The Intracellular Distribution of Influenza Virus Matrix Protein and Nucleoprotein in Infected Cells and Their Relationship to Haemagglutinin in the Plasma Membrane

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SUMMARY

Pre- and post-embedding immune electron microscopy techniques employing ferritin and large and small gold markers to detect cell surface and intracellular antigens respectively, have been combined in a study of influenza virus-infected cells. This has permitted, for the first time, the simultaneous detection of intracellular virus matrix protein (M), nucleoprotein (NP) and membrane haemagglutinin (HA). The technique facilitated an investigation of the possible physical interrelationship between these three proteins both in the infected cell, and on the infected cell membrane. Electron-dense bodies uniformly labelled by antibody to M protein were observed in the nucleus and cytoplasm. Similarly, NP was detected in both the nucleus and cytoplasm. Approximately 50% of the nuclear NP was located in close proximity to the M protein-containing dense bodies but mainly on the perimeter of the structures. A similar relationship of NP to the M-containing dense bodies was observed in the cytoplasm. M protein and NP were readily detected in sections of budding virions. Labelling of these proteins was also observed on the cytoplasmic face of the plasma membrane but the density of labelling only occasionally approached that of newly formed virions. These findings suggest that budding occurs very quickly after the internal proteins arrive at the plasma membrane. Double labelling experiments on the cell surface indicate that NP and HA behave as independent molecules and do not form tight complexes with each other.

INTRODUCTION

Investigations into the distribution of influenza virus nucleoprotein (NP) and matrix protein (M) in infected cells have relied mainly on immunofluorescence and cell fractionation techniques. There is general agreement that NP accumulates in the nucleus (Watson & Coons, 1954; Liu, 1955; Taylor et al., 1969; Lazarowitz et al., 1971; Flawith & Dimmock, 1979) and also appears at a later stage on the cell surface (Virelizier et al., 1977) where it can serve as a target antigen for cytotoxic T cells (Townsend et al., 1984a, b; Yewdell et al., 1985). The precise intracellular location of M is more controversial, with some reports claiming that it can be found in both the nucleus and the cytoplasm (Gregoriades, 1973; Oxford & Schild, 1975; Hay & Skehel, 1975) whilst others suggest that it is absent from the nucleus (Lazarowitz et al., 1971; Krug & Etkind, 1973). It is not clear whether these inconsistencies reflect variation with different virus strains and cells or whether, in investigations employing cell fractionation procedures, there is cytoplasmic contamination of the nuclear preparations. In the present study, by employing ultrastructural immunolabelling techniques, the nuclear accumulation of M protein is now clearly demonstrated.

The mechanism of budding of enveloped viruses has been reviewed by Simons & Garoff.
For influenza virus budding, both the M protein and the ribonucleoprotein complex (RNP) must migrate to the cytoplasmic face of the plasma membrane and locate the two virus-coded glycoproteins, haemagglutinin (HA) and neuraminidase (NA). It is not known whether these internal virus components migrate independently or form complexes before locating the viral glycoprotein. In the present investigations a new approach to this problem has been adopted in which the intracellular viral proteins M and NP, and the plasma membrane-associated HA have been detected simultaneously in infected cells. This was achieved by immune electron microscopy employing three distinct markers, namely ferritin and small and large colloidal gold particles. The data obtained suggest that M and NP form a 'loose' association in the nucleus and cytoplasm and that virus budding occurs rapidly after assembly of internal virion components on the inner face of the plasma membrane.

**METHODS**

**Virus.** The influenza A reassortant virus X-47 [parental strains A/PR/8/34 (H1N1) and A/Victoria/3/75 (H3N2)] and A/PR/8/34 were grown in embryonated eggs (Dowdle & Schild, 1975).

**Reagents.** Antisera against purified HA and M proteins were prepared in goats. Viral NP was detected in fixed cells with a rabbit anti-NP serum (Schild et al., 1979). Cell surface NP of unfixed preparations was detected using a mixture of two mouse monoclonal anti-NP antibodies. A monoclonal antibody (MAb) to M was supplied by W. Gerhard (Wistar Institute, Philadelphia, Pa., U.S.A.). Rabbit anti-goat Ig was conjugated to ferritin by a one-step coupling procedure using glutaraldehyde as described by De Petris & Raff (1972). Colloidal gold (15 to 20 nm) was prepared by the reduction of chloroauric acid with sodium citrate (Frens, 1973). Chloroauric acid was reduced with a combination of tannic acid and sodium citrate to produce 5 nm colloidal gold (Mulpfordt, 1982). The amount of Protein A (Pharmacia) required for the complete stabilization of the colloidal gold was determined according to the procedure of Horrisberger & Rosset (1977) and conjugated following the method described by Slot & Geuze (1981).

**Synthesis of HA.** The time of appearance of HA on the infected cell plasma membrane was monitored by haemadsorption with 0-5% guinea-pig erythrocytes. Additionally, pulse-labelling studies were performed to detect intracellular synthesis of the HA, NP, non-structural (NS1) and M polypeptides (Patterson et al., 1979).

**Cells and infection procedure for immune electron microscopy.** Madin–Darby canine kidney (MDCK) cells were cultured on 5 cm diameter Petri dishes in Eagle's MEM containing 4% tryptose phosphate broth, 10% newborn calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Confluent cell monolayers were adsorbed with virus (approximately 100 p.f.u./cell) for 1 h on ice, overlaid with warm medium and incubated at 37 °C for 1, 2, 3, 4, 5, 6, 7 or 17 h. Both infected and uninfected cells were fixed for 1 h at 20 °C with 1% glutaraldehyde in 0-1 M-cacodylate buffer pH 7-2 containing 5% sucrose. Fixed cells were washed in buffer and treated with 0-5 M-ammonium chloride for 4 h at 20 °C before immunolabelling. Plasma membrane-associated virus HA was detected with a goat anti-HA serum and visualized by labelling with a ferritin-conjugated rabbit anti-goat Ig reagent. Samples were then dehydrated and embedded in Lowicryl K4M as described by Bendayan & Orstavik (1982). Ultrathin sections were cut, mounted on Formvar-coated copper grids, and screened for cytoplasmic viral antigens by immunogold labelling.

For single gold labelling experiments grids were preincubated in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 5 min followed by 2 h with antibody diluted in PBS containing 5% BSA. The dilution of antibody that gave maximum specific labelling with the minimum of non-specific labelled background was determined for each reagent. After five 5 min washings by floating grids on drops of PBS/BSA the site of antibody binding was detected by incubating with a 15 to 20 nm colloidal gold–Protein A conjugate. The above procedure was employed in the initial step of double immunogold labelling experiments except that the first antibody was detected with a 5 nm colloidal gold–Protein A conjugate. Before labelling with the second antibody, sections were incubated with free Protein A (100 mg/ml) for 1 h to block any unoccupied Protein A-binding sites on the first antibody. The second antibody was then applied and detected with the larger Protein A-gold conjugate as described above. Control experiments in which the second antibody was omitted or substituted for an antibody that does not react with cytoplasmic components in infected cells were carried out in parallel. Controls for the specificity of labelling included uninfected cells and infected cells to which cycloheximide (100 μg/ml) was added after 90 min incubation at 37 °C.

Some experiments were designed to look for possible physical interrelationships between membrane HA and NP expressed at the cell surface. At 5-5 h post-infection (p.i.) cells were incubated for 30 min on ice with a mixture of two anti-NP MAbS in the presence of sodium azide (10-2 m) and binding was subsequently detected with a 5 nm colloidal gold Protein A reagent. They were then fixed in buffered 1% glutaraldehyde as previously described, washed and incubated in normal rabbit serum overnight. HA was detected with goat anti-HA and a ferritin-
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Conjugated rabbit anti-goat Ig. Controls were included which omitted the goat antibody. These cells were post-fixed in osmium tetroxide, dehydrated and embedded in Spurr's resin.

Additional experiments were performed using Vero cells and the A/PR/8/34 (H1N1) strain of influenza virus since this system was studied in an earlier immunoelectron microscopy investigation (Beesley & Campbell, 1984).

RESULTS

By haemadsorption HA was first detected on the membrane at 4 h p.i. Pulse labelling techniques with [35S]methionine also detected HA first at 4 h. Maximum production of HA, NP and M polypeptides was noted at 7 h. By 5 h p.i. approximately 90% of the cells had HA on the plasma membrane as determined by immunoferritin labelling.

Electron microscopy

Intracellular viral antigens were detected by immunogold labelling of ultrathin sections of influenza virus-infected cells embedded in Lowicryl K4M resin. In this technique only those antigenic determinants which are exposed at the surface of the section are accessible to the antibody reagents (Bendayan, 1984). Thus some viral structures seen in sections are unlabelled because they lie below the section surface. To provide an indication of the sensitivity of the procedure, counts were made on the number of newly formed particles labelled with the two anti-M protein antibodies. The monoclonal and polyclonal reagents labelled 63% and 78% of the extracellular virus respectively. The density of labelling with the polyclonal antibody was generally higher than that for the MAb.

X-47-infected MDCK cells

Nuclear viral antigens

Virus-infected cells were fixed at various times up to 17 h p.i. and ultrathin sections of the cells were immunolabelled to detect M and NP. In experiments to monitor the possibility that input virus was being detected, infected cultures were treated with cycloheximide (100 μg/ml) 90 min after warming to 37 °C. Neither drug-treated nor uninfected cells (Fig. 1) were labelled by the

![Fig. 1. Uninfected MDCK cell labelled with an anti-M MAb and 15 to 20 nm Protein A–gold conjugate. Very little non-specific binding is observed. Nu indicates nucleolus, Nm the nuclear membrane and C the cytoplasm. Bar marker represents 1 μm.](image-url)
immunogold reagents. Additional controls in which sections of infected cells were labelled with either normal serum or an antiserum raised against an unrelated antigen were also negative. Labelled M protein was detected at 4 h p.i. in the nucleus and was specifically associated with electron-dense structures found in infected but not uninfected cells. Only very rarely was the gold label observed in the more electron-lucent areas of the nucleus. The quantity of M protein increased with time to a maximum at 17 h p.i. but its distribution in the nucleus was unchanged (Fig. 2a).
Fig. 3. A higher power micrograph of the nucleus of an influenza virus-infected MDCK cell at 17 h p.i. It is labelled with antibodies to M (monoclonal) and NP, whose binding is shown by the distribution of 5 and 15 to 20 nm gold particles respectively. Nm indicates the nuclear membrane and C the cytoplasm. Control experiments showed that less than 5% of the large Protein A-gold conjugate was bound to the anti M-reagent. Bar marker represents 200 nm.

Substantially less NP than M was detected throughout the course of the infection but the time of its appearance closely paralleled that of M. However, the distribution of NP and M in the nucleus of the infected cell was different (Fig. 2b). Approximately 50% of the NP was associated with the M-positive, electron-dense bodies whereas the remainder was distributed in the electron-lucent areas of the nucleus. NP associated with dense bodies was mainly located at their peripheries. M labelling showed a more uniform distribution over the whole structure. Neither M nor NP were found closely associated with the nuclear membrane. A low level of NP labelling was observed in the nucleolus, particularly after 17 h p.i. M protein, on the other hand, was not detected in the nucleolus at any time. The differential intranuclear distribution of the two proteins is clearly illustrated in double labelling experiments employing two different sizes of colloidal gold (Fig. 3).

Cytoplasmic viral antigens

M and NP were first detected in the cytoplasm at 4 h p.i. and increasing amounts were detected at later times with a maximum at 17 h p.i. Both proteins were frequently associated with electron-dense structures and, as was observed in the nucleus, NP was again located predominantly at the edge of these bodies (Fig. 4b). The majority of the antigens were distributed in the cytoplasm on the apical side of the nucleus and only rarely were they detected in the cytoplasm on the basal side of the cell. The proportion of NP that was located within close proximity to M was approximately the same as was observed in the nucleus.

Association of internal viral NP and M antigens with HA glycoproteins on the cell surface

HA on the plasma membrane was first detected at 4 h by immunoferritin labelling. Label was distributed along the plasma membrane (Fig. 5a) but virus in the process of budding was only rarely observed. By 5 h, when approximately 90% of the cells were labelled for membrane HA,
Fig. 4. Influenza virus-infected MDCK cells at 17 h p.i. with the distribution of M and NP shown by 5 and 15 to 20 nm gold particles respectively. (a) Well labelled newly budded virions (arrowheads) whereas only small amounts of M (labelled with MAb) and NP are detected immediately under the plasma membrane (Pm). (b) Well labelled newly formed virions (arrows) and little M (labelled with MAb) and NP immediately under the membrane but in addition this panel shows that deeper in the cytoplasm M and NP are readily detected. (c) Labelling of M with polyclonal antibody and 15 to 20 nm gold particles. Virus particles are well labelled while cytoplasm underlying the plasma membrane (arrows) is only sparsely labelled. Bar markers represent 200 nm.
virus budding, frequently as elongated rather than as rounded particles, was readily detectable. The HA was now more frequently seen in patches, usually within the vicinity of budding virions; this was in contrast to the more random distribution over the whole membrane observed at earlier times (Fig. 5b).

Budding of enveloped viruses is believed to be triggered by the assembly of internal virion components on the cytoplasmic face of the plasma membrane which contains virus-coded glycoproteins (Simons & Garoff, 1980) although no details have been reported previously for influenza virus-infected cells. Therefore, extensive searches for M and NP proteins, either alone or as a complex detected by double labelling, on the cytoplasmic side of the plasma membrane were made. At 5, 6 and 7 h p.i. when the budding process was well under way these proteins were detected under the plasma membrane but the density of label was usually less than that observed for newly formed viruses. Furthermore, cytoplasm immediately underlying stretches of plasma membrane that contained newly synthesized HA, detected by immunoferritin labelling, were often totally devoid of M and NP (Fig. 5). Even at 17 h p.i. when there was very extensive budding and dense labelling for nuclear and cytoplasmic M and NP, there was no evidence that large amounts of these proteins accumulated under the plasma membrane (Fig. 4). Extensive searching did reveal a few cells with increased amounts of M protein and NP lying immediately beneath ferritin-labelled HA in the plasma membrane (Fig. 6). In one of these cells (Fig. 6a)
Fig. 6. Two influenza virus-infected cells at 5 h p.i. These micrographs show relatively rare examples of cells showing both NP and M proteins immediately under the HA-containing membrane. (a) Electron-dense material containing M and NP, labelled with 5 and 15 to 20 nm gold respectively, is shown underlying plasma membrane with ferritin-labelled HA (arrows) and budding virions (V). C indicates cytoplasm. (b) M protein and NP underlying ferritin-labelled HA in the plasma membrane. Bar markers represent 200 nm.

electron dense M- and NP-containing material, which was commonly observed deeper in the cytoplasm, was positioned directly under the plasma membrane. Overall, however, there was little evidence for an accumulation of large concentrations of either protein on the cytoplasmic face of the plasma membrane. In contrast, and as expected, M and NP were readily detected in newly budded virions or virus in the process of budding (Fig. 4).

To look for a possible association between the influenza HA and NP molecules on the surface of infected cells, double pre-embedding labelling experiments were performed at 5-5 h p.i. At this time after infection both NP and membrane HA were readily detected on the cell surface
but there was significantly more HA than NP. In addition, relative to HA, the NP appeared to be randomly distributed over the cell surface and, therefore, not physically linked to HA (Fig. 7). Although some gold-labelled NP did appear in close proximity to ferritin-labelled HA, NP was very frequently located in areas of the plasma membrane devoid of HA and thus there was no strong evidence for the formation of HA–NP complexes.

**A/PR/8/34-infected Vero cells**

Cells infected for 5, 7 and 17 h were labelled with the polyclonal anti-M protein antibody and M was again detected in the nucleus. The relative distribution of M labelling under the plasma membrane and in newly formed virions was similar to that observed in X-47-infected MDCK cells. Only rarely were accumulations of M protein detected on the cytoplasmic face of the plasma membrane (Fig. 8a). At sites of budding low levels of gold label under the plasma membrane were observed (Fig. 8b), but this was no more frequent than areas of budding in which no M was detectable (Fig. 8c).

**DISCUSSION**

There are conflicting reports in the literature concerning the localization of influenza virus M protein in infected cells (Gregoriades, 1973; Oxford & Schild, 1975; Hay & Skehel, 1975; Lazarowitz et al., 1971). The apparent presence of M in the nucleus has been attributed by some workers to cytoplasmic contamination of the nuclear fractions during the fractionation procedure or to the binding of M to the nuclear membrane. However, immune electron microscopy employed in the present investigations shows unequivocally that M accumulates in the nucleus of MDCK and Vero cells infected with the X-47 reassortant and A/PR/8/34 strains of influenza virus respectively. This is in agreement with the recent investigations of Beesley & Campbell (1984) who employed immunoelectron microscopy on ultrathin cryosections and also located M in the nucleus. In studies of xenopus oocytes injected with mRNA from influenza virus-infected cells Davey et al. (1985a) showed that M could be pelleted from nuclear lysates by centrifugation for 5 min at 12000 g and suggested this may be due to M binding to the outer face of the nuclear envelope. Our investigations suggest that this is not the case, as M protein was
Fig. 8. The plasma membranes of Vero cells 17 h after infection with the A/PR/8/34 strain of influenza virus. M protein is detected by labelling with goat antiserum followed by 15 to 20 nm colloidal gold-Protein A conjugate. The micrographs show (a) a relatively scarce dense accumulation of M, (b) sparsely distributed label and (c) no label under membrane in which there is budding virus. Arrows indicate plasma membrane. Bar markers represent 200 nm.

never found in close association with the nuclear membrane, but that its precipitation by relatively low speed centrifugation could rather reflect its accumulation in the nucleus as large dense structures.

In contrast to M the presence of NP in the nucleus of infected cells has been well documented using a number of techniques (Watson & Coons, 1954; Liu, 1955; Taylor et al., 1969; Lazarowitz et al., 1971; Flawith & Dimmock, 1979). However, in the present study using readily differentiated markers of small and large gold particles, we were able to investigate for the first
time the possible associations between M and NP in undisrupted infected cells. Approximately 50% of nuclear NP was found in close proximity to the electron-dense M protein-containing structures whilst the remainder was distributed in the electron-lucent areas. M protein, on the other hand, was almost exclusively associated with the dense structures. Analyses of influenza virus RNPs prepared from infected cells have shown that they are composed of NP and M (Rees & Dimmock, 1981) and this thus provides further evidence that these two proteins can form cellular complexes. It is not known whether viral RNA is present within the dense nuclear structures. However, the technology to answer this question is now available since in situ hybridization can be performed on Lowicryl sections (Binder et al., 1986).

Experiments employing vaccinia virus vectors to introduce single influenza virus genes into cells have shown that, in the absence of other virus-coded proteins, M is distributed throughout the cell whilst NP is concentrated in the nucleus (Smith et al., 1987). These observations can be explained by transfection studies employing deletion mutants of cloned NP which show that amino acids 327 to 345 are responsible for the nuclear accumulation of NP (Davey et al., 1985b). The dense NP- and M-containing complexes that we observed in both the cytoplasm and nucleus suggest that the cellular distribution of NP may be modified by its association with M.

Mature influenza virus particles form by budding through the plasma membrane (Bächli, 1969; Compans & Dimmock, 1969). Thus it would be anticipated that the internal virion components would be assembled on the cytoplasmic face of the plasma membrane containing the viral HA and NA glycoproteins. Indeed, immunoferritin labelling studies on paramyxovirus-infected cells have shown that nucleocapsids associate only with membrane containing a patch of viral glycoproteins (Choppin et al., 1971; Lenard & Compans, 1974). Similarly for influenza virus, fractionation studies on virus-infected cells have shown that M protein specifically co-fractionates with plasma membrane containing viral HA but is absent from cell membrane fractions lacking HA (Hay, 1974). The M protein appears to be essential for budding since studies of cells that can support only an abortive infection of influenza virus have shown that there is a close and specific correlation between reduction of budding and a lowering of M synthesis (Lohmeyer et al., 1979).

The present study has examined the distribution of cytoplasmic M and NP in relation to HA in the plasma membrane. Surprisingly, although small amounts of M and NP were found on the cytoplasmic face of membrane containing viral HA, the degree of labelling rarely approached the levels associated with newly budded particles. Preparations were examined at 5 h, a time at which a significant degree of virus budding was first apparent, and at 7 h when the process was well under way and maximum production of virus polypeptides was observed. In addition a late time in infection was included, 17 h, when there were large intracellular accumulations of virus proteins and extensive virus budding. Qualitatively similar results regarding the distribution of M protein and NP were obtained at these different stages in the infection cycle. Even late in infection when there were high concentrations of the two proteins in both nucleus and cytoplasm, probably in excess of the amounts required for the production of mature virions, there was still no evidence of their accumulation on the cytoplasmic face of the plasma membrane.

The difficulty in detecting M under the plasma membrane calls into question the sensitivity of the labelling procedure. This problem has recently been addressed by Griffiths & Hoppeler (1986) who determined the efficiency of labelling of Semliki Forest virus antigens in infected BHK cells. For sections of Lowicryl-embedded material, efficiencies of 18.4%, 6.6% and 1.2% were obtained for the detection of virus glycoprotein in the endoplasmic reticulum, Golgi apparatus and virus respectively. It was suggested that at sites of high antigen concentration, such as in mature virus particles, the efficiency of detection is reduced due to steric hindrance of the binding reagents. The level of detection is higher than may at first be expected and probably reflects the fact that the surface of a Lowicryl section is not smooth but shows specimen-related relief of 2 to 6 nm (Kellenberger et al., 1987). These observations, together with the present finding that up to 78% of the external virions are labelled by the anti-M protein antibodies, suggest that the relative scarcity of densely labelled M under the membrane is not merely a reflection of the sensitivity of the system. To explain our results we propose that budding occurs
very rapidly after the appropriate amounts of M and RNP have bound to plasma membrane containing the viral glycoproteins.

The situation with influenza virus contrasts with that seen in cells infected with paramyxoviruses, in which nucleocapsids lying under the plasma membrane can be readily detected (Choppin et al., 1971). Our ferritin labelling experiments indicate that HA molecules become aggregated into patches in the membrane. The data of Lohmeyer et al. (1979) suggest that this may be mediated by internal proteins, possibly M protein. The scarcity of any significant levels of gold label immediately under membrane containing HA would suggest that only very small amounts of the internal proteins are required to mediate HA patching.

Double labelling studies using small and large gold particles provided definitive evidence for the first time for the co-localization of NP and M in the nucleus and cytoplasm. After extensive searching a small number of cells were found that showed double labelling of M protein and NP directly under areas of plasma membrane containing HA. The finding of electron-dense material containing M protein and NP under the membrane leaves open the hypothesis that M and RNP form complexes in the cytoplasm which then migrate to the plasma membrane. However, more data are required before these ideas can be substantiated. If M and RNP do migrate to the cell surface independently, then the lag period between the binding of the two must be relatively short.

Beesley & Campbell (1984) have reported that M protein is localized in the plasma membrane of Vero cells 24 h after infection with the A/PR/8/34 strain of influenza virus. However, surprisingly, they were unable to detect M at 16 and 48 h. Employing the same strain of virus and cell type we have not observed extensive membrane labelling of M protein at 5, 7 or 17 h. Close examination of the micrographs published by Beesley & Campbell indicates that some of their labelled M protein may be on the external face of the plasma membrane. Small amounts of cell surface M protein have been detected previously at 6.5 h p.i. (Yewdell et al., 1981). The difference in the pictures obtained by immune electron microscopy at 16 or 17 h p.i. and 24 h p.i. may reflect either a sharp rise in the rate of export of M to the cell surface or secondary adsorption of the protein from disrupted cells or virions. Further work is needed to resolve this question satisfactorily.

The presence of NP on the cell surface, first located using immunofluorescence (Virelizier et al., 1977), is confirmed by immune electron microscopy in the present study. The recognition of NP by cytotoxic T cells may have important implications for cross-reactive immunity (Townsend et al., 1984a, b; Yewdell et al., 1985). The protein has no recognizable leader sequence at the NH₂ terminus (Huddleston & Brownlee, 1982) and the mechanism of expression at the cell surface is currently unknown. Cells transfected with NP cDNA express the protein at their surface thus showing that other virus-coded proteins are unnecessary for surface expression (Townsend et al., 1984b). It is not known whether NP associates with viral glycoproteins on the cell surface. However, the double labelling of NP and HA on the cell surface in our study indicated that these two proteins do not form tight complexes but behave as independent molecules.

The present immune electron microscopy experiments have clearly shown how this technique, in addition to revealing precise cellular location of viral antigens, can provide data on the interaction between internal virus-coded proteins and the external glycoproteins. By extending these studies to other influenza virus-coded proteins including NA, non-structural polypeptides and polymerase proteins, it is anticipated that a more detailed picture of the precise sequence of events in the morphogenesis of the virus will be provided.

REFERENCES


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