Monensin and FCCP Inhibit the Intracellular Transport of Alphavirus Membrane Glycoproteins

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ABSTRACT Temperature-sensitive mutants of Semliki Forest virus (SFV) and Sindbis virus (SIN) were used to study the intracellular transport of virus membrane glycoproteins in infected chicken embryo fibroblasts. When antisera against purified glycoproteins and ^{125}I-labeled protein A from *Staphylococcus aureus* were used, only small amounts of virus glycoproteins were detected at the surface of SFV ts-1 and SIN Ts-10 infected cells incubated at the restrictive temperature (39°C). When the mutant-infected cells were shifted to the permissive temperature (28°C), in the presence of cycloheximide, increasing amounts of virus glycoproteins appeared at the cell surface from 20 to 80 min after the shift. Both monensin (10 μM) and carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 10–20 μM) inhibited the appearance of virus membrane glycoproteins at the cell surface. Vinblastine sulfate (10 μg/ml) inhibited the transport by ~50%, whereas cytochalasin B (1 μg/ml) had only a marginal effect.

Intracellular distribution of virus glycoproteins in the mutant-infected cells was visualized in double-fluorescence studies using lectins as markers for endoplasmic reticulum and Golgi apparatus. At 39°C, the virus membrane glycoproteins were located at the endoplasmic reticulum, whereas after shift to 28°C, a bright juxtanuclear reticular fluorescence was seen in the location of the Golgi apparatus. In the presence of monensin, the virus glycoproteins could migrate to the Golgi apparatus, although transport to the cell surface did not take place. When the shift was carried out in the presence of FCCP, negligible fluorescence was seen in the Golgi apparatus and the glycoproteins apparently remained in the rough endoplasmic reticulum. A rapid inhibition in the accumulation of virus glycoproteins at the cell surface was obtained when FCCP was added during the active transport period, whereas with monensin there was a delay of ~10 min. These results suggest a similar intracellular pathway in the maturation of both plasma membrane and secretory glycoproteins.

The synthesis of the secretory proteins in highly specialized cells such as hepatocytes and pancreas acinar cells takes place at rough endoplasmic reticulum (RER) and is coupled to the segregation of the proteins into the cisternae of RER (for reviews see references 19 and 42). A specific, hydrophobic, signal sequence seems to be involved in the transmembrane transfer, and the secretory glycoproteins are released in soluble form into the RER cisternae (3–5). The primary glycosylation of the glycoproteins apparently occurs during the transmembrane segregation (34, 36, 50) and is followed by their transfer from RER to the Golgi complex in which the oligosaccharide side chains are completed (12, 32, 40). Finally, the glycoproteins are transported from the Golgi complex to the plasma membrane and are excreted to the cell exterior by exocytosis (19, 42). The transport of secretory glycoproteins both from RER to the Golgi complex and from Golgi to the cell surface seem to be discontinuous processes involving specific transitory vesicles and requiring energy (19–21, 42).

The subcellular transport of membrane glycoproteins is far less understood (41). However, it has been commonly thought that the processing and transfer of plasma membrane glycoproteins take place either in an analogous manner or even coupled to the secretory processes in cells (41, 43). Accordingly, in secretory cells a substantial amount of membrane material, resembling the plasma membrane in composition (38), is cotransported and integrated to the cell surface during the secretion process.

Cells infected with enveloped viruses provide many advantages in studying the synthesis, processing, and intracellular transfer of membrane glycoproteins in nonsecretory cells (23, 33). Only one or a few virus membrane glycoproteins are synthesized in large amounts in the infected cells after an almost complete shut off of host protein synthesis. By use of enveloped viruses as models, it has been demonstrated that the viral membrane glycoproteins, such as the vesicular stomatitis virus (VSV) G-protein (18, 35) and the alphavirus envelope protein p62 (pE2) (6, 13, 64) also contain a signal sequence required for their transmembrane transfer into the lumen of RER. Unlike secretory proteins, the membrane glycoproteins remain anchored in the RER membrane by their hydrophobic portions, which at least in some cases penetrate to the cytoplasmic side (6, 13, 65). From RER the membrane glycoproteins of VSV and alphaviruses are transported to the host cell plasma membrane (33). Recently, it has been reported that the transfer of VSV G-protein to the cell surface apparently occurs in clathrin-coated vesicles (47).

Previously, we have characterized temperature-sensitive mutants of Semliki Forest virus (SFV) and Sindbis virus (SIN) which have reversible defects in the transport of virus glycoproteins to the plasma membrane (48, 49). In the present study, we have used these mutants and drugs known to interfere with the translocation of secretory proteins to elucidate the pathway of intracellular transport of virus membrane glycoproteins

MATERIALS AND METHODS

Viruses

The origin and propagation of the wild-type SFV and the temperature-sensitive mutants isolated from it have been described previously (25). The heat-resistant (HR) strain of SIN and the temperature-sensitive mutants isolated from it (8) were kindly provided by Dr. E. F. Pfefferkorn. The passage of SIN HR strain and the mutants in our laboratory has been described elsewhere (27).

Virus Infection

Secondary, specific pathogen-free chick embryo fibroblasts (CEF), grown as confluent monolayers (2×10^6 cells) in 35-mm plastic dishes as described previously (25, 26), were infected with 50 plaque-forming units/cell. After a 1-h absorption at 39°C (restrictive temperature), the virus inoculum was removed, and the cells were washed three times with Hank's balanced salt solution at 39°C. Eagle's minimum essential medium supplemented with 0.2% bovine serum albumin (BSA) and 20 mM HEPES, pH 7.2, was added, and incubation was continued at 39°C. In temperature-shift experiments, cultures incubated for 5–6 h at 39°C, were treated with 100 μg/ml of cycloheximide for 5 min, whereafter the medium was replaced by a new medium at 28°C containing the same amount of cycloheximide and the incubation was continued at 28°C (permissive temperature) as indicated.

Antisera

The preparation of antisera against isolated envelope protein octamers (15) of SFV and SIN has been described previously (48, 49).

Labeling of Cells with ^{125}I-Protein A

Infected monolayers were washed twice with prewarmed (28° or 39°C) Dulbecco's phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in 0.1 phosphate buffer, pH 7.2, for 15 min (at 28° or 39°C). The fixed cells were washed three times with PBS containing 0.5% BSA and treated with antienvelope serum (300 μl/dish). After a 30-min incubation at 37°C, the cultures were washed three times with PBS containing 0.5% BSA, and 250 μl of ^{125}I-protein A in the above buffer was added to the cultures. The iodination of *S. aureus* protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed according to Dorval et al. (10). After agitation for 30 min at 20°C, the iodinated protein A was removed and the cultures were washed four times with PBS containing BSA. Finally, the cells were scraped in 0.5 ml of 2% SDS, and the radioactivity was counted. The efficiency of scraping as monitored with γ scintillation meter type 540 was >90%. To test the specificity of the assay we had the following controls: (*a*) Nonspecific binding was measured by treating the infected cultures with normal rabbit serum and ^{125}I-protein A. This background was 1–5% of that seen with immune serum and was subtracted from experimental values. (*b*) Separate mock-infected cultures, when treated with both antienvelope serum and normal rabbit serum, gave the same background values as in *a*. (*c*) The specificity of ^{125}I-protein A was tested in competition experiments with purified, unlabeled protein A obtained through the courtesy of Dr. Olavi Mäkelä (Department of Bacteriology and Immunology, University of Helsinki). A 50% inhibition was obtained with concentrations 0.2–0.3 μg/ml. (*d*) To test the leakage of intracellular antigens, we used anticapsid serum. It gave background values like the normal rabbit serum, indicating that the intracellular nucleocapsids were not exposed to the antibody.

Fluorescence Microscopy

The indirect immunofluorescence microscopy using anti-SFV and anti-Sindbis viral envelope antibodies was done as described earlier (30, 48, 49). As a second antibody sheep anti-rabbit IgG antibodies coupled to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) were used. In double-staining experiments concanavalin A (Con A) and wheat germ agglutinin (WGA) coupled to FITC and TRITC were used at a concentration of 100 μg/ml.

To visualize actin- and tubulin-containing structures, we fixed the cells in acetone (−20°C) or methanol (−20°C), respectively. For actin visualization, human antiactin antibodies were used as described earlier (39). For tubulin visualization, rabbit antitubulin antibodies were used. These antibodies have been raised against isolated calf brain tubulin and purified in affinity chromatography using calf brain tubulin-Sepharose 4B column as described earlier (2). Fluorescence microscopy was carried out using either Leitz Dialux 20 microscope and filters for FITC and TRITC-fluorescence or a Zeiss Universal microscope equipped with an epi-illuminator III RS and filters as above. Photographs were taken on Agfapan Professional 400 film.

Materials

Monensin, obtained through the courtesy of Dr. R. Hamill (The Lilly Research Laboratories, Indianapolis, Ind.) was dissolved in ethanol at a concentration of 10 mM. Vinblastine sulfate (Eli Lilly & Co., Indianapolis, Ind.) was dissolved in PBS (1 mg/ml). Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FFCP: (a kind gift of Dr. M. Wickström, Department of Medical Chemistry, University of Helsinki) was in ethanol at a concentration of 5 mM. The tubulin antibodies were obtained through the kind gift of Dr. R. A. Badley (Unilever Research Laboratories, Sharnbrook, U. K.). FITC-Con A was from Miles Laboratories (Elkhart, Ind.) and TRITC-WGA from Vector Laboratories (Burlingame, Calif.). ^{125}I-Na was obtained from the Radiochemical Centre (Amersham, U. K.).

RESULTS

Measuring the Virus Glycoproteins at the Cell Surface Using Iodinated Protein A

We first studied the appearance of virus membrane glycoproteins at the host cell plasma membrane during the SFV wild-type virus infection. SFV-infected cells were harvested at different times after infection, fixed with paraformaldehyde, and thereafter treated with antiserum against purified envelope protein octamers. By this method the plasma membrane remains intact (30, 63) and the antibodies bind to viral antigens at the cell surface (48). The cells were washed and ^{125}I-labeled protein A was added to the cultures for 30 min. After extensive washing the cells were scraped from the dishes and cell-bound radioactivity was determined. There was a clear increase of cell-bound radioactivity corresponding to the development of infection as shown in Fig. 1 *A*. In this experiment, saturation point was not reached, suggesting that iodinated protein A was not used in sufficient quantities.

Antiserum dilutions giving linear decrease of ^{125}I-protein A binding (Fig. 1 *B*) should give a better estimate for the amount of glycoproteins at the cell surface early in infection. Using antiserum dilution 1:320, we obtained saturation of protein A binding, as depicted in Fig. 1 *C*. Although this antiserum dilution was not sufficient for the quantitation of glycoproteins at the cell surface at late stages of infection (Fig. 1 *A*, solid line) it proved to be suitable for the studies with transport defective, temperature-sensitive mutants (see below). The binding of ^{125}I-labeled protein A to cells treated with antienvelope antibodies was specific for the virus glycoproteins. Negligible amounts of

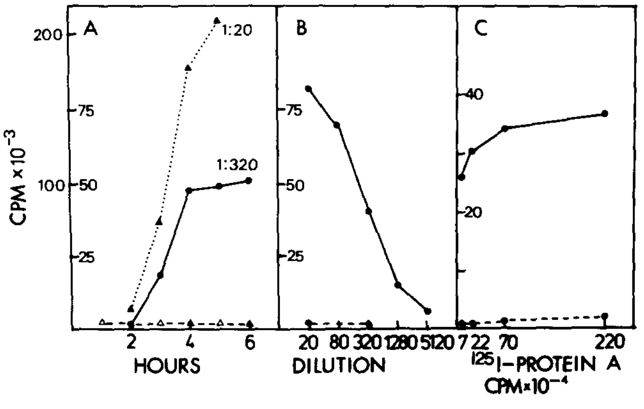

FIGURE 1 Parameters affecting the binding of ^{125}I-protein A to the surface of envelope-antiserum-treated SFV-infected CEF. Cells on 35-mm dishes infected with SFV and incubated at 37°C for indicated times (*A*) or for 5 h (*B* and *C*) were fixed in paraformaldehyde and treated with SFV envelope-antiserum followed by ^{125}I-protein A as described in Materials and Methods. (*A*) Appearance of virus glycoproteins to the cell surface during the course of SFV infection. Antiserum dilutions 1:20 (▲) (outer scale), 1:320 (●), (inner scale), normal rabbit serum 1:20 (△). (*B*) Effect of antiserum dilution on ^{125}I-protein A binding (●—●); normal rabbit serum (●- - -●). (*C*) Effect of the dose (cpm × 10^{-4} of ^{125}I-protein A: antiserum dilution 1:320 (●—●), normal rabbit serum (●- - -●). Radioactivity is expressed per 2 × 10^6 cells.

TABLE I

Specificity of the Antisera *

Antienvelope serum	^{125}I-protein A bound to cells infected with: SIN	SFV	Mock
		cpm	
SIN (1:80)‡	83,500	11,300	2,300
SFV (1:320)	4,600	53,000	900

* 35-mm dishes of CEF cells infected with HR and SFV were harvested at 6 and 5 h postinfection, respectively. The mock-infected cultures were harvested at 6 h. The cells were fixed with paraformaldehyde and treated with the respective antienvelope sera, followed by ^{125}I-protein A (220,000 cpm/dish) as described in Materials and Methods.

‡ Antiserum dilution.

labeled protein A were bound to uninfected cells or virus-infected cells treated with normal rabbit serum (Fig. 1). Treatment of SFV and SIN wild-type infected cells with heterologous antienvelope antibodies, showed low levels of cross-reaction, indicating that the reaction was also type specific (Table I).

Using the above procedure, we quantitated the amount of envelope proteins at the surface of cells infected with mutants ts-1 and ts-7 of SFV, as well as Ts-10 and Ts-23 of SIN (48, 49). For this purpose, the optimal dilution of anti-SIN envelope serum (49) was also determined. The mutant-infected cells were grown at the restrictive temperature (39°C) and some of the cultures were shifted to the permissive temperature (28°C) for 60 min. Cycloheximide (100 μg/ml) was added to the culture medium at the shift moment to prevent further protein synthesis. From the four mutants ts-1 and Ts-10 gave the lowest binding of radioactivity at 39°C and the clearest increase of ^{125}I-protein A binding after shift to 28°C, and were therefore selected for further studies. The appearance of virus glycoproteins at the plasma membrane in ts-1- and TS-10-infected cells, after shift to the permissive temperature, is shown in Fig. 2. In this experiment, the infected cells were first incubated at 39°C for 5 h (ts-1) and 6 h (Ts-10), followed by a shift to 28°C. The amount of radioactivity started to increase after a lag period of ~20 min, and there was about a fivefold increase in the cell-bound radioactivity during the 2-h incubation period at 28°C. After a 2-h incubation at 28°C, the observed total amount of ^{125}I-protein bound to cells infected with SFV ts-1 was approximately one third of that bound to cells infected with SFV wild type harvested at 5 h after infection, and represented roughly the situation at 3 h in the normal infection. The corresponding value with Ts-10 was somewhat lower, being 20% of the SIN HR wild-type value. When the antisera were used in higher concentrations, the total amount of bound ^{125}I-protein A was higher, but the kinetics of labeling remained the same. The higher values are most probably caused by binding of several antibody molecules per glycoprotein. Therefore the experiments were done with lower antibody dilutions.

Intracellular Localization of the Transport of Virus Glycoproteins

For intracellular localization of virus glycoproteins in fluorescence microscopy, lectins coupled to fluorochromes (FITC and TRITC) were used in double-labeling experiments together with antienvelope antibodies. The regions of the endoplasmic reticulum (ER) and Golgi apparatus were located in paraformaldehyde fixed and detergent treated cells with FITC or TRITC conjugates of Con A or WGA, respectively. We

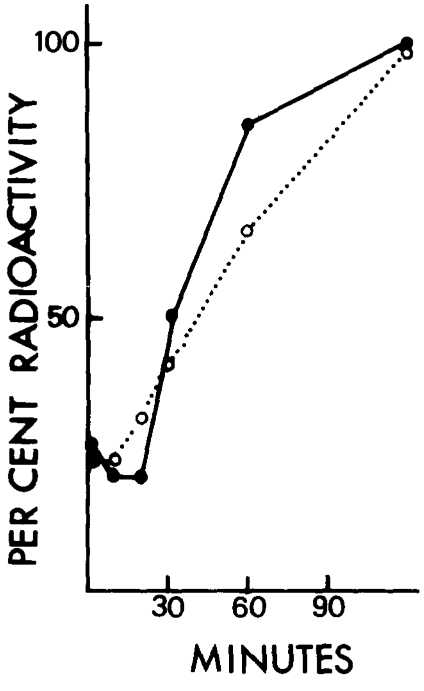

FIGURE 2 Transport of virus glycoproteins to the cell surface after shift of the mutant-infected cultures to permissive temperature. SFV ts-1 and SIN Ts-10 mutant-infected CEF cells were incubated at 39°C for 5 and 6 h, respectively. 5 min before shift, 100 μg/ml of cycloheximide was added to the medium. A prewarmed medium (28°C) containing cycloheximide was added at the shift moment and the cultures were transferred to 28°C. Two dishes were removed at the times indicated, fixed in paraformaldehyde, and treated with antienvelope antiserum. For ts-1 1:320 and for Ts-10 1:80 dilutions of SFV and SIN virus antisera were used, respectively, followed by addition of ^{125}I-protein A (220,000 cpm/dish). SFV ts-1 (●); SIN Ts-10 (○).

have recently shown that both the ER and Golgi apparatus can be highly specifically decorated with these lectins in different types of cells (63) and similar results were obtained also with CEF. Surface labeling of uninfected CEF with FITC-Con A gave an even staining over the whole cell area (Fig. 3*A*), whereas in the same cells intracellular labeling with TRITC-Con A decorated a different perinuclear cytoplasmic area, supposedly the region of ER (17, 63) in these cells (Fig. 3*B*). On the other hand, when the cells were double-stained intracellularly with FITC-Con A and TRITC-WGA, the perinuclear cytoplasmic staining seen with FITC-Con A (Fig. 3*C*) distinctly differed from the juxtanuclear reticular fluorescence obtained with TRITC-WGA (Fig. 3*D*) which was used as a marker for the region of Golgi apparatus (63).

As immunoglobulins are glycoproteins and react in some instances with lectins (29), the applicability of the lectin-staining methods in combination with immunofluorescence stainings was also studied (see also reference 63). Accordingly, uninfected CEF were first reacted with unlabeled Con A or WGA, followed by FITC/TRITC coupled anti-rabbit IgG. After these stainings no fluorescence was seen in the cells. On the other hand, cells first stained with FITC/TRITC-Con A or FITC/TRITC-WGA followed by normal rabbit serum and TRITC/FITC coupled anti-rabbit IgG showed no IgG-specific fluorescence in double staining, although the lectin-specific fluorescence could now be seen. This is shown for TRITC-WGA fluorescence in Fig. 3 (*E* and *F*).

When the ts-1-infected cells, maintained at the restrictive temperature throughout the infection, were stained for virus envelope proteins in indirect immunofluorescence, a cytoplas-

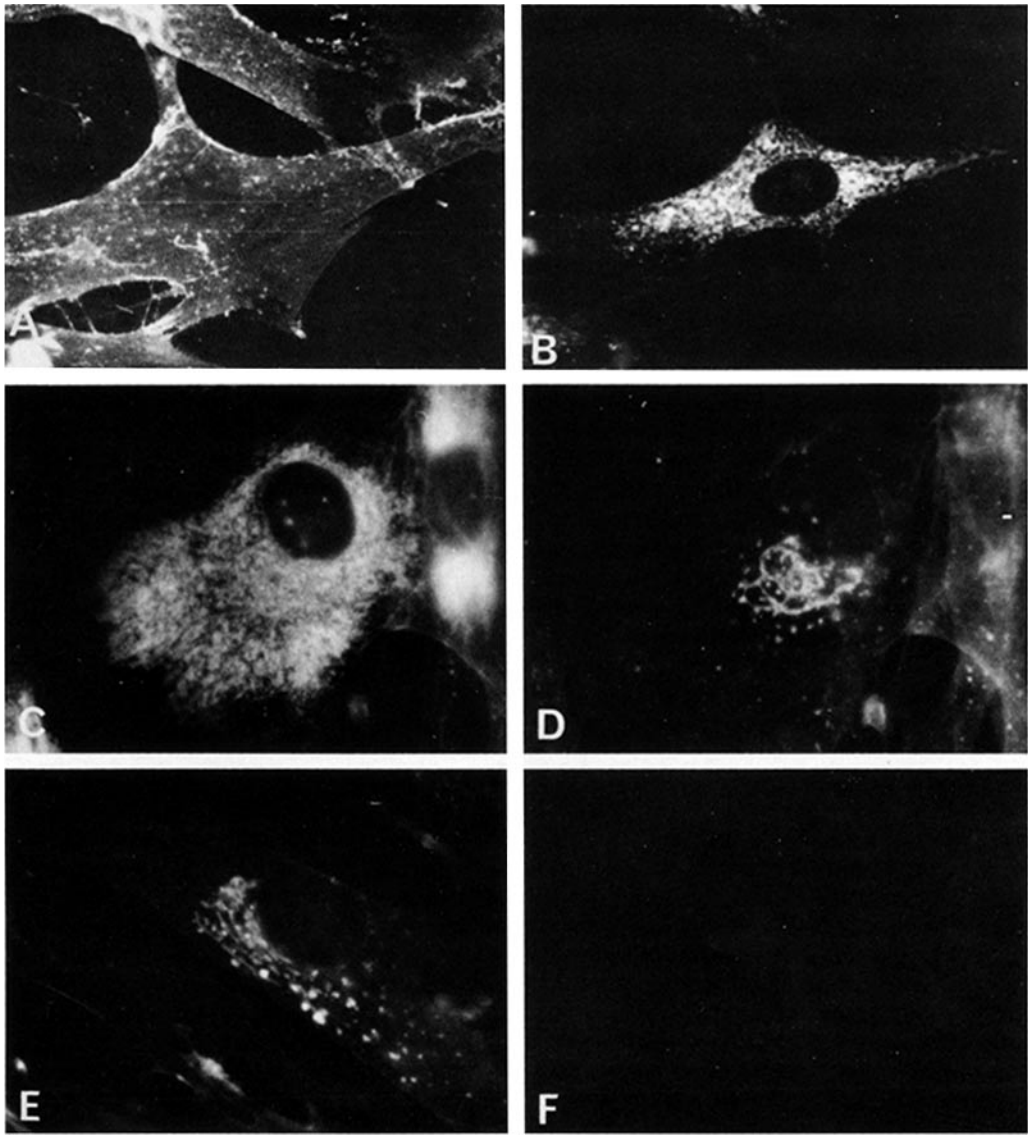

FIGURE 3 Subcellular distribution of lectin-binding sites in uninfected CEF. CEF cells, fixed in paraformaldehyde and permeabilized with 0.05% Triton X-100 (except in *A*) were labeled with lectins (Con A/WGA) coupled to fluorochromes (FITC/TRITC). (*A*) Staining of the cell surface with FITC-Con A. (*B*) In the same cells Triton X-100 treatment followed by TRITC-Con A staining, which should reveal the perinuclear ER. (*C*) The same structure visualized by FITC-Con A staining. (*D*) Localization by TRITC-WGA of a juxtanuclear structure which should correspond to the Golgi apparatus (63) in the same cell. (*E*) The same structure (Golgi complex) is decorated in cells labeled with TRITC-WGA. (*F*) The same cells treated with normal serum after TRITC-WGA followed by staining with FITC-conjugated anti-rabbit IgG. No nonspecific binding of IgG takes palce to WGA. × 480.

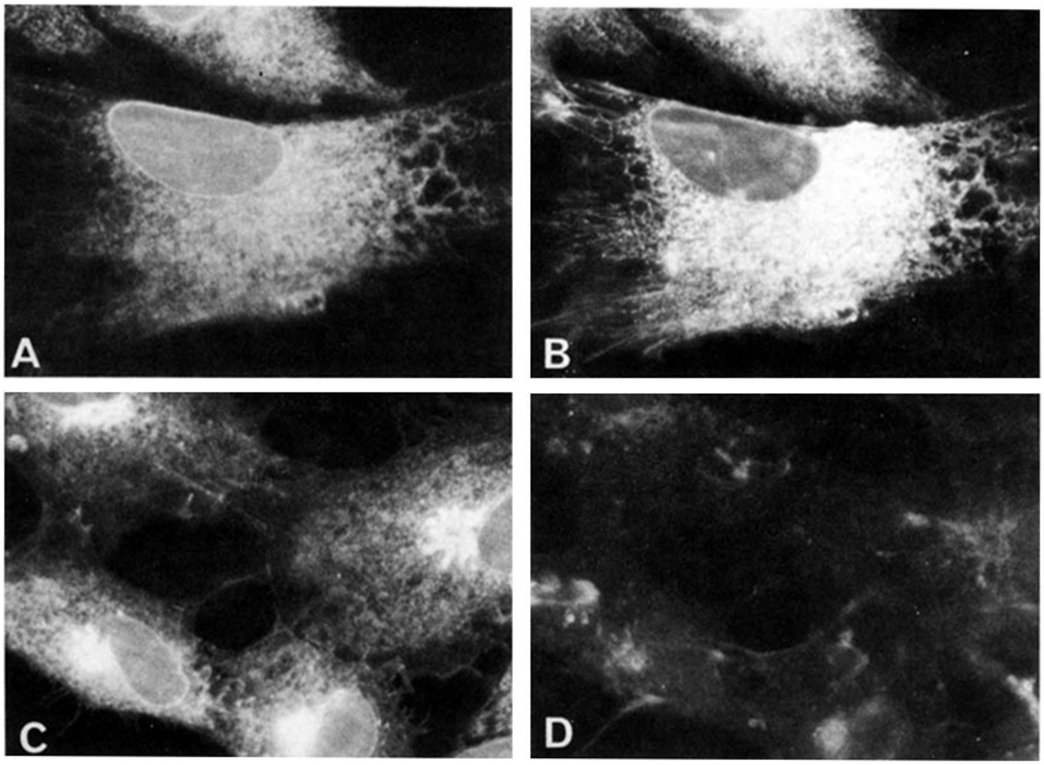

FIGURE 4 Intracellular localization of virus glycoproteins in SFV ts-1-infected cells maintained at the restrictive temperature (39°C) or shifted to the permissive temperature (28°C). Cells infected with ts-1 were maintained at 39°C for 5 h after infection (*A* and *B*) or shifted to 28°C for 60 min (*C* and *D*). Cycloheximide, 100 μg/ml, was added 5 min before shift down. The cells were fixed in paraformaldehyde and treated with 0.05% Triton X-100 before labeling. (*A*) In cells maintained at 39°C, labeling with SFV envelope antiserum followed by TRITC-conjugated anti-rabbit IgG gives an intracellular reticular fluorescence together with the staining of the nuclear periphery. (*B*) In the same cells an identical intracellular fluorescence is obtained with FITC-Con A. (*C*) In ts-1-infected cells, shifted to 28°C for 60 min in the presence of cycloheximide, labeling with envelope antiserum and FITC-conjugated anti-IgG localizes the juxtanuclear reticular structure as evidenced by colabeling the same cells with TRITC-WGA (*D*). × 700.

mic reticular fluorescence was seen together with a bright staining on the nuclear periphery (Fig. 4*A*). In double staining the immunofluorescence codistributed with that obtained with FITC-Con A (Fig. 4*B*), suggesting that the ts-1 glycoproteins were localized mainly in the membranes of the RER in cells maintained at the restrictive temperature. Similar intracellular immunofluorescence was also seen in SIN Ts-10-infected cells, incubated at 39°C (Fig. 6*A*).

When the ts-1-infected cultures were shifted to the permissive temperature (28°C) for 1 h in the presence of cycloheximide, a different distribution of the intracellular immunofluorescence was seen. In ~80% of the infected cells, a bright juxtanuclear reticular immunofluorescence was visualized which coincided with TRITC-WGA staining in double-fluorescence studies (Fig. 4*C* and *D*). This result would suggest that the ts-1 glycoproteins synthesized at 39°C, were transported to the Golgi complex when the infected cells were shifted to the permissive temperature.

Effect of Drugs on the Transport of Virus Glycoproteins

According to the above results, the virus glycoproteins of SFV ts-1 and SIN Ts-10 remain in RER when the infected cells are incubated at the restrictive temperature. The transport defect is reversed and the proteins are transported to the plasma membrane when the cultures are shifted to the permissive temperature. The temperature shift experiments were used to study the effect of drugs on the intracellular transfer of virus membrane glycoproteins. We tested the effect of cytochalasin B and vinblastine sulfate, drugs that affect the integrity of the cytoskeleton. We also tested two other agents, monensin and FCCP, which have recently been shown to inhibit the intracellular transport of secretory proteins (55, 58–61). The above drugs were added to the mutant-infected cultures 30 min before the shift to the permissive temperature. In all cases cycloheximide (100 μg/ml) was also present in the medium during the low temperature incubation. The drug-treated cells were harvested after a 2-h incubation at 28°C and the amount of ^{125}I-protein A bound to the cells was determined as described above.

The effects of cytochalasin B and vinblastine sulfate treatments were confirmed in indirect immunofluorescence microscopy using specific antibodies against actin and tubulin, respectively. Cytochalasin B treatment disrupted all the microfilaments, whereas vinblastine sulfate treatment resulted in a disruption of microtubules (results not shown).

As shown in Table II, cytochalasin B had only a marginal effect on the appearance of virus glycoproteins to the cell surface, whereas vinblastine caused an inhibition of 50–60%. The most effective inhibitors were the carboxylic ionophore monensin and FCCP, an uncoupler of oxidative phosphorylation. These two agents inhibited the transport of virus glycoproteins by 80–100%.

Steps of Intracellular Transport Effected by Monensin and FCCP

Addition of monensin (10 μM) to the culture medium at the moment of shift to 28°C, resulted in intensive staining of the juxtanuclear region in 85% of the ts-1-infected cells (Fig. 5 *A*). In double-labeled cells the immunofluorescence coincided with that of rhodamine-WGA (Fig. 5 *B*). Negligible surface immunofluorescence was demonstrable in the monensin-treated cells (Fig. 5 *C*). Similar results were obtained with monensin in the Ts-10-infected cultures, as demonstrated by intracellular immunofluorescence in Fig. 6. These results would mean that the virus glycoproteins were transported to the Golgi apparatus but not to the cell surface in the presence of monensin.

TABLE II

Effect of Drugs on the Transport of Virus Glycoproteins to the Surface of SFV ts-1- and SIN Ts-10-infected Cells after Shift of the Cultures to the Permissive Temperature

Inhibitor	Concn	Binding of ^{125}I-protein A* ts-1	Binding of ^{125}I-protein A* Ts-10
		% of control	
Monensin	10 μM	9	24
FCCP	10 μM	0	13
Vinblastine	10 μg/ml	52	42
Cytochalasin B	1 μg/ml	83	97

* Infected cultures were incubated at 39°C and the drugs were added to one set of the cultures 30 min before the shift of the cultures to 28°C at 5 h (ts-1) or 6 h (Ts-10) after infection. All cultures were harvested after a 2-h incubation at 28°C in the presence of 100 μg/ml of cycloheximide. The amount of radioactivity bound to the cells at 39°C was substrated from the value obtained at 2 h after shift for both the nontreated and drug-treated cultures to give an estimate for the amount of transported proteins.

Similar studies with FCCP (10 or 20 μM) showed a faint immunofluorescence of the juxtanuclear reticular structure only in ∼20% of ts-1-infected cells that had been shifted to the permissive temperature (Fig. 5 *D*). Neither was surface immunofluorescence seen in the drug-treated cultures. This was true also for SIN Ts-10-infected cultures. On the basis of the above results, it seems that the migration of glycoproteins from the ER is inhibited by FCCP.

According to the intracellular immunofluorescence, the virus glycoproteins could migrate to the Golgi complex in the presence of monensin, but probably not further as has been shown for the secretory IgM (58). We reasoned that by delaying the addition of monensin to the ts-1-infected cultures until after they had been shifted to 28°C, it might be possible to estimate the time of transport of virus glycoproteins from the Golgi complex to the cell surface. For this purpose, a more accurate time-course of the appearance of glycoproteins to the cell surface was required (Fig. 7). The ts-1-infected cultures were fixed at 10-min intervals after shift to the permissive temperature. Monensin was added to some of the cultures before shift and at different times after the shift to 28°C. The difference between ^{125}I activity at 120 min at 28°C and that before shift was taken as a measure of the total transported glycoproteins.

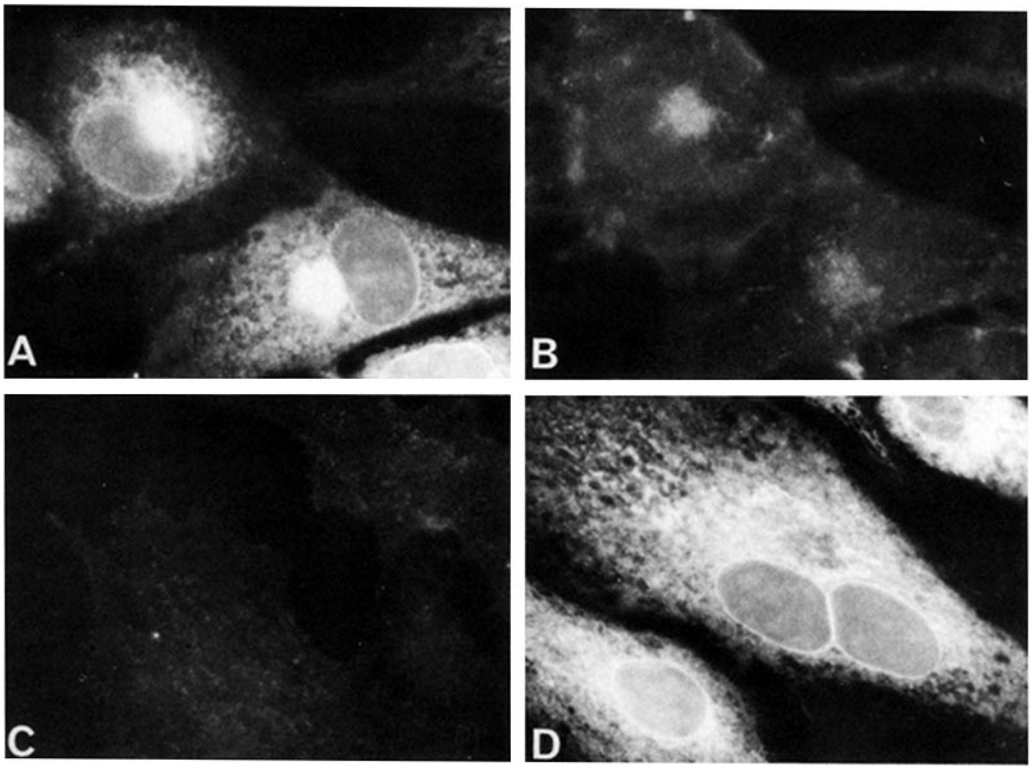

FIGURE 5 Effect of monensin and FCCP on the intracellular distribution of SFV ts-1 mutant glycoproteins. Cells infected with ts-1 were incubated at 39°C for 5 h. All the cultures were shifted to 28°C for 60 min in the presence or absence of the drugs. After fixation in paraformaldehyde, the cells were treated with 0.05% Triton X-100 (except *C*). (*A*) ts-1-infected cells 60 min after shift to 28°C in the presence of 10 μM monensin labeled with envelope antiserum followed FITC-conjugated anti-IgG. Note the bright juxtanuclear fluorescence that in double staining coincides with TRITC-WGA (*B*), used to localize the Golgi complex. (*C*) Lack of surface immunofluorescence of ts-1-infected cells shifted to 28°C in the presence of monensin. (*D*) ts-1-Infected cells 60 min after shift to 28°C in the presence of 20 μM FCCP. Labeling with envelope antiserum and FITC-conjugated anti-IgG gives a wide perinuclear fluorescence as well as the staining of the nuclear periphery.

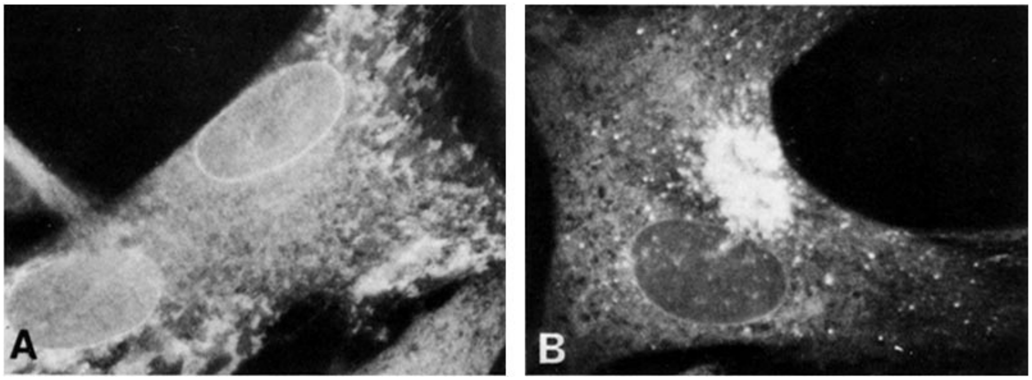

FIGURE 6 Effect of monensin on the transport of SIN Ts-10 mutant glycoproteins. Infected cells were incubated at 39°C for 6 h (*A*) followed by shift to 28°C for 60 min (*B*). The cells were fixed with paraformaldehyde followed by treatment with Triton to reveal the intracellular antigens. (*A*) Ts-10-infected cells after a 6-h incubation at 39°C stained with FITC-conjugated anti-IgG after treatment with SIN envelope antiserum. Note the wide intracellular reticular fluorescence and the staining of the nuclear periphery. (*B*) Ts-10-infected cells 60 min after shift to 28°C in the presence of 10 μM monensin. Note the bright juxtanuclear fluorescence, which in double staining coincided with that obtained with TRITC-WGA (not shown). × 700.

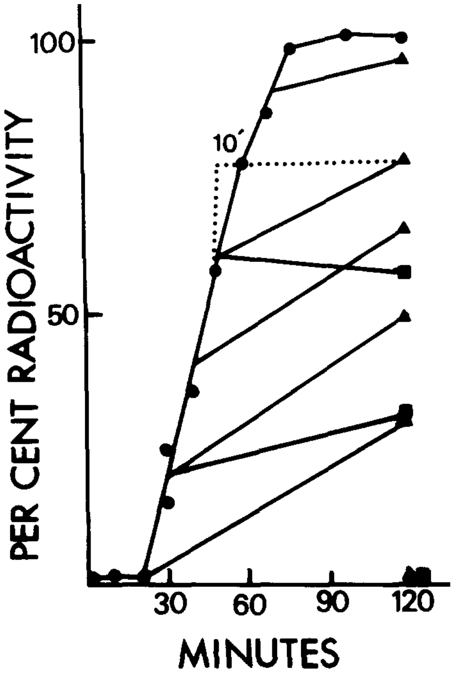

FIGURE 7 Effect of monensin and FCCP on the time-course of appearance of virus glycoproteins to the cell surface after the ts-1-infected cultures had been shifted from 39° to 28°C. ts-1-Infected cultures were incubated at 39°C for 5 h and shifted to 28°C in the presence of cycloheximide (100 μg/ml). At 10-min intervals, two dishes were fixed in paraformaldehyde. To another set of similarly treated cultures monensin (10 μM, ▲) or FCCP (20 μM, ■) was added at the indicated times and the cultures were incubated up to 2 h before fixation and treatment with envelope antiserum (1:320) followed ^{125}I-protein A. The delay of monensin inhibition on the appearance of glycoproteins was determined as shown for the 50-min time point. When monensin was added 50 min after shift down, the radioactivity continued to increase, reaching the level obtained at 60 min without the drugs. ●, No drug.

The time-course without monensin (Fig. 7) indicates that the glycoproteins appear at the cell surface between 20 and 80 min with an almost constant rate. The later monensin was added, the more glycoproteins were transported to the cell surface. Monensin seemed to stop the transport within 10–15 min after addition. This was estimated as shown for the 50-min time point in Fig. 7. The ionophores such as monensin start their action very rapidly (45), and thus the 10- to 15-min delay gives a rough estimate for the transport time from the Golgi complex to cell surface.

When FCCP was added 30 and 50 min after the ts-1-infected cultures were shifted to 28°C, the appearance of glycoproteins at the cell surface stopped almost instantaneously (Fig. 7).

DISCUSSION

In alphavirus-infected cells, the virus envelope glycoproteins are synthesized at the RER as parts of a polyprotein that contains all the sequences of the structural proteins of the virus (for reviews see references 23 and 57). The synthesis of the envelope proteins p62 (precursor virus glycoproteins E2 and E3) and E1 is coupled with their segregation into the cisternae of RER (6, 13, 65). The initial glycosylation of p62 and E1 apparently takes place when the nascent proteins protrude through the RER membrane (13, 23, 24, 31, 33, 51, 52), and the proteins remain anchored in the membrane by their hydrophobic parts (13, 14, 62). In normal infection, the virus glycoproteins are transported to the cell surface as dimers (7, 22, 56) and attain their complete oligosaccharide chains during this process (24, 53, 54).

Our previous studies using surface immunofluorescence showed that in cells infected with certain alphavirus temperature-sensitive mutants, the glycoproteins failed to be transported to the host cell plasma membrane when the cultures were incubated at the restrictive temperature. Once the mutant-infected cells were transferred to the permissive temperature, the preformed proteins appeared at the cell surface (48, 49). Radioimmunoassay with iodinated protein A was used in the present study to quantitate virus glycoproteins at the plasma membrane. By this method, small amounts of virus proteins were detected in the mutant-infected cells maintained at the restrictive temperature. This is probably caused by leakiness of the transport defect, which was not revealed by conventional immunofluorescence (48, 49).

In temperature shift experiments with SFV ts-1 and SIN Ts-10, there was a lag period of ~20 min before the glycoproteins started to appear at the cell surface. This time period is similar to the observed time of transport of virus membrane glycoproteins from their site of synthesis to the cell surface (1, 28). The lag period was followed by a transport period of ~60 min. During this time the rate of accumulation of the glycoproteins at the plasma membrane was almost constant.

Previous studies have suggested that microfilaments and especially microtubules have important roles in the intracellular transfer of secretory proteins (9, 11, 37, 44). Our results with cytochalasin B indicate that the actin-containing microfilaments are not required for the transport of virus membrane glycoproteins. The partial inhibition obtained with vinblastine could be a secondary effect caused by the loss of the integrity of the Golgi complex, which is probably maintained by the microtubular system (11, 37). Richardson and Vance (46) have shown before that colchicine and dibucaine do not inhibit the transport of SFV glycoproteins to the cell surface.

In contrast to the cytoskeleton-disrupting agents, both the carboxylic ionophore monensin and FCCP, an uncoupler of oxidative phosphorylation, efficiently inhibited the appearance of virus membrane proteins to the cell surface. Consequently, it appears that the transport of virus membrane glycoproteins is sensitive to energy depletion (FCCP) or Na/K equilibration (monensin) in cells, as is the transport of secretory glycoproteins both in regulated and nonregulated cells (58–60). Based on their studies of IgM secretion in plasma cells, Tartakoff and colleagues have suggested that the inhibition of energy production in the cells by CCCP (a substrate related to FCCP) inhibits the exit of IgM molecules from RER. Furthermore, they propose that monensin affects the intracellular transport at the level of the Golgi apparatus by inhibiting the release of secretory vesicles from Golgi membranes that become dilated because of the effect of the drug (58, 60). We examined the effect of these drugs on the intracellular transport of SFV glycoproteins in double-fluorescence experiments utilizing lectins (17) as markers for subcellular compartments (30). As has recently been demonstrated for many types of cells, lectins can be used in intracellular localization of the perinuclear ER and the juxtanuclear Golgi apparatus, respectively (17, 63, 66). Here we used these techniques for the tentative intracellular localization of virus glycoprotein in infected CEF. The lectins did not react noticeably with immunoglobulins under the conditions used, and could therefore be used in double-staining experiments together with the indirect immunofluorescence technique.

According to the double-fluorescence studies, the glycoproteins in ts-1 and Ts-10 infected cells remain at RER in cells maintained at the restrictive temperature. When the infected cells were shifted to the permissive temperature, the appearance of the virus glycoproteins at the cell surface was accompanied by a bright immunofluorescence of the Golgi apparatus, suggesting that the proteins had been transported to this organelle. When the shift was carried out in the presence of FCCP, the distribution of the intracellular immunofluorescence did not markedly change and no concentration of immunofluorescence was seen in the Golgi complex. When ts-1- and Ts-10-infected cultures were shifted to the permissive temperature in the presence of monensin, an intensive immunofluorescence was observed in the cellular location double-stained with WGA-fluorochrome, suggesting that the glycoproteins had accumulated in the Golgi complex. In conclusion, these results support the idea that the intracellular transport of alphavirus membrane proteins is inhibited by monensin and FCCP in a manner very similar to that observed with secretory proteins (58–60).

FCCP also appeared to inhibit a later stage in the intracellular transfer of virus glycoproteins. Even when the drug was added as late as 50 min after shift of ts-1-infected cultures to the permissive temperature, the addition of FCCP resulted in an immediate cessation of the accumulation of glycoproteins at the cell surface. This could not be caused by an inhibition of the transport of proteins from RER to the Golgi apparatus because the transport process had already taken place for 50 min before the addition of the drug. These results with FCCP are very similar to those reported earlier for pancreas acinar cells (20, 21). In these cells, respiratory inhibitors block the transfer of secretory proteins both from RER to the Golgi apparatus as well as from the Golgi apparatus to the plasma membrane. In both cases the effect may be related to the inhibition of the fusion of secretory vesicles with target membrane, an event that requires energy from ATP (19). FCCP has been reported to inhibit internalization of lectins into neuronal GERL, a process dependent on fusion of vacuoles to Golgi apparatus membranes after endodytosis (16). In experiments where monensin was added at different times to cultures shifted to the permissive temperature, a delay in the action of the drug corresponding to 10 min was observed. Monensin does not inhibit the secretion of IgM molecules carrying distal galactose and fucose residues, implying that the further transport of molecules already released from the Golgi apparatus is not inhibited by the drug (60). Assuming that the same would also hold for membrane glycoproteins, the observed delay time gives an estimate for the time required for the transport of virus glycoproteins from the Golgi apparatus to the plasma membrane.

Two alphavirus temperature-sensitive mutants have been used to study the transport of the viral membrane glycoproteins. The intracellular immunofluorescence studies suggested that the transfer of the virus membrane glycoproteins takes place from RER through the Golgi complex to the plasma membrane. Different steps of this process could be inhibited by FCCP and monensin. According to our results, the transport of viral membrane glycoproteins and secretory proteins bear many similarities, among them two energy-dependent steps. These steps could be the fusion of virus glycoprotein carrying coated vesicles to the Golgi and plasma membranes, respectively (47).

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