The Sendai virus matrix protein appears to be recruited in the cytoplasm by the viral nucleocapsid to function in viral assembly and budding

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The matrix (M) protein is viewed as the regulator of paramyxovirus particle assembly and budding. Accordingly it was observed to be mutated, and/or decreased in amount, in cases where virus particle production was significantly reduced. Here, a non-productive [non-defective and defective interfering (DI)] Sendai virus infection of COS cells is presented where virus particle production is abolished in the presence of a normal amount of intracellular M protein. In this infection the haemagglutinin–neuraminidase envelope glycoprotein is shown to be dispensable for virion production, and the fusion (F₀) envelope glycoprotein behaves as in a productive infection. The M protein is shown to accumulate in perinuclear patches within the cytoplasm. In contrast, localization in the plasma membrane is observed in productive infections. However in both productive and non-productive infections a significant fraction of M protein is found in association with cellular membranes. The M protein–membrane association is shown to take place in the absence of any other viral component, and the M protein–membrane complex exhibits properties similar to those observed for the integral membrane protein F₀. However these properties are distinct from those of the phosphoprotein, which is thought to associate with membranes in a non-specific manner. Concomitant with the cytoplasmic accumulation of M protein and the reduction of virus particle production in this non-productive infection, DI nucleocapsids are shown not to associate with cellular membrane fractions. This is a property which coincides with their poor envelopment in virus particles. Taken together, these data indicate the need for M protein to be recruited at the perinuclear membranes by the nucleocapsids to participate in viral assembly and budding. This view is consistent with a process of viral assembly taking place on internal cytoplasmic membranes rather than at the plasma membrane.

Introduction

Paramyxovirions are spherical enveloped particles. The envelope is derived from the host cell plasma membrane and surrounds the viral genome which is composed of a helical nucleocapsid containing a single-stranded RNA of negative polarity (15384 nucleotides), tightly wrapped in about 2600 units of NP protein. Also associated with the nucleocapsid are the large phosphoproteins which make up the RNA polymerase complex. Projected from the lipid envelope are two glycoproteins, the haemagglutinin–neuraminidase (HN) and fusion (F₀) proteins, which are involved in cell to cell transfer of the virions. The matrix (M) protein, located on the inner face of the envelope and organized in a leaflet structure, holds together the envelope and the nucleocapsid, probably by interacting with both the glycoproteins and the nucleocapsid. The interactions of the viral components in the virion are likely to reflect interactions during assembly.

It is generally accepted that virus particle assembly takes place at the cellular plasma membrane. The M protein, considered as the central organizer of assembly, as well as a limiting factor, forms a paracrystalline array with defined periodicity on the inner side of the cell plasma membrane (for a recent review see Peeples, 1991). The HN and F₀ glycoproteins reach the plasma membrane as homodimers or tetramers, following the constitutive secretory pathway. There they are anchored by their transmembrane cytoplasmic domain, with their ectodomain exposed in the extracellular environment. They are believed to move freely in the plane of the membrane until they come into contact with an array of M proteins. They then gather in patches from which cellular proteins are excluded (for a recent review see Morrison & Portner, 1991). Finally the viral nucleocapsid attaches to this modified region of the plasma membrane to form a structure which will eventually bud off from the cell membrane to yield a newly formed virion (for a review, see Ray et al., 1991).

This general scheme of virus particle assembly has been built up from (i) physical analysis of virus particle structure by electron microscopy or cross-linking experi-
Fig. 1. Comparison of M protein fate in BHK and COS cell infections. (a) BHK or COS cell samples were infected with ND SV virus or a mixture of ND and DI H4 viruses (SDI). Twenty hours post-infection, the cells were pulse-labelled with a $^{35}$S labelling mixture for 30 min and chased for 7 h. At the end of the pulse (P) or the chase (C), the cells were collected, and the cellular extracts immunoreacted with the Rab-vir serum ($\alpha$-SV). At the end of the chase the virus particles in the supernatants were also purified (VP). Equal samples of immunoprecipitates and virus particles were finally analysed by SDS-PAGE and autoradiographed. (b) The amounts of M protein recovered from cellular extracts in four separate experiments similar to, but not including, that described in (a). After the pulse (Mpulse) or after the chase (Mic) or in virus particles after the chase (Mvp), M protein amounts were estimated by scanning the autoradiograms. The fraction of M recovered in virus particles (Mvp/Mpulse) was then plotted as a function of the fraction of M remaining in the cells after the chase (Mic/Mpulse). (i) BHK cells infected with ND SV virus (○), or SDI (△). (ii) COS cells infected with ND SV virus (●) or SDI (◇).

Previously we have shown that the restriction of Sendai virus (SV) particle production correlates with a decrease in intracellular M protein concentration due to a faster turnover. This correlation was made in BHK cell mixed virus infections [co-infection with non-defective (ND) virus and defective interfering (DI) virus; SDI infection], as well as long-term BHK cell persistent infections (Roux & Waldvogel, 1982; Tuffereau & Roux, 1988). The data were in agreement with the model in which M protein is needed for virion budding and represents a limiting factor in this process.

Here we describe a mixed virus infection in COS cells where virus particle production is reduced markedly in the presence of normal amounts of M protein. In this case M protein is found in perinuclear aggregates, unlike the situation in productive ND virus infection where M protein is seen at the plasma membrane. However, both in the absence and in the presence of virion budding, M protein is associated with cellular membranes. This M protein–membrane association takes place in the absence of any other viral component. Finally, we demonstrate a drastic difference between ND and DI nucleocapsids in their ability to associate with cellular membranes. This ability correlates with the budding efficiency of the nucleocapsids. We propose that these data indicate the need for M protein to be recruited in the cytoplasm by
the viral nucleocapsids, to function in virion budding. In turn, this suggests a scheme of viral particle assembly which accounts for the transport of M protein and the nucleocapsids to the plasma membrane.

**Methods**

**Virus and cells.** BHK-21 and COS cells were cultured in MEM supplemented with 5% fetal calf serum (FCS) under 5% CO₂. SV ND H strain and DI H4 strain viral stocks (containing a copy-back DI RNA of about 7000 nucleotides) were infected with vTF7-3 (m.o.i. of 5) in balanced salt solution for 1 h at 37 °C. Infectious medium was then replaced with a transfection mixture. This was composed of 2 ml of MEM, 10 µg of pGem plasmid DNA and 30 µl of TransfectAce (Gibco), and was resuspended in 300 µl of ice-cold 10% (w/v) sucrose containing 10 mM-Tris HCl pH 7.5, 1 mM-EDTA (TE buffer), 2 mM-PMSF and 2% aprotinin. Cells were disrupted by Dounce homogenization (60 strokes). The cellular extracts were clarified (2 min, 1000 r.p.m.), resuspended in 300 µl of ice-cold HEN buffer, and 10% sucrose (1 part). After centrifugation (Beckman SW55 rotor, 160000 g, 18 h, 4 °C), fractions were collected and the bottom of the tubes. For immunoprecipitation, the fractions were diluted fivefold with detergents before reaction with the antibodies.

**Indirect immunofluorescence staining.** COS cells were seeded at low density on Lab-Tek 8 chamber glass slides (Miles Scientific). About 16 h later they were infected with ND virus or ND plus DI virus, or mock-infected. Thirty hours post-infection the cells were rinsed with calcium-magnesium-free PBS (PBS minus), fixed with 2% formaldehyde for 15 min, rinsed with PBS minus, and treated with acetone (−20 °C) for 3 min. After three washes with PBS minus, and one wash with 10 mM-HEPES pH 7.4, 1 mM-EDTA, 100 mM-NaCl (HEN buffer), the cells were incubated for 60 min at room temperature with normal horse serum (NHS) diluted 1:10 in HEN containing 3% BSA (HEN-BSA). The NHS was then replaced by a 1:25 dilution of α-MsDs, α-NpsDs, α-NpsDs and α-FsDs rabbit sera were raised against the proteins extracted after SDS-PAGE (Tuffereau & Roux, 1988). These data are shown in Fig. 1 (a). The amount of viral proteins synthesized during a pulse of [35S]-labelled amino acids (P lanes), and recovered virus particles (VP lanes), is shown. In contrast to the situation in BHK cells, in DI infected COS cells the amount of M protein recovered after the chase (lane C)

**Results**

In COS cells Sendai virus budding is restricted in the presence of normal amounts of M protein

Sendai virus SDI infections of BHK cells were shown to lead to severe restriction of virus particle production. Concomitantly, the M protein turned over rapidly, which significantly lowered its intracellular concentration (Tuffereau & Roux, 1988). These data are shown in Fig. 1 (a).
corresponds to that synthesized during the pulse (lane P). Despite the availability of M protein, virus particle production is abolished in COS cell SDI infection as in BHK cells (lane VP in both). This observation is reproduced in Fig. 1(b), where the results of four separate experiments, distinct from those in Fig. 1(a), are graphically presented. The absence of virion budding (Mvp/Mpulse is close to zero) correlates with a 90% reduction (by degradation, Tuffereau & Roux, 1985b) of intracellular M protein in BHK SDI-infected cells, but only with a mean 40% reduction in COS SDI-infected cells. Therefore virus particle budding is abolished in COS cell SDI infections, even though more than 60% of the M protein is still available. This M protein is found in both the phosphorylated and unphosphorylated forms (data not shown). Moreover, when reacted with monoclonal antibodies that discriminate between the two M protein isoforms (de Melo et al., 1992) no difference in the amount of the two forms is observed relative to an ND infection (data not shown).

The difference in turnover observed for M protein between BHK and COS cells does not apply to the HN glycoprotein which is largely absent in both cell types after SDI infections (Fig. 1a). This is presumably due to more rapid degradation, shown previously in BHK cells (Tuffereau et al., 1985b; Stricker & Roux, 1991).

\[ \text{F}_{0} \text{ protein is fully available and functional in the absence of virion budding} \]

In Fig. 1 poor SDS–PAGE resolution prevents any conclusion being drawn about the availability of the \( \text{F}_{0} \) protein. Therefore the experiment was repeated (Fig. 2a) using a specific anti-\( \text{F}_{0} \) protein antibody in parallel with the antiserum raised against the whole virus. There is no evidence for disappearance of the \( \text{F}_{0} \) protein in non-productive infections. Moreover Fig. 2(b) shows that \( \text{F}_{0} \) protein is efficiently expressed at the cell surface in both types of infections. The amount of \( \text{F}_{0} \) protein recovered on the cell surface with an antiserum raised against native \( \text{F}_{0} \) protein (\( \alpha_{-}\text{F}_{\text{vac}} \)) is close to that recovered after cell disruption, indicating that most of the \( \text{F}_{0} \) protein synthesized is expressed at the cell surface. To control the conditions of cell surface immunoprecipitation, an antiserum raised against the \( \text{F}_{0} \) protein cytoplasmic tail (\( \alpha_{-}\text{F}_{\text{Tail}} \)) was also used. The \( \alpha_{-}\text{F}_{\text{Tail}} \) does not precipitate \( \text{F}_{0} \) protein in cell surface reactions, although it recognizes very efficiently the protein after cell disruption. These observations demonstrate that the behaviour of the \( \text{F}_{0} \) protein in infected cells is independent of the level of virion production.

In COS cells, as in BHK cells, HN protein is not required for virus particle budding

As can be seen in Fig. 1, intracellular HN protein appears to be markedly reduced under conditions where viral budding is restricted (SDI infections), both in BHK and in COS cells. In BHK cells HN protein has been shown to be dispensable for virus particle budding (Stricker & Roux, 1991), but the requirement for HN protein in virus budding from COS cells is open to question. The budding of an SV temperature-sensitive mutant (ts 271; Portner et al., 1975), with a lesion in the gene coding for HN, was therefore investigated in COS cells. The mutant virus, when grown at its non-permissive temperature (39 °C), shows a rapid HN protein turnover before transport to the cell surface (Tuffereau et al., 1985b). COS cells were therefore infected with ts 271, and virus particle budding at 39 °C was estimated (Stricker & Roux, 1991). The production of ts 271 virions

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**Fig. 2. Fate of \( \text{F}_{0} \) protein in COS cells.** (a) Extracts of SDI and ND infected COS cells were incubated with both Rab-vir antiserum (\( \alpha_{-}\text{SV} \)) and an anti-\( \text{F}_{0} \) protein monoclonal antibody (\( \alpha_{-}\text{F}_{0} \); M38, a gift from Allen Portner) as described in the legend to Fig. 1. (b) COS cells infected as in (a) were pulse-labelled with a ^35S labelling mixture for 20 min at 24 h post-infection and then chased for 45 min. After the chase, total cell extracts (In lanes) or intact cells (Out lanes) were incubated with an antiserum raised against the \( \text{F}_{0} \) protein's cytoplasmic tail (\( \alpha_{-}\text{FT}_{\text{Tail}} \)), or with an antiserum against the native \( \text{F}_{0} \) protein (\( \alpha_{-}\text{F}_{\text{vac}} \)). The immune precipitates were analysed by SDS–PAGE and autoradiographed.
lacking HN protein did not decrease (data not shown), showing that, in COS cells as in BHK cells, HN protein appears dispensable for virion production. Therefore lack of HN protein availability in COS cell SDI infections is unlikely to account for the absence of virus particle production.

**Association of viral proteins with cellular membrane fractions**

Assembly of viral components at the cell plasma membrane is generally viewed as a step preceding virion budding. It is therefore likely that the absence of viral budding results from disruption of the assembly process, presumably due to the lack of association of some viral component(s) with cellular membranes. This association was therefore investigated, in both productive and non-productive infections, by flotation sucrose gradients. In these types of gradients, cytoplasmic extracts of the infected cells are loaded in 80% sucrose at the bottom of the gradient. The extracts are then overlaid with 65% and 10% sucrose. During centrifugation at equilibrium the cellular membranes, with their associated components, move up through the 65% sucrose and are recovered at the interface between the 65% and 10% layers. The cytosolic material forms density-dependent bands in the 80% sucrose layer. Fig. 3(a) shows the results obtained using this test for productive infections.
and Fig. 3(b) the results for non-productive infections. No significant differences were observed between the two types of infections. In both, a significant fraction of the viral components were found associated with the membrane. Surprisingly, the only minor difference observed was that a greater proportion of the M protein was found in the membrane fraction during non-productive infection (68% compared to 49%).
Localization of the M protein in non-productive infection

It is important to remember that the sucrose flotation gradients do not discriminate between plasma and intracellular membranes. To obtain more information on the actual localization of the viral proteins, immunofluorescence staining was performed. Fig. 4(a) shows that, in productive infections, M protein is found as a halo at the cell periphery. In contrast, in non-productive infections (Fig. 4b) M protein is localized internally in patches, and is particularly dense around the nucleus. These images are reproducible from cell to cell and from experiment to experiment. However the cells exhibit a morphology that is dependent on the type of infection. In productive infection, cells become round due to the cytopathic effect (Fig. 4a and d), whereas non-productive infection is non-cytopathic and is therefore associated with a flattened cell appearance (Fig. 4b and e). Different cell morphology however does not appear to be directly responsible for the different cellular localization, since in both types of infections NP protein is found in patches in the cytoplasm (Fig. 4d and e).

The viral glycoproteins and their potential role in M protein localization

Fig. 3 and 4 show that, in the non-productive infection, M protein associates with the membrane fractions but remains in the perinuclear region. In productive infection however M protein migrates to the plasma membrane. This suggests that the participation of M protein in virion budding requires its recruitment from the cytoplasm to the plasma membrane. Recent published data suggests that the viral glycoproteins, and in particular F0 protein, could be involved in the transport of M protein to the cell membrane, because F0 protein has been shown to promote M protein association with cellular membranes (Sanderson et al., 1993). In the system used here, HN protein appears dispensable for virion budding and is therefore unlikely to be the M protein recruiting partner. The similar behaviour of F0 protein in both productive and non-productive infections suggests that it may not mobilize M either. To verify this hypothesis, the potential role of F0 protein was investigated.

Membrane association of M protein in the absence or presence of F0 protein was estimated using flotation gradients. Fig. 5(a) shows that a large fraction (58%) of M protein associates with cellular membranes in the absence of any other viral partners. No demonstrable effect of F0 protein is observed (Fig. 5b), even when the F0:M molar ratio is close to 10, and under conditions where most of the F0 protein is membrane-associated. Similar results were observed in HeLa and BHK cells (data not shown). To exclude possible interference by the vaccinia virus infection used in this transfection system, M protein was also expressed using a simian virus 40-derived plasmid under the control of the cytomegalovirus promoter. Similar results were obtained (data not shown). This appears to exclude a role for F0 protein in favouring M protein association with cellular membranes, and is surprising in regard to the results of Sanderson et al. (1993). Therefore, the properties of the M protein–membrane complex were further investigated in parallel with those of the F0 protein–membrane complex (F0 is an integral membrane protein), and those of the P protein–membrane complex [about 30% of P protein is found in the membrane fraction when expressed alone (data not shown), but this association is predicted to be non-specific]. Fig. 6 shows that the M
Fig. 6. Properties of the viral protein-membrane complex. Four hours post-transfection, 4 x 10^7 COS cells transfected with 10 μg of pGem4-SVP/C, pGem4-SVF0 or pGem4-SVM were labelled with a ^35S labelling mixture for 18 h. Cytoplasmic extracts were then prepared and sedimented through flotation sucrose gradients (Beckman SW41 rotor). Fractions were collected and those containing membranes (top fractions 8 to 11) were pooled, diluted with TE and centrifuged (SW41 rotor, 1 h, 35 000 r.p.m., 4 °C) to pellet the membranes. Pellets were resuspended in TE and divided into five identical samples (equivalent to 8 x 10^6 cells). These samples were (i) mock-treated, (ii) resuspended in 2 M-KCl, 10 mM-Tris-HCl pH 7.4 and incubated for 1 h at 25 °C, (iii) resuspended in TE and incubated for 1 h at 25 °C, (iv) resuspended in 100 mM-sodium carbonate pH 11.0 and incubated at 0 °C for 30 min or (v) resuspended in 1% Triton X-114, 150 mM-NaCl, 10 mM-Tris-HCl pH 7.4 (see b). After incubation, samples (i) to (iv) were adjusted to 80% sucrose and recentrifuged in flotation sucrose gradients (SW55 Beckman rotor). (a) The four bottom and four top fractions were pooled, diluted with detergent and divided into three. These were incubated with either α-Psvs (P), α-Fsv (F0) or Msv (M) to estimate the fraction sedimenting as free protein (F) or as membrane-associated protein (M) respectively. These fractions were given as percentages below each column. (b) Sample (v) was partitioned into aqueous (A) or detergent (D) phases by warming at 37 °C followed by freezing according to Bordier (1981). Both phases were finally adjusted to the same salt and detergent concentrations. These were divided into protein–membrane complex exhibits the same properties as the F0 protein–membrane complex. These are drastically different from the properties exhibited by the P protein–membrane complex. The majority of the complexed M protein (80%) is resistant to high salt (2 m-KCl) and to EDTA treatment (Fig. 6a). Only base treatment (pH 11-0) substantially disrupts the complex. However this result is difficult to interpret because the pH conditions approach the isoelectric point of M protein (pH 10.2), which may affect the solubility of the protein. In Fig. 6(b), partitioning of the proteins between detergent and aqueous phases shows again that M protein behaves like F0 protein, and partitions in the detergent phase, whereas P protein is found mainly in the aqueous phase.

**Differential association of defective and non-defective viral nucleocapsids with cellular membranes**

SDI infections coincide with a massive preferential replication of DI nucleocapsids over ND nucleocapsids (Roux & Waldvogel, 1981). In BHK cells this replicative advantage of DI nucleocapsids was postulated to be responsible for disruption of the budding process, resulting in rapid HN and M protein turnover and intracytoplasmic accumulation of DI nucleocapsids. It was claimed that DI nucleocapsids were unable to stabilize the prebudding structure assembled at the plasma membrane (Tuffereau & Roux, 1988). In COS cells the actual data also point to a lack of viral assembly at the plasma membrane, since M protein accumulates in perinuclear regions of the cytoplasm. This may indicate that DI nucleocapsids never assemble with plasma membranes. Such a prediction was verified when the association of viral nucleocapsids with membranes was investigated (Fig. 7). About 20% of the DI nucleocapsids were found in the membrane fractions (Fig. 7a, DI H4-RNA), in contrast to over 90% of the ND nucleocapsids (Fig. 7a, SV-RNA). In Fig. 7(b), the bottom and top fractions of flotation gradients are pooled to facilitate the presentation of more samples, including different DI nucleocapsids. The results were similar. Interestingly, DI nucleocapsids of only half the size of ND nucleocapsids (DEL-7 samples, DEL-7 RNAs) behave similarly to DI H4 nucleocapsids, which are one-tenth the size of ND nucleocapsids. Moreover, membrane association of ND nucleocapsids takes place under conditions where none is observed for DI nucleocapsids (see SV percentage in
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Fig. 7. Association of viral nucleocapsids with cellular membranes. (a) Samples of 10⁷ COS cells were infected with ND (St) or SDI virus. Forty hours post-infection, the cells were collected, cytoplasmic extracts prepared and fractionated through flotation sucrose gradients (SW55 Beckman rotor). The collected fractions (450 μl) were diluted to 4 ml with TE and spun for 2 h (SW60 rotor, 50000 r.p.m., 4 °C). The pellets were resuspended in TE containing 0.2% sarkosyl and 10 μg of yeast tRNA, then phenol-extracted and ethanol-precipitated. The precipitates were resuspended in formamide EDTA and analysed by agarose formaldehyde gel electrophoresis followed by Northern blotting (see Methods). SV-RNA and DI H4-RNA refer to viral RNAs present in ND and DI nucleocapsids respectively. (b) The same analysis was performed as in (a) with two modifications. Firstly analysis of mock and DEL-7 DI virus-infected cells was also performed. Secondly the bottom (1 to 4) and top (8 to 11) fractions of the flotation gradients were pooled to estimate the amount of free (B) and membrane-associated (T) nucleocapsid RNAs. Note that analysis of the Northern blot by a PhosphorImager allowed monitoring of the amounts of SV- and DI RNAs in each fraction. This is presented below the corresponding lane.

Table 1. Discrimination against DI RNAs in the budding process

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* BHK or COS cells were infected with either a mixture of ND and DEL-7 viruses or a mixture of ND and DI H4 viruses. Twenty-four hours after infection, the viral RNAs present in intracellular viral nucleocapsids (IC) and in virus particles shed into the medium (VP) were purified. After separation by agarose gel electrophoresis, the relative representation (molar ratio) of ND and DI RNAs in each sample was estimated by Northern blotting and PhosphorImager scanning as described in Methods.

† RF, Restriction factor. This expresses the budding restriction of the DI RNAs relative to that of the ND RNA in the same sample. The value is obtained by dividing the intracellular DI:ND RNA molar ratio by that measured in the virus particles (IC:VP).

Lack of nucleocapsid membrane association correlates with budding restriction

The restriction of virion production from mixed virus-infected BHK cells was previously shown to reflect mainly a restriction of DI nucleocapsid envelopment relative to ND nucleocapsid. This indicates that, in BHK cells under conditions of limited virion production, the budding process discriminates between the two types of nucleocapsids (Mottet & Roux, 1989). The same is true in COS cells (Table 1). DI DEL-7 and DI H4 nucleocapsids are restricted in budding relative to ND nucleocapsids by 16- and 15-fold, respectively. In consequence, the degree of nucleocapsid association with cellular membranes appears to correlate directly with budding efficiency. Therefore, nucleocapsid–membrane association is a useful means of evaluating the assembly and budding potential of nucleocapsids. Differences in the range of the budding restriction factors are observed between BHK and COS cells, particularly for DI H4 RNAs. This reflects the efficiency of replication of the DI RNAs in the two cell types. It was indeed shown that the extent of budding restriction of DI nucleocapsids was associated virus particles. Although such particles would be found at the top of the flotation gradients, these would account for only a minor fraction of the nucleocapsids, since in some cell lines less than 10% of the total nucleocapsids are ever found in virus particles (Tuffereau & Roux, 1988). Indeed, Fig. 4(d) supports this conclusion, since the NP staining at the cell periphery (which would reflect cell-associated virus) is minimal compared to the amount of intracytoplasmic NP staining.
directly proportional to their efficiency of replication (Mottet & Roux, 1989).

Discussion

Consistent with its role as the central organizer of virion assembly and budding, M protein has been found to be decreased in amount, or has been shown (or assumed) to be mutated, under conditions of decreased virion production (Roux & Waldvogel, 1982; Hall & Choppin, 1979; Haase et al., 1985; Cattaneo et al., 1986, 1987; Johnson et al., 1981; Wechsler et al., 1979; Young et al., 1985; Wild & Bijlenga, 1981; Tuffereau et al., 1985a).

This supports the notion that M protein is the limiting factor for virus particle production. SV SDI infection in COS cells represents a novel situation where virion production is restricted in the presence of a normal amount of M protein. This situation probably arises because the COS cell proteolytic system does not process the M protein as rapidly as that of BHK cells. In the absence of virion budding M protein tends to accumulate in perinuclear regions, mainly in association with membranes. This suggests that for active participation in assembly and budding M protein has to be recruited by factors that are not effective in SDI infections. Recent published data suggest that M protein association with cellular membranes and its cellular localization correlate with the F₀ protein localization (Sanderson et al., 1993), indicating that the viral glycoproteins could be the recruiting partners. However in the infection system used here the HN glycoprotein has been shown to be dispensable for viral budding. On the other hand, the F₀ glycoprotein behaves similarly in conditions of productive and non-productive infections and therefore appears unable to modulate the budding process. However, we observed that DI nucleocapsids, in contrast to ND nucleocapsids, associate poorly with cellular membranes and this parallels M protein cytoplasmic accumulation and restriction of viral budding. Therefore ND nucleocapsids, but not DI nucleocapsids, could represent the viral partner that recruits M protein on internal membranes to bring it to the plasma membrane.

Our experiments show that a large fraction of M protein binds to membranes in the absence of any other viral component. These results differ from those of Sanderson et al. (1993), who reported that M protein associates with membranes only in the presence of F₀ protein. The reason for this discrepancy is not clear. It was, however, sufficiently disturbing to require a detailed analysis of the M protein–membrane complex to rule out a possible artefact. The complex was observed in different cell types (CV1, COS, HeLa, BHK cells) using different expression systems (vaccinia virus or Simian virus 40 vectors). The complex was found to be resistant to high salt and to EDTA treatment, and the membrane-associated M protein was shown to partition in Triton X-114. These results demonstrate that, under our experimental conditions, the M protein association with the membrane fractions obeys the criteria used to define specific protein–membrane interactions.

On the other hand, the association of M and F₀ proteins during the intracellular transit proposed by Sanderson et al. (1993) is compatible with our results if a third partner, the nucleocapsid, is introduced into the complex. Together the results of both studies suggest a scheme of viral assembly where M protein associates with intracytoplasmic membranes. There it interacts with both the nucleocapsid and the glycoproteins, at a stage of glycoprotein maturation after the point blocked by monensin which prevents M protein–glycoprotein association (Yoshida et al., 1986; Sanderson et al., 1993). This complex would then travel to the plasma membrane on constitutive secretory vesicles which would eventually fuse with the plasma membrane. The present data suggest that ND nucleocapsids are required for the complex to be stable, and that F₀ protein is needed for adequate delivery of the complex (Sanderson et al., 1993). This role of F₀ protein in assembly is in agreement with another observation. In cases of defective measles virus budding, such as in subacute sclerosing panencephalitis, not only is its M protein mutated but also the cytoplasmic portion of its F protein frequently exhibits alterations (point mutations, frameshift mutations, deletions; Cattaneo & Rose, 1993). Similar alterations are never observed on the corresponding region of the H protein.

This scheme of viral assembly would account for the transport to the plasma membrane of M protein and nucleocapsids, as well as the glycoproteins. It predicts the existence of secretory vesicles containing F₀ proteins with their cytoplasmic tails protruding to contact M protein, which in turn interacts with a nucleocapsid. It also predicts that the stable attachment of ND nucleocapsids to membranes depends on the presence of both M and F₀ proteins. Attempts to verify such predictions are under way.

This assembly process agrees with the observation that, in polarized cells, the site of virus budding corresponds to the site of surface expression of viral glycoproteins (Rodriguez-Boulan & Pendergast, 1980; Roth et al., 1983). As the information for protein sorting is contained within the glycoprotein structure (Roth et al., 1983; Jones et al., 1985; Stephens et al., 1986; Puddington et al., 1987), polarized budding can be viewed simply as the result of transport of M protein and nucleocapsids orchestrated by the glycoprotein.

The drastic difference in binding to membranes exhibited by DI and ND nucleocapsids is important in
that it suggests a specific mechanism for the efficient and stable interaction of the two components. This cannot simply be a matter of nucleocapsid length, since DEL-7 nucleocapsids (half the size of ND nucleocapsids) bind as poorly as do DI H4 nucleocapsids (one-tenth the size of ND nucleocapsids). Neither can it be related to RNA end sequences, since DEL-7 and ND RNAs share the same 3' and 5' ends (Mottet & Roux, 1989). Now that replication and envelopment of an SV DI RNA expressed from plasmid DNA is feasible (Calain et al., 1992; Calain & Roux, 1993), the investigation of a putative internal RNA sequence conferring efficient and stable association of nucleocapsids with the membrane fraction is open to experimentation. The issue is relevant in that nucleocapsid association with membranes, as measured by flotation gradients, appears to be a valid test for budding efficiency.

In conclusion, this paper provides data which support a scheme of paramyxovirus assembly and budding in which assembly takes place on intracytoplasmic membranes. This model accounts for transport of M protein and nucleocapsids to the plasma membrane, a step in viral assembly which still remains somewhat obscure, and has lead to testable prediction.

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