EFFECT OF CYTOCHALASIN B ON THE MATURATION OF ENVELOPED VIRUSES

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A variety of animal viruses acquire an envelope by a process of budding at the surface of the infected cell. The resulting virions contain lipids which closely reflect those of the host plasma membrane, whereas most cellular membrane proteins are effectively excluded from the viral envelope. Thus, the maturation process requires specific interactions among the viral proteins which are incorporated into the cellular plasma membrane (1–3). Reports of the packaging of cellular actin within virions of several groups of enveloped viruses (4), as well as electron microscopic evidence of microfilaments in close association with budding virions (5), have suggested that the cytoskeletal network may be actively involved in the assembly and budding of these viruses. It seems plausible, therefore, that both the interactions among viral components and the action of cellular contractile elements could be required for the maturation of enveloped viruses.

Cytochalasin B (CB), a metabolite of the mold Helminthosporium dematoides, affects several functions of cells in culture. It has been shown to inhibit the association of actin with membrane-associated actin-binding protein, thereby preventing actin polymerization (6). The drug also inhibits microfilament-dependent cellular activities including cell division, motility, and phagocytosis (7, 8). Other effects of CB include inhibition of transport of sugars and inhibition of endocytosis (9–13).

We have utilized CB to investigate the requirement for an intact cytoskeletal network for the maturation process of two enveloped viruses. The results described in this report indicate that CB treatment does not significantly impair virus assembly but does impair the glycosylation of viral glycoproteins.

Materials and Methods

Virus and Cells. For preparation of virus stocks, confluent Madin-Darby bovine kidney (MDBK) cell monolayers in 150-mm Petri dishes were infected with the A/WSN (H1N1) strain of influenza virus (14) or the Indiana serotype of vesicular stomatitis virus (VSV) at a...
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multiplicity of ~0.1 plaque-forming unit (PFU)/cell. After a 2-h adsorption at 37°C, 6 ml of reinforced Eagle's medium (15) containing 2% newborn calf serum (NCS) (Biocell Laboratories, Los Angeles, Calif.) was added to each dish and incubation was continued at 37°C for 48 h. Culture fluids were harvested, clarified by centrifugation at 1,500 g for 20 min, mixed with 1/5 vol of 5% bovine serum albumin (BSA) in Eagle's medium, and stored at ~70°C.

Plaque Assays. Plaque assays for influenza virus were performed on Madin-Darby canine kidney (MDCK) cell monolayers (16), and for VSV on BHK21-F cell monolayers (17).

Hemagglutination Titters. Hemagglutination titers were determined in microtiter plates using chicken erythrocytes, as described previously (18).

Purification of Virus. Culture fluids were harvested and centrifuged at 8,000 g for 15 min to remove cellular debris; virus was precipitated with 7% polyethylene glycol plus 2.3% NaCl (19), purified by centrifugation on a 5–40% continuous potassium tartrate gradient (20), and dialyzed vs. 0.01 M sodium phosphate, pH 7.2.

Radiolabeling of Viral Components. MDBK cell monolayers were infected with WSN strain of influenza virus at a multiplicity of ~10 PFU/cell. After a 2-h adsorption at 37°C, the cultures were washed twice with warm phosphate-buffered saline (PBS) and overlaid with serum-free reinforced Eagle's medium containing either [3H]leucine (10 μCi/ml) or [3H]glucosamine (10 μCi/ml) plus 14C-amino acids (1.5 μCi/ml). Incubation was continued in this medium for 24 h at 37°C, after which the virus was harvested and purified as described above. All radioisotopes used in these experiments were obtained from Schwarz/Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.

Polyacrylamide Gel Electrophoresis. Samples in 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol were boiled for 1 min, after which glycerol (1%) and bromophenol blue (0.005%) were added to each sample before layering them onto gels.

Samples labeled with [3H]leucine were electrophoresed on 10% polyacrylamide slab gels with a 3.6% acrylamide stacking gel (3-mm thickness) for 18-20 h at 10 mA. Unlabeled samples were electrophoresed on 10% polyacrylamide slab gels with a 5% acrylamide stacking gel (1-mm thickness) for 18-20 h at 5 mA. Slab gels were run in a discontinuous Tris-glycine buffer with 5% SDS (21). Unlabeled gels were stained with 0.2% Coomassie Brilliant Blue.

Samples labeled with [3H]glucosamine and 14C-amino acids were electrophoresed for 18-20 h at 5 mA on 7.5% polyacrylamide tube gels (5-mm internal diameter) with a 2.5% acrylamide stacking layer (22). Tube gels were run in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS.

Preparation of Gels for Determination of Radioactivity. Tube gels were frozen on dry ice, sliced into 1-mm thick disks, and prepared for liquid scintillation counting as described previously (23).

Assay of Neuraminidase Activity. 400 μg of purified virus were incubated with excess fetuin as a substrate in 0.015 M sodium phosphate, pH 5.9 containing 0.18 mM CaCl₂ for 2 h, and the acid butanol-soluble reaction product examined spectrophotometrically at 549 nm, as described by Warren (24). Activity is expressed as neuraminidase units, calculated according to a formula described previously (25).

Electron Microscopy. Virus-infected cells were fixed in 1% glutaraldehyde in PBS, postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in an epoxy resin mixture. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.) (26).

Results

Effects of Cytochalasin B on Virus Release. Confluent monolayers of MDBK cells were infected with influenza virus under single cycle conditions and incubated in the presence or absence of varying concentrations of CB (Sigma Chemical Co., St. Louis, Mo.). In all cases in which CB was added to the maintenance medium, it was present during the entire incubation period. Because CB was dissolved in a final concentration of 0.5% dimethyl sulfoxide (DMSO), the same concentration of DMSO was added to control cultures. After a 12- to 24-h incubation, hemagglutination (HA) titers were
Table I

Effect of Cytochalasin B on Yield of Influenza Virus

| Treatment | CB  µg/ml | N'ase U/ml | Virus yield HAU/ml | Percentage of control |
|-----------|-----------|------------|-------------------|----------------------|
|           | 0         | 0          | 1,024             | 100                  |
|           | 10        | 0          | 256               | 25                   |
|           | 25        | 0          | 128               | 12.5                 |
|           | 50        | 0          | 16                | 1.6                  |
|           | 0         | 5          | 1,024             | 100                  |
|           | 50        | 5          | 512               | 50                   |

WSN strain of influenza virus grown in MDBK cells (multiplicity of infection [m.o.i.] = 20 PFU/cell) in the presence of varying concentrations of CB with or without V. cholera N'ase. HAU, hemagglutinating units; N'ase, neuraminidase.

determined on medium from each culture. The results, shown in Table I, revealed that HA titers were reduced in proportion to CB concentration. At a concentration of 50 µg/ml, >98% reduction was observed in titers of influenza virus grown in the presence of CB as compared to controls. These data indicate that the drug has a dramatic effect on some aspect of influenza virus production. The results of infectivity titrations show that the reduction in HA titers is paralleled by a drop in infectious virus production (see below).

Electron Microscopy of CB-Treated Cultures. To further characterize the effects of CB on influenza virus infected cells, we examined drug-treated and untreated cells by phase contrast and by thin-section electron microscopy. Examination by phase contrast showed that 30 min after CB treatment, cells were morphologically distinct from untreated cells in that they were rounded and less well spread on the plastic surface. Electron micrographs showed fewer microvilli on surfaces of CB-treated cells and disappearance of the dense mesh of cytoskeletal elements along the plastic-bound surfaces of these cells.

The pattern of virus maturation and release at plasma membranes of control and drug-treated cells was also markedly different. In control cultures, individual virus particles were observed on cell surfaces or in the process of budding (Fig. 1 A). In contrast, cells from CB-treated cultures showed large clusters of virus adhering to their surfaces (Fig. 1 B). These results indicated that the CB-induced reduction in virus titers was not entirely a result of the failure of particles to be produced by CB-treated cells, but rather, a result of the failure of many particles to be released into the culture medium.

Effects of Neuraminidase on Virus Release. The large aggregates of virus particles on surfaces of CB-treated cells were strikingly similar to those observed previously (27) with ts mutants of influenza virus defective in neuraminidase activity, and to virus aggregates observed upon treatment of influenza virus-infected cells with 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA), a synthetic neuraminidase inhibitor (28). These results had suggested that an active viral neuraminidase was probably required to cleave neuraminic acid (the HA receptor) from viral and cell surfaces, and that a defect in the enzyme resulted in aggregation of the virus by binding of hemagglutinin to neuraminic acid residues (27, 28). Addition of purified Vibrio cholera
neuraminidase to cultures infected with the $ts$ mutants resulted in dispersal of the aggregates with a concurrent enhancement in virus titers.

If virus produced in the presence of CB were lacking neuraminidase activity, then the addition of purified bacterial neuraminidase to CB-treated cultures should
enhance particle release. Influenza virus-infected cultures containing 0 or 50 μg/ml CB were therefore treated with 5 U/ml of purified V. cholerae neuraminidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). As a result of this treatment, titers of CB-grown virus rose to within 2-fold of those of untreated controls. Control virus titers were unaltered by neuraminidase treatment (Table I, lines 5 and 6). The recovery of high levels of virus from CB-treated cells in the presence of neuraminidase indicates that the disruption of the cytoskeletal network per se did not significantly alter the maturation of these viruses, but that CB treatment resulted in a defect in viral neuraminidase activity.

Infectivity of Virions Produced in the Presence of CB. To further characterize the virus particles produced in the presence of CB, we assayed the infectivity of virions produced under these conditions. MDBK cells were infected with 20 PFU/cell of influenza virus or VSV in the presence of 0 or 50 μg/ml CB and the infective virus yields were determined by plaque assay. As shown in Fig. 2, the titer of VSV grown in the presence of CB was 84.5% of the nontreated control. Influenza virus yields from CB-treated cells were <5% of control virus. However, when CB-treated influenza virus-infected cells were treated with 5 U/ml of exogenous neuraminidase, there was a 20-fold increase in infectious virus and the resulting titers were >50% of control values. These results indicate that CB-treated cells produce high yields of infectious influenza virus particles, if neuraminidase is added to overcome the block in virus release.

Assay of Neuraminidase Activity. We determined the neuraminidase activity (Materials and Methods) of purified influenza virus grown in the presence and in the absence of CB. Table II shows that CB treatment of cells resulted in a 10-fold reduction in specific activity of neuraminidase of purified virions obtained from these cells. Because exogenous neuraminidase was added to all cultures to enhance virion release, we used VSV, a nonneuraminidase containing virus, as a control to ensure that our purification procedure removed the exogenous bacterial neuraminidase from
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Table II
Effect of CB on Production of Influenza Viral N'ase

| Virus          | CB treatment | N'ase activity* | Percentage of control |
|----------------|--------------|-----------------|-----------------------|
|                | µg/ml        | NAU/mg viral protein | %                     |
| Influenza A/WSN| 0            | 2.84            | 100                   |
| Influenza A/WSN| 50           | 0.29            | 10.0                  |
| VSV            | 0            | 0.12            | 4.2                   |

* Specific N'ase activity of purified virus grown in MDBK cells. 1 neuraminidase unit (NAU) is defined as the amount of enzyme which releases neuraminic acid to produce an absorbance of 1.0 at a wavelength of 549 nm, after a 1-h incubation.

Fig. 3. Autoradiograph of polypeptides of influenza A/WSN grown in MDBK cells which were treated with 50 µg/ml of CB (lane A) or grown in the absence of drug (lane B). Virus was grown in serum-free medium containing 10 µCi/ml [3H]leucine, and purified and analyzed by SDS PAGE as described in Materials and Methods. Designations of viral components are described in the text.

the virus preparations. The lack of detectable neuraminidase activity in VSV indicates that the purification procedure effectively removed the exogenous bacterial neuraminidase.

To rule out the possibility that CB was capable of directly inhibiting neuraminidase activity, we determined the neuraminidase activity of purified influenza virions with concentrations of 0–50 µg/ml CB in the assay mixture (not shown). CB did not inhibit the active neuraminidase of purified control virions. These results indicate that virions produced in drug-treated cells have dramatically impaired neuraminidase activity, and that CB does not directly inhibit the enzymatic activity of the neuraminidase glycoprotein (NA) of control virions.

Analysis of Viral Polypeptides. It was possible from the results described above that
virions grown in CB-treated cells contained an inactive form of the viral NA glycoprotein, or that NA was absent from these virions, possibly because an intact cytoskeletal network could be necessary for assembly of this glycoprotein with other viral components at the plasma membrane. To determine whether the NA glycoprotein could be detected on CB-grown influenza virus, we analyzed the polypeptides of purified virions by SDS-polyacrylamide gel electrophoresis (PAGE). Influenza virus-infected cells were grown in medium containing [3H]leucine with or without 50 μg/ml CB. Cell monolayers were washed twice with serum-free medium before and after virus adsorption, and serum was excluded from the growth medium to prevent the cleavage of HA into HA1 and HA2, which would interfere with resolution of the NA glycoprotein (29). 5 U/ml of neuraminidase were included in the maintenance medium to enhance the release of virus from CB-treated cells. The purified virions were then examined by SDS-PAGE and autoradiography as shown in Fig. 3. Four major structural proteins could be resolved in control as well as CB-grown virions. The membrane protein (M) and the nucleoprotein subunit (NP) were unaltered by CB treatment. However, the two glycoproteins, HA and NA, from CB-grown virus
Fig. 5. SDS-PAGE of polypeptides of influenza A/WSN virions grown in untreated MDBK cells (right lane) or in MDBK cells treated with 50 μg/ml of CB (left lane). Drug was added 6 h before infection and was also present post infection. The protein bands are stained with 0.2% Coomassie Brilliant Blue. The band designated “A” corresponds to the component identified as actin. The stained HA band in CB-grown virions is more heterogeneous and has a faster electrophoretic mobility than the control band, as described for Fig. 3.

(lane A) migrated more rapidly and were more heterogeneous than those of untreated control virus (lane B). These results suggested that glycosylation of viral glycoproteins might be impaired in the presence of CB. To further examine this possibility, we grew influenza virions in medium containing [3H]glucosamine and 14C-amino acids in the presence or absence of 50 μg/ml CB. Again, serum-free conditions were maintained to prevent the cleavage of the HA glycoprotein, and exogenous neuraminidase was included to enhance virion release from CB-treated cells. The polypeptides of purified virions were analyzed on 7.5% SDS polyacrylamide tube gels (Fig. 4). Again, M and NP proteins of CB-grown and control virions were similar in relative amounts and electrophoretic mobilities, whereas the HA and NA glycoproteins in CB-grown virus (panel B) migrated more rapidly and were more heterogeneous than those of control virus (panel A). [3H]glucosamine was specifically incorporated into both the HA and NA glycoproteins of control virions; however CB treatment nearly abolished incorporation of glucosamine label into the viral glycoproteins. These results provide further evidence that glycosylation of the viral glycoproteins is impaired by CB-treatment.

Presence of Actin in CB-Grown Virions. It seemed possible that the disruption of
mirofilaments with CB may prevent the packaging of actin by influenza virus, which
would enable us to examine a possible role for actin within virions. We examined
purified virions grown in the presence of CB for their ability to incorporate cellular
actin. Because actin was not labeled under conditions used to examine virion
polypeptides in Figs. 3 and 4, influenza virus grown in the presence or absence of 50
µg/ml CB was purified and analyzed on polyacrylamide slab gels, which were stained
with Coomassie Blue. In gels of both CB-treated and untreated virions (Fig. 5) there
was a stained band at ~45,000 daltons, corresponding to the component identified as
actin (4). In several experiments in which CB was added to cells at different times
before or after influenza virus infection, a higher level of actin was reproducibly
associated with virions from CB-treated cells than with virions from untreated control
cells (data not shown). These results indicate that CB treatment did not inhibit the
association of actin with enveloped virions. Therefore, the incorporation of actin
within enveloped virus particles, like the maturation of these particles, is independent
of an intact cytoskeletal network.

Discussion

Several lines of evidence have suggested that the cytoskeletal network, and specifi-
cally actin microfilaments, may be required for some aspect of the assembly of viral
components at the plasma membrane or for the budding of enveloped viruses. Wang
and coworkers (4) have found cellular actin in purified preparations of influenza,
Sendai, Newcastle disease, and RNA tumor viruses, and Damsky and coworkers (5)
observed microfilaments in close association with budding murine mammary tumor
virus. Tyrrell and Ehrnst (30) found that CB in low concentrations (2 µg/ml) did not
inhibit the cocapping of membrane-associated matrix protein and glycoprotein of
measles virus in the presence of viral glycoprotein-specific antibody, but that the
nucleocapsid did not cap under these conditions. These authors suggested that
polymerized actin may be required for the association of nucleocapsid with matrix
protein. The experiments outlined in this paper were undertaken to determine whether
or not an intact cytoskeletal network is required for the functional association of viral
components and for the budding of enveloped viruses.

To disrupt actin microfilaments we employed the drug CB, which inhibits the
polymerization of contractile elements and impairs cellular functions which are
dependent upon that process (6–9). This drug is also known to inhibit hexose transport
across the plasma membrane (9–13), resulting in decreased glycosylation of both
cellular and viral glycoproteins. The decrease in influenza virus yields which resulted
from CB treatment in our studies was proportional to the concentration of the drug
added; 50 µg/ml caused a 100-fold reduction in virus titers. We found that this
apparent reduction was not a result of an inhibition of virus particle production but
rather a result of the formation of large aggregates of particles adhering to cell
surfaces, with impaired release of particles into culture medium. The formation of
aggregates and the failure of influenza virus to be released into culture medium under
conditions in which viral neuraminidase activity was impaired has been described
previously. Both influenza virus mutants with a ts defect in NA (27) and wild type
influenza virus in the presence of FANA, a neuraminidase inhibitor (28), exhibit
aggregation similar to that which we observed in CB-treated cultures. In both previous
reports, the addition of purified bacterial neuraminidase resulted in disruption of
virus aggregates and a concurrent elevation in virus titers. It was proposed from those results that the HA glycoprotein binds to neuraminic acid on viral and cell surfaces, and that virus release occurs only when the NA glycoprotein cleaves neuraminic acid, the HA receptor, from these surfaces. In our studies, the addition of exogenous neuraminidase to CB-treated, influenza virus-infected cultures also resulted in a greater than 30-fold increase in HA titers. Our results provide evidence that particle production in drug-treated cells was not significantly impaired, whereas neuraminidase activity in virions from such cells was reduced by 90%.

Because of the report (30) that nucleocapsids may not become associated with matrix protein of measles virus when microfilament function is inhibited, it was of utmost importance to determine if virions released from CB-treated cells were complete, i.e., were not lacking the viral genome. We found that upon neuraminidase treatment of CB-grown influenza virus, the increase in the infectivity titers roughly paralleled the increase in HA titers. This result indicates that the process of assembly of functional enveloped virions is not dependent on cytoskeletal elements.

Examination of the structural polypeptides revealed that influenza virions produced in CB-treated cells contained glycoproteins altered in electrophoretic mobility and unlabeled by [3H]glucosamine. This result was not surprising, because CB is known to inhibit the uptake of carbohydrate precursors. Dix and Courtney (31) and Marciano-Cabral et al. (32) found that treatment of herpes simplex virus-infected cells with 50 or 100 μg/ml CB resulted in a 96% reduction in the yield of infectious virus and an accumulation of nucleocapsids in the nuclei of treated cells. It was suggested that CB-induced inhibition of glycosylation of viral membrane components may inhibit recognition of the envelopment site by the herpes virus nucleocapsid. In our studies, it was of interest that such inhibition may inactivate the NA glycoprotein but did not noticeably impair HA activity or the envelopment of the viral genome. Previous results with several inhibitors of glycosylation differ from our results with CB. Hemagglutinin activity of influenza virus from cells treated with high concentrations of glucosamine or with the aberrant sugar 2-deoxy-D-glucose is markedly reduced, as is the infectivity of these virions (33-37). Fluorosugars also inhibit production of active hemagglutinin in Semliki forest virus and in fowl plague virus (38). Tunicamycin, an antibiotic which completely inhibits glycosylation, abolishes the HA activity of influenza virus, and noninfectious virions are produced (37, 39). In general, inhibition of incorporation of labeled sugar precursors by the above compounds appears to parallel the estimated degree of inhibition of glycosylation, as indicated by shift in electrophoretic mobility of the hemagglutinin glycoprotein. Complete inhibition of glycosylation by tunicamycin results in production of a nonglycosylated hemagglutinin polypeptide designated HA₀ (mol wt ~63,000), which migrates as a sharply defined band in SDS-polyacrylamide gels (37). In contrast, CB causes complete inhibition of the incorporation of labeled glucosamine into viral glycoproteins, but probably only partially inhibits glycosylation, which results in the heterogeneous population of HA molecules observed in electropherograms. Because of these contrasting results, it seems likely that the effect of CB on glycosylation is a secondary effect of its inhibition of sugar transport, resulting in a depletion of the intracellular pool of carbohydrate precursors. The partial glycosylation of glycoproteins synthesized in CB-treated cells is suggested by the electrophoretic heterogeneity of the two influenza glycoproteins as well as the apparently unaltered HA activity; such partial
glycosylation may enable incorporation of neuraminic acid into CB-grown virions. Our results also suggest that neuraminidase activity is more sensitive to this degree of glycosylation inhibition than is hemagglutinin activity; however, the possibility cannot be excluded that formation of inactive neuraminidase is a result of another effect of CB.

It is apparent from our results that the maturation of enveloped viruses is not a microfilament-dependent process. In fact, the disruption of microfilaments does not appear to prevent the incorporation of actin into enveloped virions, indicating that this process is also independent of a polymerized cytoskeleton.

Summary

The maturation of two enveloped viruses, influenza and vesicular stomatitis, occurs in cells treated with cytochalasin B. Virions produced in the presence of 50 \( \mu \)g/ml cytochalasin B (CB) appear to be as infectious as those from control cells, indicating that polymerized actin is not required for the assembly of functional viral components. CB inhibits the release of influenza virus from treated cells, a phenomenon which appears to be a result of the synthesis of an aberrant neuraminidase (NA) glycoprotein; virions grown in CB-treated cells had a 90% reduction in specific enzymatic activity.

We found that both influenza viral glycoproteins (NA and Hemagglutinin glycoprotein) had faster electrophoretic mobilities and were more heterogeneous in CB-treated cells as compared with controls. We also observed complete inhibition of incorporation of labeled glucosamine into viral glycoproteins in the presence of the drug. It was of interest that CB-induced inhibition of glycosylation appeared to cause loss of neuraminidase function, whereas hemagglutinating activity was not noticeably impaired. The presence of altered glycoproteins did not significantly diminish the infectivity of either influenza virus or vesicular stomatitis virus.

Our results indicate that no step in the maturation of enveloped viruses is dependent upon an intact cytoskeletal network.

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