Site-specific Maturation of Enveloped Viruses in L Cells Treated with Cytochalasin B

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ABSTRACT Treatment of infected L cells with 10 \( \mu g/ml \) cytochalasin B (CB) was found to promote a rapid relocalization of viral glycoproteins on the cell surface. Whereas the vesicular stomatitis virus G protein and the influenza virus hemagglutinin were uniformly distributed on the surface of untreated cells, in CB-treated cells, they were strikingly concentrated at cell extremities in the regions of clustered blebs. Glycoprotein concentration at cell extremities was accompanied by preferential maturation of virus particles from the same sites; both vesicular stomatitis and influenza viruses budded predominantly from the vicinity of clustered blebs. This effect of CB was completely reversible. Removal of CB from the cell growth medium resulted in a return of viral glycoproteins to the uniform distribution characteristic of untreated cells and to uniform virus budding. The results of this study are interpreted in terms of a model that suggests that preferential budding of viruses from the regions of bleb clusters is due to the concentration of viral glycoproteins at these sites.

The cytochalasins have a profound effect on the surface morphology of a wide variety of cultured mammalian cells. Within minutes after sensitive cells are treated with effective doses of cytochalasin B (CB) or cytochalasin D, the cytoplasm contracts and blebs (also called blisters, zeiotic knobs, or knobby protuberances) appear on the cell surface (1, 6, 8, 14–16, 22, 23). Over the next 30–120 min the blebs aggregate to form clusters (also called rosettes, crowns, or bouquets) on the upper surface of substrate-adherent cells or at one pole in the case of suspension cells (14, 17). Adherent cells may react further by acquiring an “arborized” (20, 21, 26) or macrovacuolated (2, 3) morphology after 12–48 h in the presence of the drug. The cellular response to the cytochalasins is temperature-dependent (14), and it requires an input of metabolic energy (16, 22). Colchicine does not affect the cytoplasmic contraction or bleb formation promoted by the cytochalasins, but it blocks aggregation of blebs into clusters (17, 27). All morphologic effects of the cytochalasins are reversible when the drug is withdrawn from the cell growth medium (22, 26).

Sundquist and his colleagues (27, 28) have demonstrated that CB-induced changes in cell surface morphology are accompanied by an equally dramatic relocalization of certain cell surface proteins. The case of surface \( \beta_2 \)-microglobulin in Lu106 (transformed human lung) cells is illustrative. Whereas \( \beta_2 \)-microglobulin is uniformly distributed on the surface of untreated cells, it is relocalized into a “cap” at the site of clustered blebs in cells treated with 10 \( \mu g/ml \) CB. The CB-induced redistribution of \( \beta_2 \)-microglobulin is reversible and it is inhibited by colchicine. CB is found to promote a similar relocalization of H-2 antigens in transformed 3T3 cells (28), \( \beta_2 \)-microglobulin in HeLa cells, and measles virus hemagglutinin in persistently infected Lu106 cells (27).

The studies described below have focused on the effect of CB on the surface distribution of viral glycoproteins in infected cells. The project was undertaken to test the idea that if CB were to restrict the distribution of viral glycoproteins to particular areas of the infected cell membrane (as it does in the case of the measles virus hemagglutinin), then virus particles should mature only from those glycoprotein-containing sites. Viruses should bud preferentially from membrane regions containing their specific envelope glycoproteins. Our experimental tests involved infecting L-929 cells with vesicular stomatitis virus (VSV) or with influenza virus and treating the infected cells with CB. Cells were then examined for the surface distribution of virus-specific glycoproteins and for the sites of virus maturation.

MATERIALS AND METHODS

Cell and Virus Growth: L-929 (mouse) cells were propagated in suspension at 37°C in Eagle’s minimal essential medium modified for suspen-
ception cultures (Flow Laboratories, Inc., Rockville, MD) containing 3% fetal calf serum and antibiotics as previously described (12). Experiments involving adherent cells were carried out with subconfluent monolayer cultures prepared by allowing suspension cells to attach overnight to glass or plastic surfaces in Dulbecco's modified minimal essential medium or in Eagle's basal medium containing 3% fetal calf serum and antibiotics. The cell reaction to CB was comparable on glass and on plastic surfaces, and it did not depend on the identity of the medium employed. CB (Sigma Chemical Co., St. Louis, MO) was added to cell cultures from a 1 mg/ml stock solution prepared in ethanol. Solvent in the absence of drug did not affect cell morphology or glycoprotein localization.

Stock cultures of VSV-Indiana (Mudd-Summers strain) were grown on monolayer cultures of baby hamster kidney-21 cells as previously described (11). L cells were infected at a multiplicity of infection of 2 in cell growth medium (containing 1.5% rather than 3% fetal calf serum). The virus was allowed to attach to cells for 1 h at room temperature, and infection was continued for a total of 6–10 h at 37°C. Cultures showing marked cytopathologic effects (such as cell rounding or detachment from the substrate) were not employed for cytochalasin experiments. The WSN strain of influenza A (the gift of Dr. W. Young and Dr. G. Nichols, University of Virginia) was grown on Madin-Darby canine kidney (MDCK) cells and employed to infect L cells as described above for VSV.

Light and Electron Microscopy: L-cell monolayers were prepared for phase-contrast microscopy by growing them in 25 cm² plastic tissue culture flasks. Flasks of cells were drug-treated as described in the text and photographed at various intervals, in a Leitz Diavert inverted, phase-contrast tissue culture microscope (E. Leitz, Inc., Rockleigh, NJ), which was maintained in a warm (37°C) environment.

Scanning electron microscopy was carried out with L-cell monolayers prepared and drug-treated on round (2.2 cm in diameter) glass coverslips. Cells were fixed with 2% glutaraldehyde, critical point-dried, and coated with gold as described previously (4). Photographs were taken on a JEOL 70T scanning electron microscope (JEOL USA, Electron Optics Div., Peabody, MA) operated at 25 kV.

Transmission electron microscopy was carried out with monolayer cultures of L cells prepared in glass petri dishes of 10 cm diam. After experimental operations (such as virus infection and drug treatment) were complete, cells were fixed immediately for 10 min at room temperature in PBS (Dulbecco's phosphate-buffered saline) containing 2% glutaraldehyde and then removed from the dish by scraping. Thereafter, cells were centrifuged (at 1,000 g for 1 min) into a hard pellet, fixed overnight at 4°C in 2% glutaraldehyde-PBS, postfixed in 1% OsO₄-PBS for 1–2 h at room temperature and stained en bloc for 1 h with 1% uranyl acetate. Samples were then dehydrated, embedded in plastic (Poly/Bed 812; Polysciences, Inc., Warrington, PA), sectioned, examined in a JEOL 100cx electron microscope, and photographed at a magnification of 3,300.

Indirect Immunofluorescence: Indirect immunofluorescence experiments to localize the VSV G protein and the influenza hemagglutinin were performed with cells grown and infected on 1.8 x 1.8-cm glass coverslips. Cells were fixed for 15 min at room temperature in 3% paraformaldehyde (freshly prepared) in PBS. They were then washed thoroughly in PBS and exposed sequentially to primary (mouse monoclonal) antibody and to 20–50 μg/ml affinity-purified, fluorescein isothiocyanate-labeled, goat anti-mouse IgG (Sigma Chemical Co.). Both incubations were for 60 min at room temperature. After the secondary antibody treatment, cells were washed in PBS, mounted (inverted) on microscope slides in 90% glycerol/10% PBS, and photographed on Kodak Tri-X film (Eastman Kodak Co., Rochester, NY) in a Leitz Ortholux epifluorescence microscope (x63 oil objective). Primary antibodies were the following: VSV G protein, 0.5–1.0 μg/ml antibody (purified by protein A-Sepharose chromatography [9]) from anti-G hybridoma clone No. 18 (lgG2a; a gift of Dr. W. Volk [29]); influenza WSN hemagglutinin, supernatants (diluted 1:20 in PBS containing 1.0% bovine serum albumin) from antihemagglutinin

**Figure 1** Appearance and movement of blebs on the surface of CB-treated L cells. Monolayer cells were exposed to medium containing 10 μg/ml CB and phase-contrast photographs were taken at the indicated times (in minutes) thereafter. The arrows identify blebs whose movement can be followed in successive panels.
FIGURE 2  Scanning electron microscope photographs of a control L cell (top) and a similar cell that had been treated with 10 µg/ml CB for 1 h at 37°C (bottom). Bleb clusters are clearly visible in the bottom panel.

FIGURE 3  Dependence of bleb cluster formation on CB concentration. Monolayer cultures of L cells were treated for 1 h at 37°C with medium containing CB at the indicated concentration. Cells were then viewed in the phase-contrast microscope and scored for the presence of bleb clusters. Each point shown represents the result obtained from counting at least 50 cells.

RESULTS

Treatment of L cells with CB produced a prompt effect on cell surface morphology. In the first 1–3 min after exposure to 10 µg/ml CB, substrate-attached cells contracted, their microvilli were reabsorbed, and blebs appeared on the cell

hybridoma clones A13-1R1 (IgG2a) and C5-1R1 (IgG2b) obtained from Dr. W. Gerhard (Wistar Institute). None of the three primary antibodies gave significant staining with uninfected cells.
surface. During the next 30–60 min the blebs moved peripherally to form clusters at cell extremities as shown in Figs. 1 and 2. 80% or more of the cells acquired bleb clusters, and the clusters were stable at cell extremities for 12 h or more in the presence of drug. Nonadherent (suspension) cells responded similarly except that blebs aggregated to form a single large cluster (1, 15) instead of two or more smaller ones as was observed in monolayer cells. Infection with VSV or with influenza virus did not alter the overall morphologic response to CB. Blebs formed normally and migrated into clusters at cell extremities in infected cells as they did in uninfected ones.

The maximum effect of CB on cell morphology was observed at doses of ~6 μg/ml or greater as shown in Fig. 3. Lower doses produced a diminished response in a smaller proportion of cells. All morphologic effects of CB were reversible within 90 min at 37° when CB was removed from the cell growth medium.

The effect of CB on the cell surface distribution of viral glycoproteins was examined by infecting monolayer cells with VSV or with influenza virus for 6–10 h and then adding CB to the medium. Cells were fixed at various times thereafter and the surface distribution of viral glycoproteins was probed

![Image](http://example.com/image.jpg)
by indirect immunofluorescence. The results demonstrated that in untreated cells the VSV G protein was found in small speckles or patches more or less uniformly distributed over the entire cell surface as shown in Fig. 4a. CB treatment had a significant effect on this distribution. As blebs were forming during the first 1–3 min of treatment, the speckled pattern of stain was lost and G protein accumulated in the blebs, particularly at the base or "collar" of each bleb (Fig. 4b). G protein

![Figure 5](image_url)
remained associated with collars as blebs moved peripherally to form clusters at cell extremities as shown in Fig. 4c. When mature clusters had formed (after 45–60 min), therefore, all or most of the G protein was found concentrated in the regions of clusters as shown in Fig. 4d. This polarized distribution, which was observed in ≥70% of the cells, did not change significantly during an additional 3–4 h of incubation in the presence of CB. The behavior of the VSV G protein was quite similar to that observed for the influenza hemagglutinin (HA). HA was found in a speckled distribution on the surface of untreated cells and polarized in the regions of bleb clusters in treated ones as shown in Fig. 4, e and f. Both the VSV G protein and the influenza HA reverted to the speckled distribution characteristic of drug-free cells when treated cells were returned to normal growth medium for 90 min or more at 37°C.

Transmission electron microscopy was carried out to identify the sites of virus maturation in CB-treated cells. Monolayer L-cell cultures were infected with VSV or with influenza virus for 6–10 h, treated for 60 min with 10 μg/ml CB, fixed, and then processed for electron microscopy. The results showed that virus particles in the process of maturing at this time were located preferentially in the regions of clustered blebs as shown in Figs. 5 and 6. It was very rare to see virus particles budding from other areas of the cell membrane. Most budding was observed not in the blebs or ziotic knobs themselves, but rather in the regions of membrane just proximal to the cell at the base of the blebs. This phenomenon is clearly illustrated in Fig. 5, b and c. In contrast to the situation with CB-treated cells, in untreated cells, VSV was found to bud uniformly from all areas of the cell membrane (Fig. 5a). Influenza virus matured predominantly from the upper (non-adherent) surface in untreated monolayer cells, but budding occurred from all areas of the membrane (Fig. 6a), not specifically from extreme regions as was observed in CB-treated cells.

Like the polarized distribution of the VSV G protein and the influenza HA, polarized budding of both viruses was found to be reversed when CB was removed from the cell growth medium. A uniform pattern of budding was observed in CB-treated cells that had been incubated for 60 min or more in the absence of drug.

DISCUSSION

The results described above show a clear correlation between the sites of viral maturation and the location of envelope glycoproteins. Both were found at cell extremities in CB-treated cells, but uniformly distributed on the membranes of untreated cells and of treated cells from which the drug had been removed. Our results raise the possibility that envelope glycoproteins may be functionally involved in selecting the region of membrane to be employed in viral budding. Budding could, for instance, be more rapid or more efficient from membrane sites containing specific envelope glycoproteins. This conclusion is supported by studies of virus-infected MDCK cells (24, 25). When adherent MDCK cells are infected with VSV, the G protein is found to be concentrated in the basolateral portion of the membrane from which nearly all vesicular stomatitis virions mature. Conversely, influenza

![Figure 6](https://example.com/fig6.jpg)
and Sendai viruses mature preferentially from the apical membrane where their envelope glycoproteins are concentrated (25). Neither the MDCK experiments nor the CB studies described here exclude the possibility that factors other than envelope glycoproteins may be involved in specifying the membrane sites of virus budding (24). Both, however, show a clear correlation between maturation and the location of viral glycoproteins.

CB treatment of infected cells was crucial to the experimental strategy employed here. CB treatment caused both the cellular glycoproteins, H-2 antigen and glycoprotein 0 (5), on the surface of viral glycoproteins. There is (to our knowledge) no evidence for the effect of CB on the distribution of cell surface glycoproteins (27). Neither the MDCK experiments nor the CB treatment had a similar reversible effect on the distribution of two cellular glycoproteins: H-2 antigen and glycoprotein 0 (5). The CB-induced restriction of viral glycoproteins to cell extremities was reversible when CB was removed from the cell growth medium, and CB did not appear to decrease the overall yield of virus particles maturing from the cell surface (7, 13, 18). CB treatment had a similar reversible effect on the distribution of two cellular glycoproteins, H-2 antigen and glycoprotein 0 (5), on the surface of infected L-929 cells (N. Salomonson and J. Brown, unpublished observations). It is clear, therefore, that the effect of CB on the distribution cell surface glycoproteins was not limited to an effect on viral glycoproteins (27, 28). For both the VSVG protein and the influenza HA, CB appeared to produce a restricted glycoprotein distribution by relocalization of existing glycoprotein molecules rather than by glycoprotein loss followed by selective insertion of newly synthesized material at cell extremities. There was no evidence of large-scale glycoprotein loss when infected cells were treated with CB and then examined by indirect immunofluorescence for the presence of virus-specific surface glycoproteins. Instead, existing viral glycoproteins appeared to migrate in an orderly way to cell extremities as shown in Fig. 4. Internalization of viral glycoproteins followed by reinsertion at cell extremities also seems unlikely for the same reason.

It is not at all clear at the molecular level how CB treatment can cause the striking glycoprotein relocalization observed in L-929 and other cell types (27, 28). Relocalization is unlikely to be caused by a direct interaction of CB with cellular glycoproteins. There is (to our knowledge) no evidence for such a direct interaction. Instead, the effects of CB on surface glycoprotein distribution are more probably an indirect consequence of its effects on microfilaments. These effects, such as drug binding and blockage of filament growth, have been clearly described at the molecular level in studies involving the use of purified F-actin (10, 19). It is not clear, however, how the direct cytochalasin-actin interactions observed with purified components may account for the complex set of effects, including effects on cytoplasmic microfilament distribution (16, 23) and on surface glycoprotein localization, produced by treatment of cultured cells with cytochalasin. It is clear, on the other hand, that CB treatment can be employed effectively to produce a highly polarized distribution of cell surface glycoproteins (27, 28) as described above.

We thank Charles Little, Bill Young, and Guy Nichols for help with the immunofluorescence experiments, Wes Volk and Walter Gerhard for providing monoclonal antibodies, and Bob Bloodgood for several helpful discussions of membrane-cytoskeletal interactions.

This work was supported by grant GM-34036 from the National Institutes of Health and by a grant from the American Heart Association (Virginia Affiliate).

Received for publication 2 August 1984, and in revised form 15 October 1984.

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