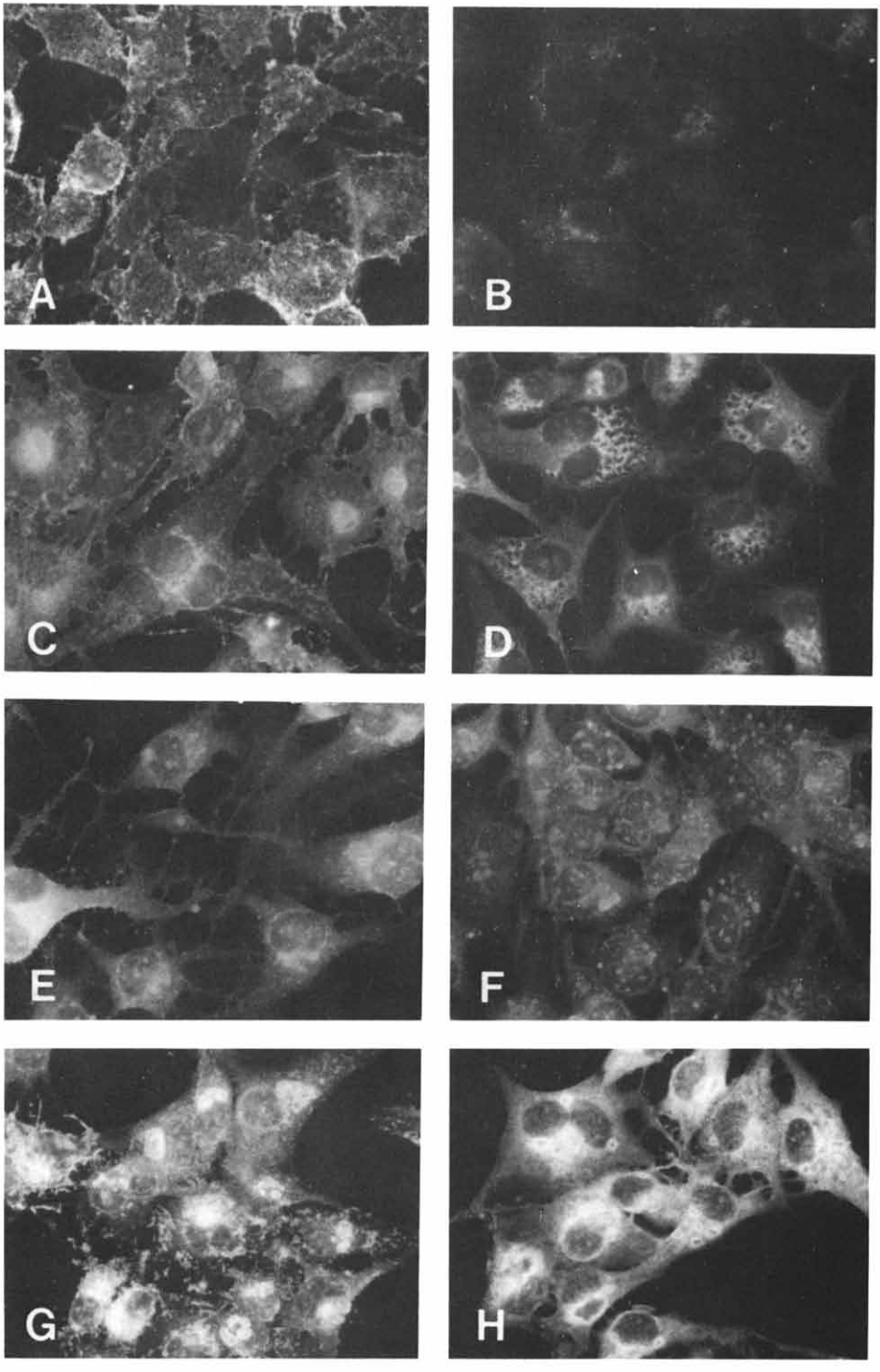
between monensin-treated and untreated cells have been observed with anti-HN serum (not shown). When stained with anti-M serum after fixation, the untreated cells showed diffuse fluorescence as well as various sizes of inclusions in the cytoplasm (Fig. 3E). Monensin-treated cells showed a similar pattern of cytoplasmic fluorescence but the number of cytoplasmic inclusions of M seemed to increase (Fig. 3F). In contrast to the glycoproteins and M protein antigens, there appeared to be little specific difference in NP antigen distribution between monensin-treated and untreated cells (Fig. 3G, H). Although the inclusions of nucleocapsid in the treated cells appeared to be hollow for unknown reasons, they might not be associated with dilated vacuoles as observed with the glycoproteins in Fig. 3D, because the fluorescent patterns of the nucleocapsids and vacuoles were quite different from each other and because the hollow fluorescence of the nucleocapsid was also observed in the untreated cells. These results taken together indicate that monensin causes extensive morphological changes in intracellular membranes, inhibits the appearance of viral glycoproteins at the plasma membrane, and blocks virion formation.

Effect of monensin on the posttranslational processing of NDV glycoproteins in BHK cells

Morrison et al. (1985) have recently shown that monensin blocks the transport of NDV glycoproteins from the *cis* to the *trans* Golgi membranes of chick embryo cells. In addition, the intracellular proteolytic cleavage, which is a phenomenon characteristic of virulent strains of NDV (Nagai et al., 1976b), was reported to be blocked by monensin (Morrison et al., 1985). We have confirmed these results in BHK cells. BHK cells infected with a virulent strain, Miyadera, were labeled with [^{35}S]methionine, [^{3}H]glucosamine or [^{3}H]galactose in the absence or presence of monensin, and analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 4, proteolytic cleavage of F_0 to F_1 and F_2 was strongly inhibited by monensin, and incorporation of galactose but not of glucosamine into HN and F_0 was significantly reduced. This latter observation indicates that the glycoprotein transport could be blocked between *cis* and *trans* stacks of the Golgi complex, because galactosyltransferase is restricted to the *trans* face of the Golgi complex (Griffiths et al., 1982; Roth and Berger, 1982), whereas removal of mannose and attachment of peripheral *N*-acetylglucosamine may occur in some or all of the remaining cisternae on the *cis* side of the Golgi stack (Dunphy and Rothman, 1983). The transport block between *cis* and *trans* Golgi membranes was also supported by the finding that monensin did not block the attachment of palmitic acid to the F_0 glycoprotein (not shown), since fatty acid addition to glycoproteins seems to occur within the Golgi complex, probably in the *cis* side (Schmidt and Schlessinger, 1980; Griffiths et al., 1983). With NDV, only F_0 (F) but not HN_0 (HN) undergoes fatty acid acylation (Chatis and Morrison, 1982).

M protein does not associate with plasma membranes devoid of viral glycoproteins

Plasma membranes were isolated as described in Materials and Methods from infected and [^{35}S]methionine-labeled BHK cells in the absence or presence of monensin, and viral polypeptides associated with those membranes were analyzed by


A
B
C
D
E
F
G
H

polyacrylamide gel electrophoresis. As shown in Fig. 5, all the viral polypeptides including M were greatly reduced in amount in the plasma membrane of monensin-treated cells. We compared the protein species contained in the ghosts from monensin-treated and -untreated cells with each other by polyacrylamide gel electrophoresis followed by Coomassie blue staining, and found that there was no marked difference except that some viral proteins (NP and M) could be additionally visualized in the untreated ghosts but not in the treated ones (data not shown). This result would eliminate the possibility that the plasma membranes from monensin-treated cells could fractionate very differently from those of untreated cells. These results confirm that monensin blocks the transport of viral glycoproteins to the plasma membrane and indicate that M and the other viral proteins also do not associate with the plasma membrane in the presence of the drug. It appears likely either that monensin blocks directly the transport of M to the plasma membrane or that viral glycoprotein insertion into the plasma membrane is a prerequisite for the association of M. Evidence available suggests the latter possibility, because M associates directly with the plasma membrane through a route different from that of viral glycoproteins (Nagai et al., 1976a; Lamb and Choppin, 1977) and thus probably insensitive to monensin.

M protein does not associate with the Golgi membranes where viral glycoproteins accumulate

To examine whether M protein can associate with the Golgi membranes where viral glycoproteins are accumulated in the presence of monensin, cell fractionation experiments were carried out. Monolayers of BHK cells were infected with NDV and then incubated in the presence of monensin. At 5 h after infection the cells were pulse-labeled for 10 min with [^{35}S]methionine, or pulse-labeled and chased for 60 min. The cells were then fractionated into subcellular membranes by centrifugation through a discontinuous sucrose gradient as described in Materials and Methods. Preliminary studies showed that monensin-treated membranes were fractionated at least morphologically like untreated membranes (not shown). Viral polypeptides in fraction 2 + 3 (intracellular smooth membranes), fraction 5 (RER) and fraction 4 (the mixture of smooth and rough membranes) were analyzed by immunoprecipitation followed by polyacrylamide gel electrophoresis. As shown in Fig. 6, HN_0 and F_0 were significantly increased in fraction 2 + 3 after a 60 min chase (lane 4) as compared with those in the same fraction of a 10 min pulse (lane 1). Table 2 summarizes the data obtained by quantitative densitometric analysis of Fig. 6. HN_0 and HN, and F_0 in fraction 2 + 3 increased to approximately 7- and 10-fold,

Fig. 3. Antibody fluorescent staining of NDV-infected cells in the absence (A, C, E, G) or presence (B, D, F, H) of 0.2 μM monensin. D26-infected BHK cells were stained 9 h after infection with anti-NDV antiserum without acetone fixation (A, B) or with various monospecific antisera to NDV proteins after acetone fixation (C–H). C and D were stained with anti-F serum, E and F with anti-M, and G and H with anti-NP, respectively.

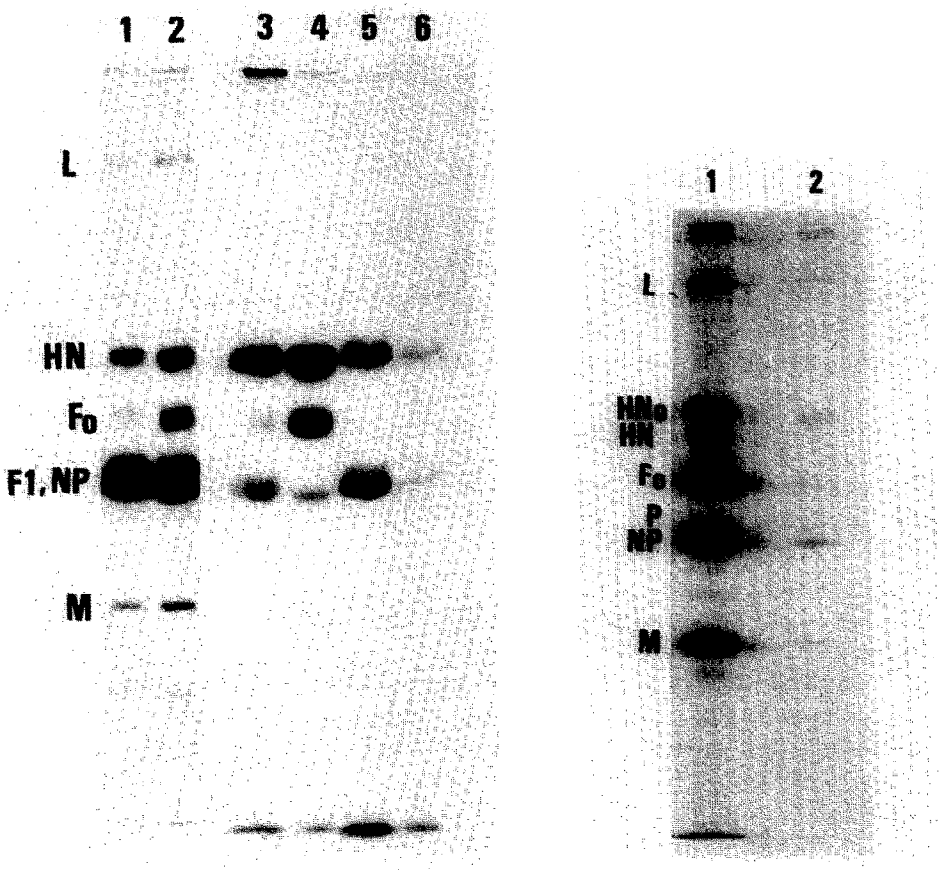


Fig. 4. Effect of monensin on the proteolytic cleavage of NDV F_0 to F_1 and F_2 and on the glycosylation of viral glycoproteins. Monolayers of BHK cells on 3.5 cm dishes were infected with NDV, strain Miyadera, and labeled for 60 min with 10 μCi/ml [^{35}S]methionine (lanes 1 and 2), 10 μCi/ml [^{3}H]glucosamine (lanes 3 and 4) or 10 μCi/ml [^{3}H]galactose (lanes 5 and 6) in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 0.2 μM monensin. The cells were processed for immunoprecipitation using anti-NDV followed by polyacrylamide gel electrophoresis and fluorography.

Fig. 5. Viral polypeptides associated with the plasma membrane of NDV-infected cells. Monolayers of BHK cells on 10 cm dishes were infected with D26 and labeled for 60 min with 10 μCi/ml [^{35}S]methionine in the absence (lane 1) or presence (lane 2) of 0.2 μM monensin 6 h after infection. Plasma membranes were isolated from those cells as described in Materials and Methods and analyzed by polyacrylamide gel electrophoresis and fluorography. The same amount of protein (100 μg), which was determined by the method of Lowry et al. (1951), was applied on each lane.

respectively, after a 60 min chase. This level of increase of the glycoproteins is far more extensive than that observed in untreated NDV-infected BHK cells (Nagai et al., 1976a), which showed approximately only 1.2–1.5-fold increase. In spite of such marked accumulation of the glycoproteins, M and NP were present only at a trace

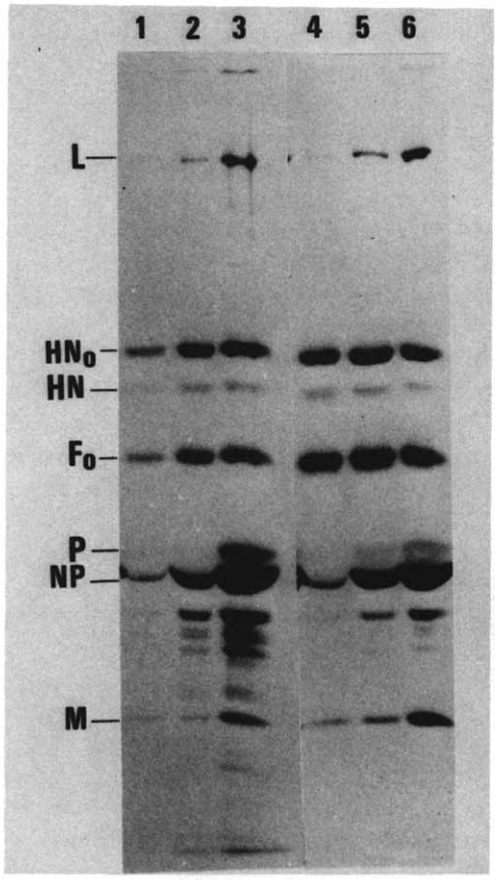


Fig. 6. Viral polypeptides associated with cytoplasmic fractions. Monolayers of BHK cells on 10 cm dishes were infected with D26 and incubated in the presence of 0.2 μM monensin. At 5 h after infection, the cells were pulse-labeled for 10 min with 10 μCi/ml [^{35}S]methionine (lanes 1–3) or pulse-labeled and chased for 60 min (lanes 4–6). The cells were then fractionated into subcellular organellae by centrifugation through a discontinuous sucrose gradient as described in Materials and Methods. Viral polypeptides in fraction 2+3 (lanes 1, 4), fraction 5 (lanes 3, 6) and fraction 4 (lanes 2, 5) were analyzed by immunoprecipitation followed by polyacrylamide gel electrophoresis and fluorography.

TABLE 2

DISTRIBUTION OF VIRAL GLYCOPROTEINS IN CYTOPLASMIC FRACTIONS OF NDV-INFECTED, MONENSIN-TREATED BHK CELLS

The values were calculated from quantitative densitometric analysis of the film shown in Fig. 6 by a densitometer, Densitron model PAN (Jookoo Sangyo Co.).

Labeling conditions	Glyco-proteins	Percent protein in membrane fractions		
		Fraction 2+3	Fraction 4	Fraction 5
10 min pulse	HN_0 + HN	4	45	51
	F_0	3	47	50
10 min pulse and 60 min chase	HN_0 + HN	29	40	31
	F_0	30	38	32

level in that fraction and did not increase at all after a 60 min chase. These results indicate that viral glycoproteins migrate from RER to the internal smooth membrane (Golgi membrane) and accumulate there in monensin-treated BHK cells, and that M protein cannot associate with such smooth membranes containing large amounts of viral glycoproteins.

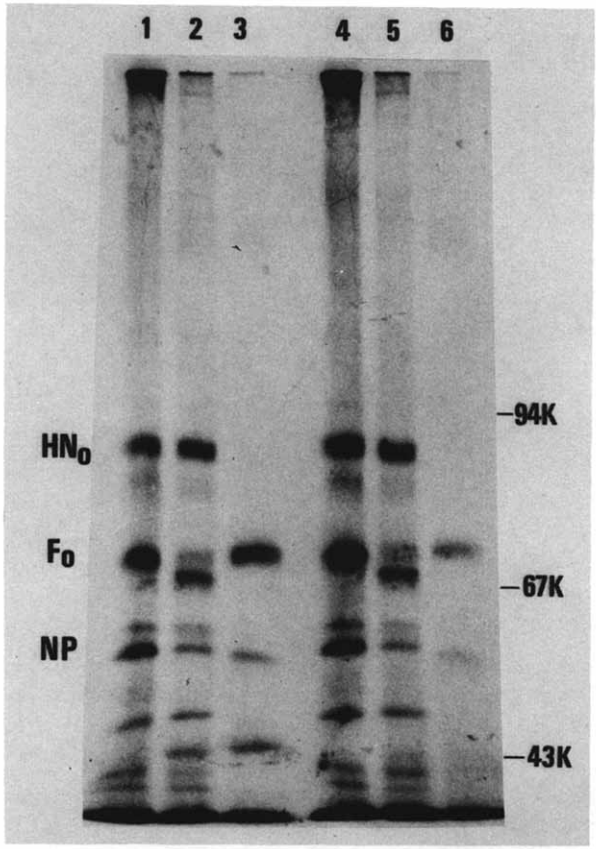


Fig. 7. Protease digestions of intracellular smooth membranes isolated from NDV-infected cells. BHK cells infected with D26 were labeled for 60 min with 10 μCi/ml [^{35}S]methionine in the presence of 0.2 μM monensin, and intracellular smooth membranes (fraction 2 + 3) were isolated from these cells as described in Materials and Methods. To remove the possible contamination with inside-out membranes or virions, which are hemadsorbable, the membrane fraction was incubated for 20 min with chicken red blood cells (final 2%) in an ice bath, and the red blood cells were sedimented by low-speed centrifugation. Intracellular smooth membrane vesicles in the supernatant were incubated in PBS at 37°C for 60 min with 200 μg/ml trypsin or with 200 μg/ml chymotrypsin. The digestion was stopped by boiling for 3 min, and the digestion products were precipitated with 3 vols. of ethanol at −20°C overnight. Electrophoresis was carried out on a 8% polyacrylamide gel. Lanes 1 and 4, undigested membranes; lane 2, trypsin-digested membranes; lane 3, 2% Triton X-100-treated and trypsin-digested membranes; lane 5, chymotrypsin-digested membranes; lane 6, 2% Triton X-100-treated and chymotrypsin-digested membranes.

Viral glycoproteins are present as transmembrane proteins on the Golgi membrane

To examine whether viral glycoproteins on the intracellular smooth membrane are transmembranous, digestion experiments of the membrane with various proteases were carried out. BHK cells infected with NDV were labeled for 60 min with [^{35}S]methionine in the presence of monensin, and intracellular smooth membranes were isolated from these cells as described in Materials and Methods. The smooth membrane vesicles were treated with trypsin or chymotrypsin, which digests only proteins exposed on the external (cytoplasmic) surface of the membrane vesicles. The digestion products were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 7, significant amounts of NP were degraded upon treatment of the vesicles with trypsin, whereas HN_0 and F_0 resisted the digestion except that the apparent molecular weight of F_0 changed from 68 500 to 66 000. Although consistently observed, the decrease of molecular weight of HN_0 by the digestion appeared to be much smaller than that of F_0. This may indicate that the cytoplasmic domain of HN_0 may be smaller or that the domain may be less sensitive to the protease used. When the organization of the vesicles was disrupted by treatment with Triton X-100, all the viral proteins were extensively digested with trypsin. Similar results were obtained upon treatment of the vesicles with chymotrypsin. These results suggest that the glycoproteins are present as transmembrane proteins on the Golgi membrane so that its cytoplasmic domain may be able to interact with M protein.

Monensin does not inhibit assembly of NDV in MDCK cells

As shown in Table 3, monensin did not inhibit the growth of NDV in MDCK cells even at concentrations much higher than those which were strongly inhibitory in BHK cells. The lack of inhibition in MDCK cells is apparently not due to possible low efficiency of the drug uptake by MDCK cells, because the growth of VSV was strongly inhibited. Thus, the effect of monensin on NDV assembly is host-cell dependent and seems not to be due to some possible direct interaction between the drug and the viral components.

TABLE 3

GROWTH OF NDV AND VSV IN MDCK CELLS IN THE ABSENCE OR PRESENCE OF MONENSIN

Monolayers of MDCK cells were infected with VSV or NDV and incubated at 37°C in the absence or presence of monensin. 1 μM of the drug was used for the VSV-infected cells and 5 μM for the NDV-infected cells. The infectivities were assayed 9 h after infection as described in Materials and Methods.

Monensin	VSV (pfu/ml)	NDV (pfu/ml)
−	3.3×10^8	2.0×10^7
+	3.5×10^6	3.2×10^7

Discussion

This paper describes the inhibition of NDV replication by monensin. The drug was suggested to block the transport of viral glycoproteins between *cis* and *trans* Golgi stacks, as judged by the mode of glycosylation and fatty acid acylation of the polypeptide chain. Due to this block in transport of the glycoproteins, their intracellular distribution was markedly changed so that internal smooth membranes were greatly enriched in the glycoproteins, whereas plasma membranes were virtually devoid of them. Under these conditions, the behavior of M protein was examined.

Previous reports have indicated that the M protein associates almost selectively with those areas of plasma membrane which already contain the viral glycoproteins (Nagai et al., 1976a). Although the site of synthesis of M protein and its pathway to the plasma membrane have not been clearly established yet, available evidence suggests that M and glycoproteins do not share a common pathway (Nagai et al., 1976a; Lamb and Choppin, 1977). Thus, it is unlikely that monensin also blocks the transport of M protein. Therefore, our present result that M protein is virtually absent in the plasma membrane of monensin-treated cells substantiates the concept that the incorporation of viral glycoproteins to the plasma membrane is the prerequisite for the association of M with the membrane, and suggests that the interaction between M and cytoplasmic domains of the glycoproteins is important for the assembly of paramyxovirus. Our data are not compatible with the concept, which was suggested for influenza virus (Gregoriades, 1980; Gregoriades and Frangione, 1981), that the interaction between M and lipid bilayer by their hydrophobicity has primary importance for the virus assembly. It is possible, however, that the interaction between M and the lipid bilayer may stabilize the association of M with viral glycoproteins at the plasma membrane (Matsumoto, 1982). Although primary amino acid sequences of F and HN of several paramyxoviruses other than NDV (Collins et al., 1984; Hsu and Choppin, 1984; Paterson et al., 1984; Hiebert et al., 1985), and of Sendai virus M protein (Blumberg et al., 1984; Hidaka et al., 1984) have been recently predicted by nucleic acid sequencing, we remain without a clear understanding of the mechanisms involved in the interaction between viral glycoproteins and M protein. To solve this problem it is necessary to determine the spatial distribution of functional domains of these proteins.

Our present results have also shown that no significant association of M with the intracellular smooth membranes and no virus budding have occurred, even when these membranes are greatly enriched with viral glycoproteins. These results contrast with those showing that Sindbis virus (Johnson and Schlesinger, 1980), Semliki forest virus (Griffiths et al., 1983), coronavirus (Nieman et al., 1982; Alonso-Caplen et al., 1984) and probably VSV (Alonso and Compans, 1981) bud from cytoplasmic vacuoles in the presence of monensin. Therefore, cellular site or component(s) appear to be important for the interaction of M with glycoproteins in the assembly of NDV. There is a possibility that the glycoproteins undergo some conformational changes during transport from the *trans* Golgi to the plasma membrane, which may be necessary for specific interaction with M protein. Alternatively, M protein may

migrate after synthesis only to the plasma membrane but not to the Golgi apparatus by an unknown mechanism.

It is also conceivable that there might be some direct interaction of the drug with viral proteins, which could result in a block of the specific interactions between M and glycoproteins. However, such a direct effect seems not to be involved, because the drug did not inhibit the interaction in MDCK cells. Some paramyxoviruses are very sensitive to the change in ionic milieu caused by ouabain, an inhibitor of Na,K-ATPase (Nagai et al., 1972), and thus the effect of monensin shown here could also be explained from this aspect. However, this appears also unlikely because NDV is relatively resistant to ouabain (Nagai et al., 1972).

The inhibition of proteolytic cleavage of virulent NDV F_0 by monensin suggests that the enzyme for the cleavage of BHK cells is present somewhere after the monensin-sensitive step in the course of viral glycoprotein transport. This result is compatible with that of Morrison et al. (1985) showing a similar intracellular localization of the proteolytic enzyme for NDV F_0 in chick embryo cells.

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