Glycosylation of measles virus haemagglutinin protein in infected cells

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Glycosylation of measles virus haemagglutinin protein in infected cells

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Processing of the measles virus haemagglutinin (H) protein was analysed by the pulse–chase method, immunoprecipitation with an anti-H monoclonal antibody and SDS–polyacrylamide gel electrophoresis, combined with the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or monensin (inhibitors of intracellular processing of secretory proteins) to cultures and digestion of the protein with endoglycosidase H or neuraminidase. The apparent M_r of the H protein was increased from 74K to 78K during the chase period. Addition of either CCCP or monensin to the chase medium inhibited the appearance of the 78K H protein, but not the immunoreactivity of the H protein or dimer formation, suggesting that these two events occur in the rough endoplasmic reticulum. The 74K H protein processed in the presence of CCCP was fully sensitive to endoglycosidase H digestion, whereas the 74K H protein processed in the presence of monensin was partially resistant to endoglycosidase H. In experiments using ^{3}H-labelled sugars, [^{3}H]galactose was incorporated into the 74K H protein in the presence of monensin. Neuraminidase treatment increased the electrophoretic mobility of the 78K H protein to 74K. Only the 78K H protein was detected on the surface of untreated cells, and it was resistant to endoglycosidase H digestion. These data suggest that after galactose addition sialic acid is added to the H protein in the trans-Golgi complex and then the mature 78K H protein is transported to the cell surface.

Introduction

Measles virus (MV) is a member of the morbillivirus subgroup of paramyxoviruses. It has two envelope glycoproteins, the haemagglutinin (H) and the fusion (F) proteins. The H protein possesses haemagglutinating activity and is analogous to the haemagglutinin–neuraminidase (HN) glycoprotein of other paramyxoviruses such as Sendai virus and mumps virus. It is generally accepted that the H protein has an essential role in the first step of infection, i.e. viral attachment to the sialic acid-containing cellular receptors. In addition, the H protein also induces host immune responses such as the production of neutralizing antibody which plays an important role in the host's defence against MV infection. It is still unclear whether the H protein elicits major histocompatibility complex-restricted cytotoxic T lymphocytes.

The H protein undergoes a series of *N*-linked glycosylation events within cells before the mature protein is ready to be incorporated into virions. In general, *N*-linked oligosaccharide chains are transferred cotranslationally to the growing polypeptide in the rough endoplasmic reticulum (RER). Processing of the high mannose oligosaccharide to the complex form begins after transport to the Golgi complex. In a stepwise, concerted set of reactions, six of the nine mannose residues are removed and *N*-acetylglucosamine, galactose, sialic acid and fucose are added one at a time to each oligosaccharide chain. Oligosaccharide processing is completed before the protein reaches the cell surface (Kornfeld & Kornfeld, 1985).

During the maturation process of the MV H protein, the electrophoretic mobility of the protein decreases, maybe reflecting the processing pathway from the high mannose to the complex type oligosaccharides (Graves, 1981; Bellini *et al.*, 1983; Young *et al.*, 1985; Kohama *et al.*, 1985). However, it is still unclear which step in oligosaccharide processing is responsible for the altered mobility. In this communication, we attempt to dissect the maturation process of MV H protein by pulse–chase methods combined with inhibitors of the intracellular processing of glycoproteins and digestion with endoglycosidase H or neuraminidase.

Methods

Cells, virus and infections. HeLa cells used in this work were cultured at 35 °C in Dulbecco's modified Eagle's medium supplemented with 5% foetal calf serum. The cells were infected with MV (Edmonston strain) at an m.o.i. of 2.

Radioisotopic labelling of infected cells. Infected cells were incubated at 35 °C for 24 h and pulse-labelled with 100 μCi/ml of [^{35}S]methionine (1070 Ci/mmol, American Radiolabeled Chemicals) for 10 or 20 min at 35 °C, then the cells were chased in the presence of 2 mM unlabelled methionine for the appropriate periods of time at 35 °C. For the labelling of infected cells with ^{3}H-labelled sugars, 100 μCi/ml of D-[6-^{3}H]glucosamine hydrochloride (25 Ci/mmol, American Radiolabeled Chemicals) or D-[6-^{3}H]galactose (20 Ci/mmol, American Radiolabeled Chemicals) was added to the medium for the appropriate period of time.

Immunoprecipitation and SDS–PAGE. Virus proteins were analysed by immunoprecipitation. For total cell immunoprecipitation, the chased cells were solubilized in radioimmune precipitation assay (RIPA) detergent buffer (Ogura *et al.*, 1987) and immunoprecipitated with anti-H monoclonal antibody (kindly provided by Professor V. ter Meulen, Würzburg, Germany) and the immunoprecipitates were subjected to SDS–PAGE. For cell surface immunoprecipitation, the chased cells were washed with cold PBS, monoclonal antibody was added and the cells were incubated at 4 °C for 15 min. The cells were washed with ice-cold PBS five times to remove excess antibody and solubilized in RIPA detergent buffer. The lysates were precipitated by Protein A–Sepharose CL-4B, and analysed by SDS–PAGE. The M_rs of the two types of H protein and its dimer were estimated by using ^{14}C-labelled marker proteins (Amersham) in preliminary experiments.

Carbonyl cyanide m-*chlorophenylhydrazone (CCCP) treatment or monensin treatment of the cells.* Infected cells pulse-labelled with [^{35}S]methionine were chased in the presence of 50 μg/ml of CCCP or 1 μM-monensin for the appropriate periods of time. The cells were then subjected to RIPA.

Endoglycosidase H digestion or neuraminidase digestion of H protein. Immunoprecipitated H protein bound to Protein A–Sepharose CL-4B was resuspended in 100 mM-sodium citrate pH 5·0, and identical samples were either digested overnight at 37 °C with 50 milliunits (mU)/ml of endoglycosidase H or were mock-treated. The samples were washed with PBS and then analysed by SDS–PAGE. For neuraminidase digestion, samples of H protein were resuspended in 100 mM-citrate–phosphate buffer pH 6·5 containing 10 mM-$CaCl_2$, and identical samples were either digested for 5 h at 37 °C with 50, 125 and 375 mU/ml of neuraminidase or were mock-treated. The samples were washed with PBS and then subjected to SDS–PAGE.

Chemicals. CCCP and monensin were purchased from Sigma. Endoglycosidase H and streptococcus neuraminidase were obtained from Genzyme and Seikagaku Kogyo, respectively.

Results

Synthesis of MV H protein in infected cells

In order to study post-translational glycosylation, MV-infected HeLa cells were pulse-labelled for 20 min with [^{35}S]methionine 24 h post-infection (p.i.) in the presence or absence of CCCP or monensin and an anti-H monoclonal antibody was used in immunoprecipitation. Immediately after labelling (Fig. 1, lanes 2 to 5) a single H protein band with an apparent M_r of 74K was detected. After 60 min of chase, an additional H protein band with an apparent M_r of 78K appeared and the 74K H protein disappeared after 120 min of chase. After 60 min of chase, the intensity of the H protein bands no longer increased. A similar shift in the electrophoretic mobility of the H protein has been observed by several investigators (Graves, 1981; Bellini *et al.*, 1983; Young *et al.*, 1985; Kohama *et al.*, 1985). We examined the effect of CCCP, an inhibitor of oxidative phosphorylation which blocks the transport of secretory or membrane proteins from the RER to the Golgi apparatus (Fries & Rothman, 1980) and monensin, a sodium ionophore which is known to disrupt the functioning of the trans-Golgi (Tartakoff, 1983), on the glycosylation of the H protein. In the presence of CCCP the intensity of the 74K H protein band increased with chase time (Fig. 1, lanes 6 to 8), suggesting that the immunoreactivity of the H protein was acquired cotranslationally in the RER. On the other hand, the 78K H protein did not appear even after 120 min of chase in the presence of monensin (Fig. 1, lanes 9 to 11). This indicates that the increased M_r of the H protein occurs in the Golgi complex.

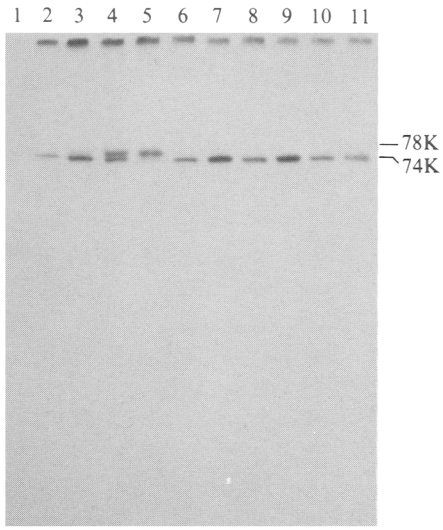

Fig. 1. Time course analysis of processing of the H protein in the absence or presence of CCCP or monensin. HeLa cells in 3·5 cm diameter Petri dishes were infected with MV at an m.o.i. of 2 and incubated at 35 °C. At 24 h p.i., the cells were labelled with [^{35}S]methionine for 20 min and chased with unlabelled methionine in the absence (lanes 2 to 5) or presence of CCCP (lanes 6 to 8) or monensin (lanes 9 to 11) for 0 (lane 2), 30 (lanes 3, 6 and 9), 60 (lanes 4, 7 and 10) or 120 (lanes 5, 8 and 11) min and then subjected to total cell immunoprecipitation using anti-H monoclonal antibody followed by SDS–PAGE. Lane 1, mock infection.

The 74K H protein in the untreated cells shifted slightly towards a faster electrophoretic mobility after 30 and 60 min of chase (Fig. 1, lanes 2 to 5 and Fig. 2*a*, lanes 2 to 5). The same tendency was also observed in the CCCP- or the monensin-treated cells (Fig. 1, lanes 6 to 11 and Fig. 2*a*, lanes 6 to 11).

In MV-infected cells the H proteins are present as dimers (Bussell *et al.*, 1974; Hardwick & Bussell, 1978;

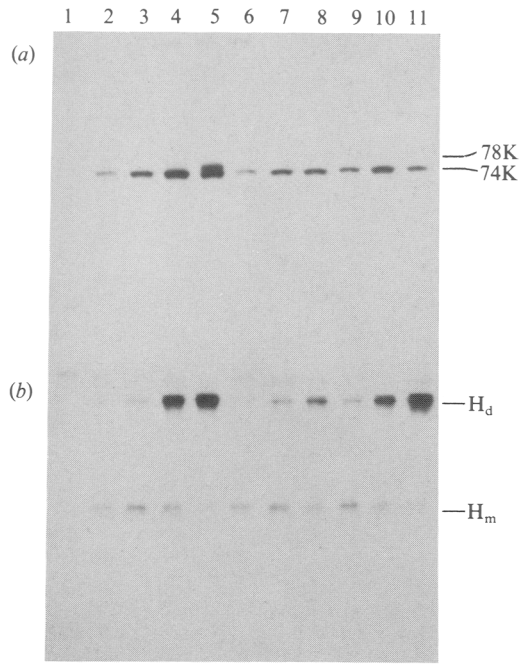

Fig. 2. SDS–PAGE of immunoprecipitated H protein under reducing or non-reducing conditions. Infected HeLa cells pulse-labelled with [^{35}S]methionine for 10 min were chased with unlabelled methionine in the absence (lanes 2 to 5) or presence of CCCP (lanes 6 to 8) or monensin (lanes 9 to 11) for 0 (lane 2), 15 (lanes 3, 6 and 9), 30 (lanes 4, 7 and 10) or 60 (lanes 5, 8 and 11) min and then subjected to total cell immunoprecipitation using anti-H monoclonal antibody. SDS–PAGE was carried out in the presence (*a*) or absence (*b*) of 2-mercaptoethanol. Lane 1, mock infection; H_d, H protein dimer; H_m, H protein monomer.

Casali *et al.*, 1981; Fujinami *et al.*, 1981). Under non-reducing conditions we also detected dimers of the H protein immediately after 10 min of pulse-labelling, and CCCP and monensin did not inhibit their formation (Fig. 2*b*). It is therefore apparent that the oligomerization of the H protein takes place in the RER.

Endoglycosidase H sensitivity of the H protein in infected cells

We examined the sensitivity of the H protein in endoglycosidase H, which is known to cleave the *N*-linked high mannose oligosaccharides attached to the protein as long as they have not been processed into complex oligosaccharides (Tai *et al.*, 1979). As shown in Fig. 3, lanes 2 to 5, the 74K H protein was resolved into three new molecules by endoglycosidase H digestion while the 78K H protein was resistant. This indicates that the 74K and 78K H proteins have high mannose oligosaccharides and complex oligosaccharides, respectively. The 74K H protein chased in the presence of CCCP was completely sensitive (Fig. 3, lanes 6 to 9), whereas the 74K H protein, chased for 120 min in the presence of monensin, contained a partially endoglycosidase H-resistant molecule (Fig. 3, lanes 9 to 11).

Incorporation of ^{3}H-labelled sugars into H protein

In order to determine whether the 74K H protein has *N*-acetylglucosamine or galactose, MV-infected cells

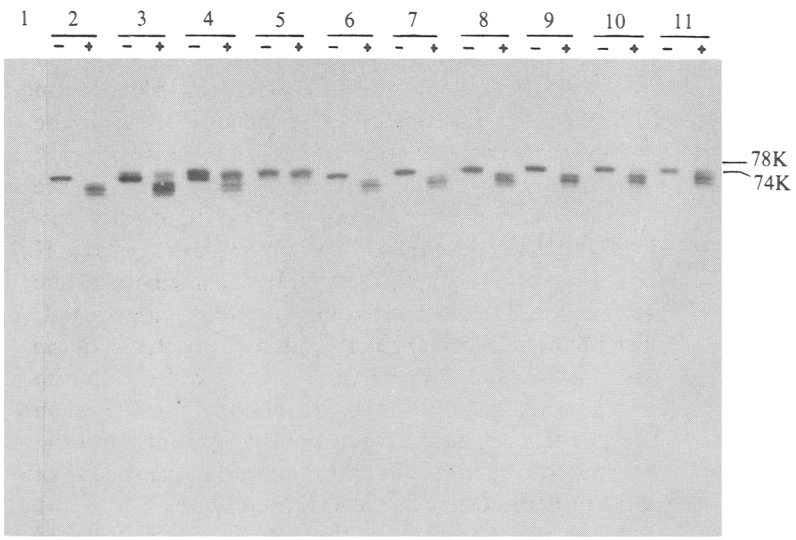

Fig. 3

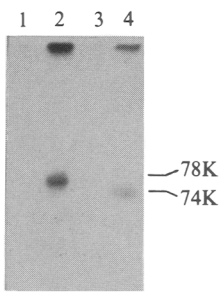

Fig. 4

Fig. 3. Sensitivity of the H protein to endoglycosidase H. Infected HeLa cells pulse-labelled with [^{35}S]methionine for 20 min were chased with unlabelled methionine in the absence (lanes 2 to 5) or presence of CCCP (lanes 6 to 8) or monensin (lanes 9 to 11) for 0 (lane 2), 30 (lanes 3, 6 and 9), 60 (lanes 4, 7 and 10) or 120 (lanes 5, 8, and 11) min. Immunoprecipitated H protein was digested overnight at 37 °C with endoglycosidase H (+) or was mock-treated (−) and then subjected to SDS–PAGE. Lane 1, mock infection.

Fig. 4. Incorporation of [^{3}H]galactose into the H protein. Infected HeLa cells were labelled with [^{3}H]galactose for 6 h in the absence (lane 2) or presence of CCCP (lane 3) or monensin (lane 4) and then subjected to total cell immunoprecipitation using anti-H monoclonal antibody followed by SDS–PAGE. Lane 1, mock infection.

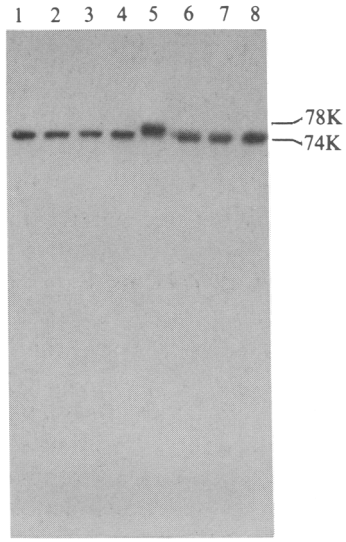

Fig. 5. Sensitivity of the H protein to neuraminidase. Infected HeLa cells were labelled with [^{35}S]methionine for 30 min and chased with unlabelled methionine for 0 (lanes 1 to 4) or 5 (lanes 5 to 8) h. Immunoprecipitated H protein was digested at 37 °C for 5 h with 0 (lanes 1 and 5), 50 (lanes 2 and 6), 125 (lanes 3 and 7) or 375 (lanes 4 and 8) mU/ml of neuraminidase and was then subjected to SDS–PAGE.

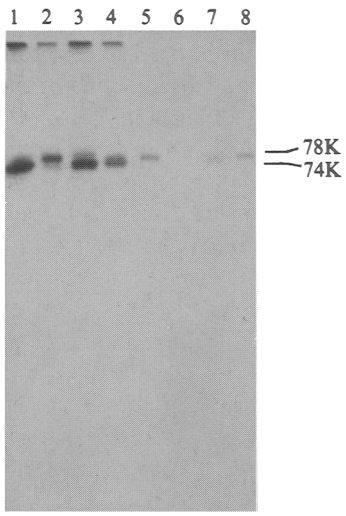

Fig. 6. Effect of CCCP or monensin on cell surface expression of H protein. Infected HeLa cells labelled with [^{35}S]methionine for 1 h were chased with unlabelled methionine for 0 (lane 1) or 6 (lanes 2 to 8) h in the absence (lanes 1, 2, 5 and 8) or presence of CCCP (lanes 3 and 6) or monensin (lanes 4 and 7) and then subjected to total cell immunoprecipitation (lanes 1 to 4) and cell surface immunoprecipitation (lanes 4 to 8) using anti-H monoclonal antibody followed by SDS–PAGE.

were labelled with [^{3}H]glucosamine or [^{3}H]galactose. Both [^{3}H]glucosamine and [^{3}H]galactose were incorporated into both the 74K and 78K H proteins (data not shown), suggesting that the shift from 74K to 78K of the protein occurs after the addition of glucosamine and galactose.

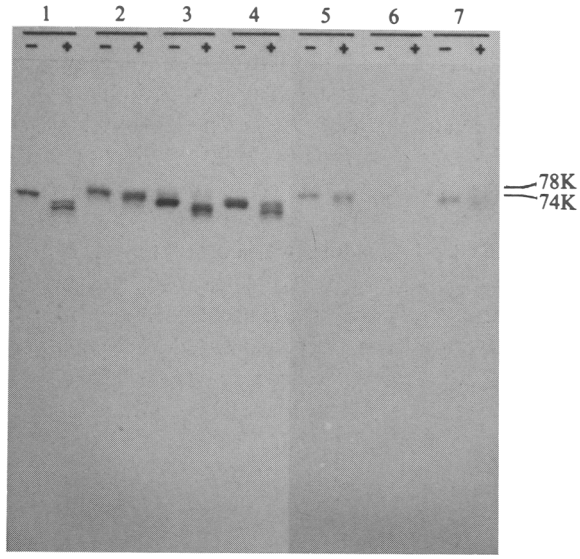

Fig. 7. Endoglycosidase H sensitivity of H protein on the cell surface. Infected HeLa cells were labelled with [^{35}S]methionine for 30 min and chased with unlabelled methionine for 0 (lane 1) or 4 (lanes 2 to 7) h in the absence (lanes 1, 2 and 5) or presence of CCCP (lanes 3 and 6) or monensin (lanes 4 and 7) and then subjected to total cell immunoprecipitation (lanes 1 to 4) or cell surface immunoprecipitation (lanes 5 to 7) using anti-H monoclonal antibody. Immunoprecipitated H protein was digested overnight at 37 °C with endoglycosidase H (+) or was mock-treated (−) and then subjected to SDS–PAGE. Fluorography exposure times for total cell immunoprecipitation and cell surface immunoprecipitation were 3 and 7 days, respectively.

In addition, CCCP completely inhibited incorporation of [^{3}H]galactose into the H protein (Fig. 4, lane 3), whereas in the presence of monensin, [^{3}H]galactose was incorporated into the 74K H protein (Fig. 4, lane 4). This indicates that monensin does not block glycosylation until after the addition of galactose.

Neuraminidase sensitivity of the H protein in infected cells

We attempted neuraminidase digestion of the 74K and 78K H proteins in order to examine the addition of sialic acid to these proteins. As shown in Fig. 5, the 74K H protein pulse-labelled with [^{35}S]methionine for 30 min was relatively insensitive to various concentrations of neuraminidase. On the other hand, the 78K H protein pulse-labelled and chased for 4 h was sensitive to neuraminidase, and its electrophoretic mobility was altered to that of the 74K H protein. These results indicate that the mobility shift of the H protein from 74K to 78K is a result of the addition of sialic acid to the 74K H protein.

Cell surface expression of H protein in infected cells

H protein expression on the cell surface was examined by cell surface immunoprecipitation. In HeLa cells infected

with MV, the 74K H protein was pulse-labelled with [^{35}S]methionine for 20 min. After 60 min of chase, only the 78K H protein appeared on the cell surface of the untreated cells and its intensity had not increased by 120 min of chase (Ogura *et al.*, 1990). The 78K H protein on the cell surface of the untreated cells was resistant to endoglycosidase H (Fig. 7, lane 5). Cell surface expression of the H protein was completely inhibited by CCCP (Fig. 6, lane 6), whereas the 74K H protein was detected on the cell surface in the presence of monensin (Fig. 6, lane 7). This monensin-treated 74K H protein on the cell surface generated three sensitive molecules and a resistant one by endoglycosidase H digestion (Fig. 7, lane 7) which is similar to the results from total cell immunoprecipitation in the presence of monensin (Fig. 7, lane 4).

Discussion

The pulse-labelling experiments presented here show that the 74K H protein rapidly acquired immunoreactivity with a monoclonal antibody, which is in contrast to the gradual maturation of the F protein (Ogura *et al.*, 1990). However, slow maturation of the HN protein was observed in paramyxoviruses such as Sendai virus (Mottet *et al.*, 1986), Newcastle disease virus (Nishikawa *et al.*, 1986) and mumps virus (Yamada *et al.*, 1988). The slightly increased mobility of the 74K H protein after the chase period was reproducibly observed in both the untreated and treated cells. It may be due to the removal of glucose from the *N*-linked oligosaccharides, which is transferred from dolichol to the growing H polypeptides in the RER. As the formation of H dimers was hardly affected by CCCP addition to the chase medium, it is conceivable that oligomerization of the H protein occurs before the protein leaves the RER.

The pulse-labelled 74K H protein had decreased in mobility after 60 min of chase when the 78K H protein appeared. The appearance of heterogeneity in H protein mobility during chase periods, which was thought to be due to the processing of oligosaccharide residues from the high mannose to the complex type (Graves, 1981; Bellini *et al.*, 1983; Young *et al.*, 1985; Kohama *et al.*, 1985), has also been observed in mumps virus (Yamada *et al.*, 1988). Monensin inhibited the heterogeneous mobility of the MV H protein and a similar observation was made in the case of mumps virus (Yamada *et al.*, 1988).

Both the 74K and 78K H proteins were found to contain glucosamine and galactose by [^{3}H]glucosamine and [^{3}H]galactose labelling experiments. The 78K H protein was reduced to 74K by neuraminidase treatment. These data indicate that the mobility shift occurs by the addition of sialic acid after the addition of galactose. Since monensin, which disturbs the trans-Golgi function (Tartakoff, 1983), inhibited the mobility shift, and galactosyl transferase is known to be restricted to the trans face of the Golgi complex (Griffiths *et al.*, 1982; Roth & Berger, 1982), this shift seems to take place in the trans-Golgi.

The incorporation of galactose into the MV 74K H protein was not inhibited by monensin, whereas the Newcastle disease virus HN and F_0 proteins were reported to be labelled with [^{3}H]glucosamine but not with [^{3}H]galactose in the presence of monensin in BHK cells (Yoshida *et al.*, 1986). This implies that monensin blocks different steps in the functions of the trans-Golgi in HeLa and BHK cells. This difference can be explained if the effect of monensin on Newcastle disease virus assembly is host-cell dependent (Yoshida *et al.*, 1986).

The 78K H protein, but not the 74K H protein, was detected on the cell surface of untreated cells by both cell surface immunoprecipitation and ^{125}I-labelling of the cell surface (data not shown). No cell surface expression of the H protein was observed in the presence of CCCP due to the blocking of its transport from the RER to the Golgi complex. On the other hand, under conditions where the transition of the 74K H protein to the 78K H protein was inhibited by monensin, the 74K H protein appeared on the cell surface. This 74K H protein was partially sensitive to endoglycosidase H, as was the intracellular 74K H protein when it was labelled in the presence of monensin. These data seem to suggest that although monensin blocks the addition of sialic acid, transport of the H protein to the cell surface is not disturbed in HeLa cells. In this respect, it is important to note that monensin had different effects on Newcastle disease virus assembly in BHK cells and MDBK cells (Yoshida *et al.*, 1986). In addition, Newcastle disease virus HN and F_0 proteins without galactose were not associated with the plasma membrane (Yoshida *et al.*, 1986). From these data, it is possible to conclude that galactose addition may play a necessary part in transport of the viral glycoproteins to the cell membrane, independent of the action of monensin. The 78K H protein without sialic acid seemed to be biologically inactive as judged by a haemadsorption assay (data not shown), suggesting the importance of sialic acid in the biological functions of the MV H glycoprotein.

The nucleotide sequences of cloned cDNAs corresponding to the full-length MV H mRNA indicate that five potential *N*-linked glycosylation sites are present on the MV H protein (Alkhatib & Briedis, 1986). Individual *N*-linked oligosaccharide chains of the simian virus 5 HN protein have recently been reported to have different roles in its folding, assembly and transport (Ng *et al.*,

1990). In order to understand the role of *N*-linked glycosylation of the MV H protein for its biological functions, it will be necessary to study the functional role of the individual oligosaccharide chains.

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