Biosynthesis of the Influenza Virus Envelope in Abortive Infection

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(Accepted 17 July 1978)

SUMMARY

Synthesis and processing of the envelope proteins of influenza A virus (fowl plague virus) have been analysed in BHK, HeLa and L cells, in which the virus undergoes abortive replication and does not form virus particles, and in the productive chick embryo fibroblast system. In abortive infection, synthesis of the M protein is specifically inhibited. The extent of this defect varies depending on the host cell and the amount of virus particles formed closely reflects the amount of M synthesized. Cell fractionation experiments demonstrated that the haemagglutinin glycoprotein HA is synthesized in abortive as well as in productive cells at the rough endoplasmic reticulum, that it migrates via smooth internal membranes to the plasma membrane and that it is cleaved by proteolysis into fragments HA_1 and HA_2 in the course of migration. Immune electron microscopy using monospecific antibodies against haemagglutinin and neuraminidase showed that both glycoproteins are exposed at the cell surface. Thus, synthesis and processing of the virus glycoproteins does not depend on the formation of the M protein. However, the M protein appears to be necessary for budding and thus for particle formation.

INTRODUCTION

Influenza virus acquires its envelope, which is composed of a lipid bilayer, external glycoprotein spikes and an inner layer of carbohydrate-free M protein, in a budding process at the cell surface. Cell fractionation experiments that have been carried out in recent years by several groups suggest that the envelope proteins arrive in the plasma membrane by different pathways. The polypeptide chains of the virus glycoproteins, i.e. the haemagglutinin and the neuraminidase, are synthesized on the rough endoplasmic reticulum. From there they migrate via smooth internal membranes to the plasma membrane (Compans, 1973a; Stanley et al. 1973; Hay, 1974; Klenk et al. 1974). In the course of migration these polypeptides undergo post-translational modifications. These involve sequential glycosylation at the rough endoplasmic reticulum and at smooth internal membranes (Compans, 1973b; Stanley et al. 1973; Klenk et al. 1978) and proteolytic cleavage which takes place at smooth internal membranes (Klenk et al. 1974) or at the plasma membrane (Lazarowitz et al. 1971; Hay, 1974). In contrast, processing and intracellular transport of the M protein has not been observed. Immediately after synthesis this protein can be detected in various cytoplasmic membrane fractions (Compans, 1973a; * Author to whom requests for reprints should be sent.

0022-1317/79/0000-3304 $02.00 © 1979 SGM
Klenk et al. 1974; Meier-Ewert & Compans, 1974) as well as the plasma membrane and it is rapidly incorporated into mature virions (Hay, 1974).

Depending on the host cell and the virus strain used, replication of influenza virus may be abortive in that formation of virus particles does not occur, even though virus-specific proteins, such as the haemagglutinin and the neuraminidase, are synthesized (Henle et al. 1955; Franklin & Breitenfeld, 1959; ter Meulen & Love, 1967). In the present study we have analysed abortive infection of fowl plague virus (FPV) in BHK21, HeLa, and L cells as compared to productive infection in chick embryo fibroblasts. We will show that in abortive infection of the kind analysed here synthesis of the M protein is specifically inhibited. Comparison of abortive and productive infection thus provides a suitable system to study the role of the M protein in synthesis and processing of the other envelope proteins and in envelope assembly.

METHODS

Viruses and cells. The Rostock strain of fowl plague virus [A/FPV/Rostock (HavlN1)] and recombinants between FPV and virus N [A/chick/Germany/49 (Hav2Neq1)] were used. Seed stocks were grown in the allantoic cavity of 11-day-old embryonated eggs and were stored as infected allantoic fluids at -80 °C. Virus replication was analysed in monolayer cultures of primary chick embryo fibroblasts (CEF; Zimmermann & Schäfer, 1960) and of BHK21, HeLa, and L cells. BHK21, HeLa, and L cells were uncloned lines originally obtained from the American Type Culture Collection and underwent about 100 passages in this laboratory. HeLa and L cells were grown in reinforced Eagle’s medium (REM; Bablanian et al. 1965) with 10% foetal calf serum. For BHK21 cells the same medium has been used supplemented with 10% tryptose phosphate broth.

Plaque assays, haemagglutination titrations and neuraminidase assays have been carried out by established procedures.

Radioactive labelling of virus-specific proteins. Pulse-chase experiments were carried out essentially as described previously (Klenk et al. 1974) except that the cells were incubated in REM prior to the labelling period. Radioactive isotopes were used at the following concentrations: 14C-protein hydrolysate, 5 μCi/ml; 3H-amino acids, 10 μCi/ml; 35S-methionine, 10 μCi/ml.

Cell fractionation. Fractions of cytoplasmic extracts have been prepared as described previously (Klenk et al. 1974, 1978). Plasma membranes of HeLa and L cells were isolated after fixation with fluorescein mercuric acetate (Warren et al. 1967). Plasma membranes of CEF were isolated by the procedure of Bingham & Burke (1972).

Polyacrylamide gel electrophoresis. Samples were dissociated with SDS and mercaptoethanol and analysed in continuous and discontinuous buffer systems. Electrophoresis in the continuous buffer system was carried out on cylindrical gels containing 10% acrylamide (Klenk et al. 1970) and on slab gels (150 × 150 × 1.5 mm) containing 15% acrylamide (Studier, 1973). For electrophoresis in the discontinuous buffer system, 10 to 20 μl samples were prepared as described (Schwarz & Klenk, 1974) and analysed on gradient slab gels (10 to 20% acrylamide; Laemmli, 1970). Cylindrical gels were sliced and counted for radioactivity as described (Schwarz & Klenk, 1974). Slab gels were dried on filter paper in vacuo. Autoradiography was carried out on Kodak film RP/R-54. Fluorography was done by the procedure of Bonner & Laskey (1974). A Transissidine General TG 2970 densitometer was used for scanning.

Radioimmune assay. Anti-FPV-immunoglobulins were purified by ammonium sulphate precipitation and subsequent chromatography on DEAE cellulose (Levy & Sober, 1960)
Biosynthesis of influenza envelope from the serum of a chicken immunized with FPV. The antibody preparation (15 mg) was tritiated in vitro following the procedure of Sanborn & Durand (1974), except that the final precipitation with ethanol was replaced by threefold precipitation with ammonium sulphate. After labelling the antibody preparation had a haemagglutination inhibition titre of 1:1000. Specific radioactivity was 10 μCi/mg protein.

The membranes of cytoplasmic extracts prepared as described above from about 5 × 10^7 cells were sedimented by centrifugation at 100,000 g for 2 h. The pellet was resuspended in 4 ml PBS, and the protein content of the suspension was determined (Lowry et al. 1951). Samples of this membrane preparation were mixed with 200 μl of the ³H-antibody solution and incubated for 45 min at 37 °C and then for 4 h at 4 °C. Unbound antibody was removed from the membranes by four cycles of sedimentation at 100,000 g for 1 h and resuspension in PBS. The final pellet was suspended in 200 μl PBS and the amount of radioactive antibody bound to the membranes was then determined by liquid scintillation.

Immune electron microscopy by the indirect ferritin-antibody labelling technique. Antisera specified against the individual FPV glycoproteins have been obtained by immunizing rabbits with recombinants of FPV and virus N (Klenk et al. 1975b) containing either FPV haemagglutinin [FPV(H)–N(N)] or FPV neuraminidase [N(H)–FPV(N)]. Immunization was done through a series of subcutaneous injections of purified virus suspension emulsified with complete Freund’s adjuvant (1:1). Hyperimmune serum collected 7 to 10 days after the booster injection was inactivated for 30 min at 56 °C and absorbed exhaustively with uninfected chick embryo fibroblasts. Immunoglobulins (IgG) were purified as described above and stored at 4 °C. The potency and specificity were determined using FPV as the reference virus. For FPV, the antiserum prepared against recombinant [FPV(H)–N(N)] had a haemagglutination inhibition titre of 1:160 and a 50 % plaque reduction titre at a dilution of 1:640; the antiserum prepared against virus strain N(H)–FPV(N) had no detectable HI or virus-neutralizing activities. However, it possessed a 50 % neuraminidase blocking titre at a dilution of 1:600. A further indicator of the specificity of the prepared antisera was the inability of anti-HA (anti-FPV(H)–N(N)] IgG to inhibit the HA activity when mixed with the recombinant [N(H)–FPV(N)]. Similarly, anti-NA [anti-N(H)–FPV(N)] IgG did not inhibit the HA activity of the recombinant FPV(H)–N(N).

Anti-rabbit IgG from sheep (a gift from Dr G. Pauli) was cross-linked to ferritin using glutaraldehyde as the coupling reagent (Kishida et al. 1975). The ferritin-antibody label obtained after two cycles of differential high speed centrifugation was kept in sterile 0.01 M-phosphate buffer, pH 7.5, at 4 °C.

Monolayer cultures of L cells and CEF in 3.5 cm plastic Petri dishes were infected with FPV as described above. Cells were sampled at 2 to 7 h after infection at 37 °C, and pre-fixed in situ with cold 0.25 % glutaraldehyde in PBS for 15 min. After several PBS washings, the cell sheet was covered with 2 % bovine serum albumin solution for 15 min. The excess albumin solution was aspirated and, after two PBS washings, the cells were covered with 0.2 ml of anti-HA or anti-NA IgG for 15 min at 25 °C. The excess antibody solution was washed off with PBS and the cells were covered with 0.2 ml of the ferritin-antibody label for 15 min at 25 °C. Untreated ferritin label was thoroughly washed with PBS. The cells were fixed with 2.5 glutaraldehyde in PBS for 1 h, scraped and pelleted by centrifugation at 1000 g for 10 min. The cell pellet was post-fixed in 1 % osmic acid, dehydrated in ethanol and embedded in Epon 812.

Thin sections were contrasted with 2 % uranyl acetate and lead citrate. A Zeiss EM 9A electron microscope was used to examine sectioned preparations.

Chemicals and isotopes. Fluorescein mercuric acetate was obtained from Nutritional
RESULTS

Growth of FPV in CEF, BHK, HeLa, and L cells

CEF, BHK, HeLa, and L cells have been infected with FPV under single cycle conditions (m.o.i. approx. 10 p.f.u./cell). At regular intervals after inoculation, infectivity, haemagglutination and neuraminidase activity have been determined in the cells and in the medium. The growth curves shown in Fig. 1 demonstrate that production of infectious virus is reduced in BHK, HeLa, and L cells: 10 h after infection the virus yields are 10 to 15 p.f.u./BHK cell, 5 to 10 p.f.u./HeLa cell, and 0.1 to 1 p.f.u./L cell, as compared to more than 500 p.f.u./CEF. Similarly, in the non-permissive systems there is a reduction of haemagglutinin and neuraminidase activity released into the medium, which is again more
extensive in the HeLa cells than in BHK cells and in L cells more extensive than in HeLa cells. In contrast, cell-bound haemagglutinin and neuraminidase titres are as high in the non-permissive systems as in the permissive systems.

These results which confirm and extend previous observations (Henle et al. 1955; Franklin & Breitenfeld, 1959; ter Meulen & Love, 1967; Gandhi et al. 1971) indicate that virus replication in BHK, HeLa and L cells is abortive because of a defect in assembly and that the extent of this defect varies with different cells. The concept of a block in assembly is supported by electron microscopic analysis of infected cells which demonstrates that, in contrast to productive cells, virus budding does not occur on non-permissive cells (Fig. 2).

**Synthesis of virus proteins**

Synthesis of the individual virus proteins has been analysed by polyacrylamide gel electrophoresis of infected cells that were labelled with $^{35}$S-methionine. After labelling by
Fig. 3. Synthesis of FPV polypeptides in (a) CEF, (b) BHK, (c) HeLa, and (d) L cells. Cells were labelled at 5 h after infection for 10 min with $^{38}$S-methionine. Electrophoresis was carried out on a 15% slab gel in the continuous buffer system. The densitometer profiles obtained after autoradiography are shown.
a short pulse, proteins P, HA, NP, M, and NS can be seen (Fig. 3). The three P proteins are not resolved under the electrophoresis conditions used in this experiment, but can be discriminated in discontinuous gel systems (data not shown). The neuraminidase protein NA does not appear as a separate peak, because it co-migrates with NP (Hay, 1974; Klenk et al. 1975a). Whereas most virus-specific proteins are synthesized in BHK, HeLa, and L cells in similar proportions as in CEF, synthesis of the M protein is clearly reduced in the abortive systems. Moreover, a comparison of Fig. 1 and 3 demonstrates that there is a striking correlation in the reduction of M and the reduction of virus assembly. In CEF, the amount of radioactivity incorporated by a 1 h pulse into M is about 1.4 times higher than that in the haemagglutinin proteins. In L cells, this ratio is only 0.05. Thus, synthesis of M is reduced in L cells by a factor of almost 30. This factor did not change regardless of whether synthesis of M was analysed 4 or 9 h after infection.

After labelling by a longer pulse (Fig. 4), HA\(_1\) and HA\(_2\) can be discriminated in pro-
Fig. 5. Association of FPV polypeptides with cytoplasmic membranes of L cells. The protein patterns obtained from smooth (A, B) and rough (C, D) membranes are shown. Cells were labelled at 5 h after infection with a mixture of \(^3\)H-leucine, \(^3\)H-valine, and \(^3\)H-tyrosine in a 10 min pulse (A, C) or in a 10 min pulse followed by a 60 min chase period with cold amino acids (B, D). Electrophoresis was carried out in the discontinuous buffer system on gradient gels that were developed by fluorography.
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Fig. 6. Association of FPV polypeptides with plasma membranes of (a) L cells and (b) CEF. Cells were labelled 5 h after infection for 90 min with a mixture of $^3$H-leucine, $^3$H-valine and $^3$H-tyrosine (●—●). Virus-specific proteins labelled in CEF with $^{14}$C-amino acids were co-electrophoresed as markers (○——○). Electrophoresis was carried out on cylindrical gels in the continuous buffer system.

As has been pointed out in the Introduction, cell fractionation experiments revealed that in productive infection the haemagglutinin migrates from the rough endoplasmic reticulum via smooth internal membranes to the plasma membrane. It could also be demonstrated that cleavage of the haemagglutinin of FPV takes place in the course of migration (Hay, 1974; Klenk et al. 1974). We have carried out here similar experiments in abortively infected productive and abortive cells indicating that the haemagglutinin is cleaved in both systems. Again, labelling of M is greatly reduced in the abortive system.

Association of virus proteins with cellular membranes

As has been pointed out in the Introduction, cell fractionation experiments revealed that in productive infection the haemagglutinin migrates from the rough endoplasmic reticulum via smooth internal membranes to the plasma membrane. It could also be demonstrated that cleavage of the haemagglutinin of FPV takes place in the course of migration (Hay, 1974; Klenk et al. 1974). We have carried out here similar experiments in abortively infected...
Fig. 7. Binding of anti-FPV-immunoglobulin to cytoplasmic membranes. Different amounts of membranes obtained from CEF (●—●) and from L cells (○—○) were exposed to a constant amount of antibody as described in the Methods.

cells. FPV-infected L and HeLa cells have been labelled either by a short pulse with radioactive amino acids or by a short pulse followed by a chase with cold amino acids. Cytoplasmic extracts have been fractionated and virus proteins were analysed in the individual cell fractions. The results were similar to those obtained previously on cell fractions of CEF (Klenk et al. 1974), except that the M protein was missing in L and HeLa cell fractions. Fig. 5 shows the polypeptides present in fraction 2, containing membranes derived predominantly from the smooth endoplasmic reticulum, and in fraction 6, containing rough membranes of L cells. The uncleaved haemagglutinin glycoprotein can be detected after the short pulse on rough as well as smooth membranes. After the chase period, NP, NS, and several non-identified proteins presumably of host origin can be seen in the rough membrane fraction, but there is very little haemagglutinin. In the smooth membrane fraction, however, the haemagglutinin is still present, but now exclusively in the cleaved form. These results demonstrate that, as in productive systems, the haemagglutinin migrates in non-permissive cells from the rough endoplasmic reticulum to smooth cellular membranes where cleavage takes place.

Fig. 6 shows the polypeptide profiles observed in plasma membranes when cells were labelled 5 h after infection for 90 min with radioactive amino acids. The plasma membrane of L cells contains mainly haemagglutinin. Similar results have been obtained when plasma membranes of HeLa cells have been analysed (data not shown). In contrast, on the plasma
The data described so far indicate that, apart from the M protein, the envelope proteins are synthesized in abortive infection in similar proportions as in productive systems and that there is no apparent difference between the systems in the post-translational processing of the haemagglutinin glycoprotein. However, the data do not provide direct information on the absolute amounts in which the proteins are present on cellular membranes. To this end, quantitative analysis has been carried out using a radioimmunoassay. Cytoplasmic extracts were prepared from infected cells, and the membranes present in these extracts

membrane of CEF, in addition to HA₁ and HA₂, a distinct amount of M can be detected as has been observed before in productive systems (Lazarowitz et al. 1971; Hay, 1974).

Fig. 8. Exposure of FPV haemagglutinin on the surface of (a) L cells and (b) CEF. L cells and CEF were subjected to the indirect ferritin-antibody labelling technique at 7 and 4 h after infection, respectively, using antiserum specific for FPV haemagglutinin. Magnification × 60000.
Fig. 9. Exposure of FPV neuraminidase on the surface of (a) L cells and (b) CEF. L cells and CEF were labelled 7 and 4 h after infection respectively using antiserum specific for FPV neuraminidase.

were exposed to an excess of radioactively labelled immunoglobulins obtained from the serum of a reconvalescent chicken. Fig. 7 shows that there is a linear relationship in the amount of antigen-containing membranes and the amount of radioactive antibody bound to these membranes. Membranes obtained from the abortive system bind almost as much antibody (10 000 ct/min/mg membrane protein) as membranes from productive cells (13 300 ct/min/mg membrane protein). Thus, the amount of virus protein associated with the membranes is similar in both systems and, since most of this protein is haemagglutinin, it can be concluded that the absolute amounts of haemagglutinin are also similar.

**Distribution of haemagglutinin and neuraminidase in the plasma membrane**

The distribution of haemagglutinin and neuraminidase on the surface of CEF and L cells was then examined using the indirect ferritin-antibody labelling technique. Labelling by this procedure was specific in that membrane-bound ferritin particles could only be detected on infected but not on uninfected cells. The haemagglutinin appeared about 2 to 3 h after infection on the plasma membrane of CEF and L cells as random, discrete patches of varying size. Later in infection the amount of haemagglutinin had increased in the plasma membranes of both cells, but there was a distinct difference between the cells in the distribution of this antigen. In L cells the entire plasma membrane was covered with a continuous dense coat of ferritin-labelled antibody (Fig. 8a), whereas in CEF the plasma membrane still contained clusters of haemagglutinin and segments which were free of the antigen
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Fig. 10. Shedding of plasma membrane vesicles of L cells infected with FPV. Cells were labelled 7 h after infection using antiserum specific for FPV haemagglutinin. Magnification x 60000.

(Fig. 8b). The neuraminidase appeared in the plasma membrane at the same time as the haemagglutinin. However, as compared to the haemagglutinin the neuraminidase was present at all times after infection only in small amounts and it had a patchy distribution on the plasma membrane of both cells (Fig. 9).

Budding virions which can be labelled with both types of antibodies are detected on CEF at about 3 h after infection and dramatically increase in number at about 4 h. In contrast, budding virions are practically absent on L cells (Fig. 2). Occasionally in L cells, shedding of the plasma membrane by exocytosis was observed which resulted in the appearance of extracellular pleomorphic particles (Fig. 10). These membrane vehicles, which showed no resemblance to mature virus except for the presence of virus envelope antigens on the surface, might be responsible for the slight increase in haemagglutinating and neuraminidase activity released from non-permissive cells late in infection (Fig. 1).

DISCUSSION

The data presented here demonstrate that most influenza virus proteins are synthesized in abortively infected cells in similar amounts as in productive cells. The only significant difference is a marked reduction in the synthesis of the M protein in abortive infection. A similar observation has been made recently by others (Bosch et al. 1978; Valcavi et al. 1978). Furthermore, comparative analysis of host cell systems differing in the extent of
abortiveness, as done in the present study, reveals that the amount of virus particles formed closely reflects the amount of M synthesized. Thus, it appears that the defect in the production of M is the direct cause for the abortiveness.

Evidence has been presented by others that the reduction in the synthesis of protein M results from reduced production of its mRNA (Bosch et al. 1978). Beyond that, however, the reason for the defective M synthesis is not fully understood. It is conceivable that there might be other underlying defects, such as faulty assembly of nucleocapsids (Caliguiri & Gerstein, 1978) or reduced RNA synthesis (Avery, 1975). In this context it should be pointed out that synthesis of M protein is a late event in influenza virus replication which appears to be tightly controlled and rate limiting for virus assembly (Lazarowitz et al. 1971; Skehel, 1972; Klenk & Rott, 1973; Hay, 1974; Meier-Ewert & Compans, 1974; Lamb & Choppin, 1976). It is therefore possible that various defects of early functions in the infection process might influence the synthesis of the M protein. Evidence has been presented in several previous studies that in abortive systems the block in virus assembly might be the result of trapping of nucleocapsid protein in the nucleus (Franklin & Breitenfeld, 1959; ter Meulen & Love, 1967; Gandhi et al. 1971). In the systems analysed here, however, this does not appear to be the mechanism of inhibition, since the amounts of nucleocapsid protein detected by cell fractionation in the cytoplasm were similar in abortive and productive infection.

Abortive infection as analysed here provides a suitable system to study the interdependence of the individual pathways by which the virus envelope proteins are synthesized and incorporated into mature virions. Our data show that there is no significant difference between abortive and productive systems with respect to the amount of haemagglutinin synthesized, its incorporation into cellular membranes, and its migration to the plasma membrane. Also, there are no indications in abortive systems for aberrations in proteolytic cleavage and, as shown previously (Klenk et al. 1975b), in glycosylation. The haemagglutinin is readily exposed at the surface of abortively infected cells and, presumably due to the lack of virus assembly and release, it is present here late after infection in even higher amounts than in productive cells. Synthesis of the neuraminidase does not appear to be altered in abortive infection, either. Thus, synthesis, processing and transport of the virus glycoproteins do not depend on the availability of the M protein.

Budding of regular virus particles is a very rare event in abortively infected cells, even if the plasma membrane is densely packed with virus glycoproteins. From the surface of such cells membrane vesicles are occasionally released by shedding. These structures, however, which might correspond to the non-infectious particles lacking M described by others (Kendal et al. 1977; Bukrinskaya et al. 1978), have very little resemblance to mature virions. Our data thus clearly demonstrate that the M protein is necessary for virus assembly. In addition, they support the concept derived from studies on a variety of enveloped viruses that M or an equivalent protein attaches to areas of the plasma membrane which already contain virus glycoproteins and that the attachment of M is the step in virus assembly that directly promotes budding (Garoff & Simons, 1974; Hay, 1974; Nagai et al. 1976; Knipe et al. 1977).

The authors thank Fräulein E. Otto for excellent technical assistance and Dr R. Rott for many valuable discussions. L. T. T. was a recipient of a fellowship from the Alexander von Humboldt-Gesellschaft. This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47, Virologie) and forms part of the thesis of J. L.
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(Received 18 May 1978)