Dissection of the Golgi Complex. II. Density Separation of Specific Golgi Functions in Virally Infected Cells Treated with Monensin

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ABSTRACT In the accompanying paper (Griffiths, G., P. Quinn, and G. Warren, 1983, *J. Cell Biol.*, 96: 835–850), we suggested that the Golgi stack could be divided into functionally distinct *cis*, *medial*, and *trans* compartments, each comprising one or two adjacent cisternae. These compartments were identified using Baby hamster kidney (BHK) cells infected with Semliki Forest virus (SFV) and treated with monensin. This drug blocked intracellular transport but not synthesis of the viral membrane proteins that were shown to accumulate in the *medial* cisternae. In consequence, these cisternae bound nucleocapsids. Here we show that this binding markedly increased the density of the *medial* cisternae and allowed us to separate them from *cis* and *trans* Golgi cisternae. A number of criteria were used to show that the intracellular capsid-binding membranes (ICBMs) observed in vivo were the same as those membranes sedimenting to a higher density in sucrose gradients in vitro, and this separation of cisternae was then used to investigate the distribution, within the Golgi stack, of some specific Golgi functions. After labeling for 2.5 min with [^{3}H]palmitate, most of the fatty acid attached to viral membrane proteins was found in the ICBM fraction. Because the viral membrane proteins appear to move from *cis* to *trans*, this suggests that fatty acylation occurs in the *cis* or *medial* Golgi cisternae. In contrast, the distribution of α_{1-2}-mannosidase, an enzyme involved in trimming high-mannose oligosaccharides, and of galactosyl transferase, which is involved in the construction of complex oligosaccharides, was not affected by monensin treatment. Together with data in the accompanying paper, this would restrict these two Golgi functions to the *trans* cisternae. Our data strongly support the view that Golgi functions have specific and discrete locations within the Golgi stack.

Understanding of the functions of the Golgi complex would be greatly facilitated if the component parts of the organelle could be resolved. There have been many attempts to do this, using the variations in the density (6), surface properties (15), and antigenicity (17) of Golgi membranes. In contrast to cytochemical results, which show sharp delimitations of activities (see references 2, 7, 12), these procedures have, at best, produced subfractions enriched in certain Golgi activities, with considerable amounts of these same activities being found in other fractions. Lack of information about the localization of any Golgi marker enzymes has made it impossible to determine whether the observed distributions of activity result from cross-contamination of the subfractions or reflect the distribution of these activities between different compartments which have been separated (1, 3).

One way to overcome this problem would be to perturb the physical properties of one specific part of the Golgi complex. This approach, using ethanol intoxication, has been tried with limited success (6, 9). If the perturbation were sufficient to cause a large change in the behavior of that particular subset of the Golgi membranes, it should be possible to measure a number of Golgi complex functions and ask whether or not they follow the change in behavior. Evidence for activities clearly following, or not following, the change would strongly suggest specific locations within the Golgi complex for those functions. Conversely, an activity partially following the change would suggest that it is spread over at least two compartments.

In the preceding paper (13), we were able to identify the site, within the Golgi complex, at which monensin blocked the

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transport of Semliki Forest virus (SFV) membrane proteins. This was possible because, although monensin blocked transport of these proteins, it did not prevent their synthesis. In consequence, they accumulated in the compartment before the block, and there bound viral nucleocapsids. These intracellular capsid-binding membranes (ICBMs) were morphologically distinct from other Golgi cisternae. Their origin was determined using a variety of biochemical and cytochemical techniques, and they were shown to be derived from one or two cisternae from the middle of the stack which we termed *medial* cisternae. We were thus able to define three distinct compartments, *cis*, *medial* and *trans*, each comprising one or two adjacent cisternae in the Golgi stack (13).

Viral nucleocapsids are very dense (19) and membranes binding them in large numbers, a property possessed uniquely by *medial* cisternae in monensin-treated, SFV-infected cells, should therefore have altered physical properties. They should, for example, have a higher equilibrium density than the other cisternal membranes. If such a difference were to allow discrimination of the two membrane populations, it would be possible to investigate the distribution between them of Golgi functions. Here we show that in SFV-infected cells treated with monensin such a discrimination between Golgi compartments is possible and can be used to localize functions within the Golgi stack.

MATERIALS AND METHODS

These were all as previously described (10), with the additions or modifications that follow.

Cell Fractionation: Confluent Baby hamster kidney BHK cells on 10-cm dishes were scraped with a rubber policeman into ice-cold phosphate-buffered saline (PBS), and all subsequent operations performed at 4°C. They were washed three times by centrifugation (1,000 *g*, 5 min) and resuspension in PBS. After the final wash, the cells were suspended in ≥10 vols of 10 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, and collected by centrifugation (1,000 *g*, 10 min). The pellet of swollen cells was suspended in 1 vol of the same buffer containing 40 μg/ml phenylmethylsulphonyl fluoride (PMSF), and the cells were disrupted with 10 strokes of an all-glass Dounce homogenizer. The homogenate was immediately adjusted to 250 mM sucrose, 20 mM PIPES, pH 7.0, 5 mM KCl, 1 mM $MgCl_2$ by addition of a 2× stock solution. Postnuclear supernatants were prepared by centrifugation (600 *g*, 5 min) and layered over continuous sucrose gradients prepared from 6 ml each of 500 mM and 2 M sucrose solutions, both containing 10 mM PIPES, pH 7.0, 5 mM KCl, 1 mM $MgCl_2$. Gradients were centrifuged at 30,000 rpm for 16 h (Beckman SW40; Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) and fractions were collected from the bottom of the tube.

Labeling with [^{3}H]Palmitic Acid: [^{3}H]Palmitic acid (20 Ci/mmol, New England Nuclear, W. Germany) was lyophilized and redissolved in ethanol. 10-cm dishes of confluent BHK cells were labeled 6 h after infection. They were washed twice with prewarmed PBS and then incubated at 37°C for 2.5 min with 5 ml of prewarmed medium containing 0.5 mCi/ml [^{3}H]palmitic acid. For monensin-treated cells, the culture medium was adjusted to 10 μM monensin 2-h postinfection. This concentration of monensin was maintained in all subsequent incubation media.

Quantitation of Radioactive Virus on Polyacrylamide Gels: [^{3}H]palmitic acid is rapidly incorporated into cellular phospholipid, giving a high background to assays for labeling of protein. To overcome this, protein was collected from sucrose gradient fractions by precipitation with trichloroacetic acid in the presence of 100 μg of gelatin carrier. The precipitates were washed with ethanol and ether, dissolved in sample buffer (10), and resolved by SDS PAGE to separate viral proteins from other labeled species. After staining, individual gel tracks were excised with a scalpel and frozen on dry ice. Each track was cut into 2-mm slices which were dissolved in 0.5 ml of 9% (wt/vol) H_2O_2, 10 mM HCl by incubation at 60°C for 48 h, and counted in 10 ml Rotizint 22 (Roth GmbH, Karlsruhe, W. Germany). Radioactivity in the viral protein was determined by comparison with [^{35}S]methionine-labeled virus and molecular-weight markers run in parallel tracks. Immunoblotting was performed by the method of Burnette (4), modified as described previously (12).

Assay of α_{1-2}-Mannosidase Activity: This was performed as described by Tabas and Kornfeld (27), except that the [^{3}H]mannose-labeled glycopeptides for use as substrate were prepared from SFV-infected BHK cells treated with monensin to prevent oligosaccharide processing (13). Labeling with [^{3}H]mannose, harvesting of the cells, proteolysis and purification of the glycopeptides were done as described (10), except that the final chromatographic step on Biogel P4 was performed using water as the eluent. The high-mannose glycopeptides were collected, lyophilized, and redissolved to 10^6 cpm/ml in 50 mM sodium phosphate, pH 6.5, 5 mM $MgCl_2$, 0.1% (wt/vol) Triton X-100 for use in the assay. As the mannosidase assay demands small volumes, some concentration of the gradient fractions was needed to obtain measurable activities. Since mannosidase is a membrane protein (27) it was selectively extracted into Triton X-114 (2). This resulted in a 20-fold increase in concentration of both galactosyl transferase and mannosidase with no loss of activity and made it possible to use almost all of the gradient fractions for the assays.

Morphometric Estimation of the Volume Density of ICBMs in Fractions from the Sucrose Gradient: The fractions from the sucrose gradient were adjusted to 0.25 M sucrose and the membranes collected by centrifugation (100,000 *g*, 60 min). The pellets were suspended in 1% glutaraldehyde in 100 mM PIPES (pH 7.0) and left at 20°C for 30 min. The fixed membranes were collected by microcentrifugation. Further processing for electron microscopy was as described previously (12). Three blocks from each pellet were randomly sectioned. Fifteen micrographs were taken for each fraction at the first grid corners where tissue was seen, at a primary magnification of × 7,000, and enlarged photographically to give a final magnification of × 23,000. All clearly recognizable ICBM structures were marked and a transparent square lattice overlay (with 0.5-cm squares) was placed over each micrograph (28). All corner points (P) which covered ICBMs were counted and related to the total number of points over the micrograph. The ratio P_{ICBM}/P_{TOTAL} gives the volume density of ICBMs in each fraction.

RESULTS

ICBMS Are Denser than Normal Golgi Cisternae

SFV-infected cells were labeled with [^{3}H]mannose for 10 min in the presence or absence of monensin. Under these conditions, only viral membrane proteins were labeled (Fig. 1) and [^{3}H]mannose incorporated into protein could therefore be used to assay the viral membrane proteins. The cells were then incubated for 30 min in unlabeled media to allow transport of the tritiated viral proteins to the Golgi complex (10). Cells were harvested and postnuclear supernatants centrifuged to equilibrium through continuous sucrose gradients. These were fractionated and assayed for [^{3}H]mannose incorporated into protein. The results showed that the Golgi membranes containing the viral proteins in monensin-treated cells equilibrated at a higher density than those from the untreated cells (Fig. 2).

These higher density membranes containing viral membrane proteins obtained from monensin-treated cells were shown to be the ICBMs seen in vivo by the following biochemical and morphological criteria.

EQUILIBRIUM DENSITY OF THE HIGH DENSITY MEMBRANE FRACTION COULD BE LOWERED BY CONDITIONS THAT SPECIFICALLY REMOVED NUCLEOCAPSIDS: ICBMs would be expected to have a higher density than normal Golgi membranes because of the dense nucleocapsids bound to the accumulated viral membrane proteins. Removal of the capsids should therefore lower the equilibrium density of the membranes. Infected cells were labeled with [^{3}H]mannose in the presence of monensin, incubated in unlabeled medium for 30 min, and fractionated on sucrose gradients. The fractions containing the labeled viral protein were pooled and the membranes collected by centrifugation after dilution of the sucrose to 0.25 M. The pellet was suspended in 0.25 M buffered sucrose and divided into two portions. One portion, with no further treatment, was re-run on a density gradient identical to that used in the original preparation. The other was adjusted to 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and refractionated on a gradient prepared in this buffer. These latter conditions are known to disrupt specifically the interaction between the nucleocapsids and the

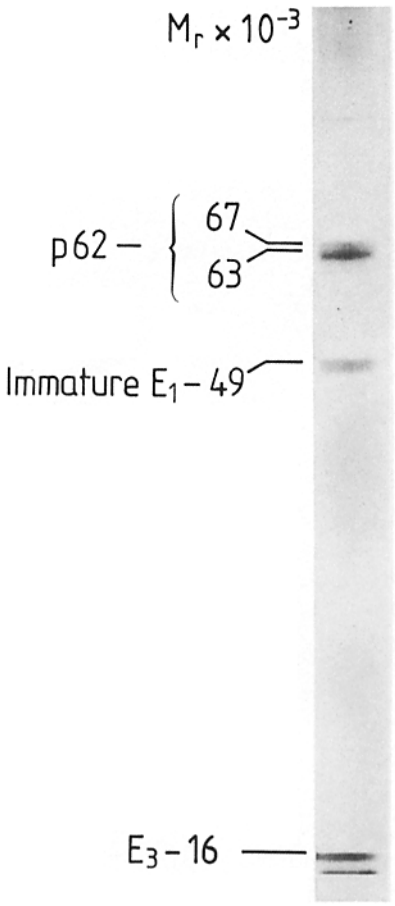


FIGURE 1 SDS PAGE of proteins from SFV-infected BHK cells labeled with [^{3}H]mannose for 10 min. Essentially only viral glycoproteins were labeled.

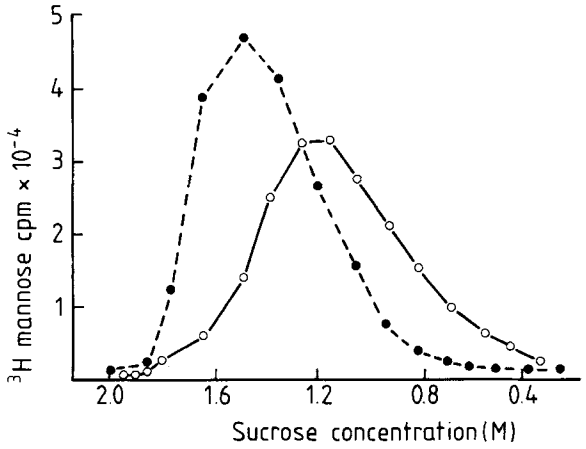


FIGURE 2 Density gradient fractionation of SFV-infected BHK cells. The cells were labeled for 15 min with [^{3}H]mannose and then incubated with excess unlabeled mannose for 30 min. Postnuclear supernatants were fractionated on linear sucrose gradients, and the fractions assayed for [^{3}H]mannose labeled protein. Monensin-treated cells (●) were treated with 10 μM monensin from a 10 mM stock in ethanol 3 h before labeling, and this concentration of monensin was maintained in all subsequent incubation media until the cells were harvested. Control cells (○) were treated with the same volume of ethanol without monensin at the same times.

viral spike proteins (14). Fractions were collected from the gradients and assayed for viral protein. Collection and centrifugation of the membranes per se had no effect on their equilibrium density. The treatment that removed nucleocapsids, however, caused the membranes to re-equilibrate at a lower density, not significantly different from that of normal Golgi membranes (Fig. 3; cf. Fig. 2).

MORPHOLOGY OF THE HIGH DENSITY MEMBRANE FRACTION: A preparation of infected BHK cells treated with monensin was fractionated and the membranes enriched in spike proteins were collected, fixed, and prepared for electron microscopy. Fig. 4 is a low magnification field from an Epon-embedded sample. By comparison with Fig. 5 which shows the appearance of ICBMs in vivo, it is clear that the fraction contained many profiles easily identifiable as ICBMs (arrows) by the nucleocapsids bound to the cytoplasmic face and the virus particles budded into the lumen. Only clearly identifiable ICBM structures are indicated. There are also significant numbers of vacuoles which we believe are ICBM structures that have been damaged during the fractionation.

MORPHOMETRY: To confirm that the distribution of morphologically recognizable ICBMs across the gradient was the same as that found for labeled viral membrane proteins, we fractionated infected BHK cells treated with monensin on sucrose gradients. Each fractionation was prepared for electron microscopy and the volume densities of ICBMs in each were determined by morphometric analysis. An approximation of the relative pellet volumes was obtained by measurement of total protein. The product of the two values thus reflects the relative numbers of ICBMs in each fraction. The results showed an excellent correlation between the distribution of clearly recognizable ICBMs and that of labeled viral protein (Fig. 6). We concluded, therefore, that the high density membranes containing viral membrane proteins, found in monensin-treated SFV-infected BHK cells, were the ICBMs observed in the intact cells.

Localization of Golgi Functions

Since the density of the ICBM fraction, which derives from the *medial* cisternae of the Golgi complex stack, is greater than that of normal Golgi cisternae, at least two parts of the Golgi complex stack can be discriminated in vitro and activities thus assigned to locations within the stack of Golgi cisternae.

FATTY ACID ADDITION: The kinetics of fatty acid addition to newly synthesized viral membrane proteins suggest that it

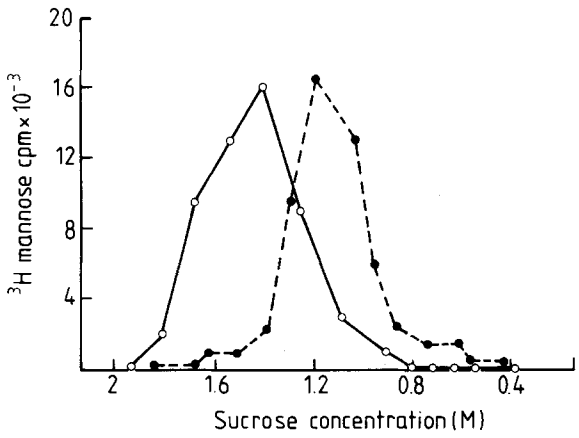
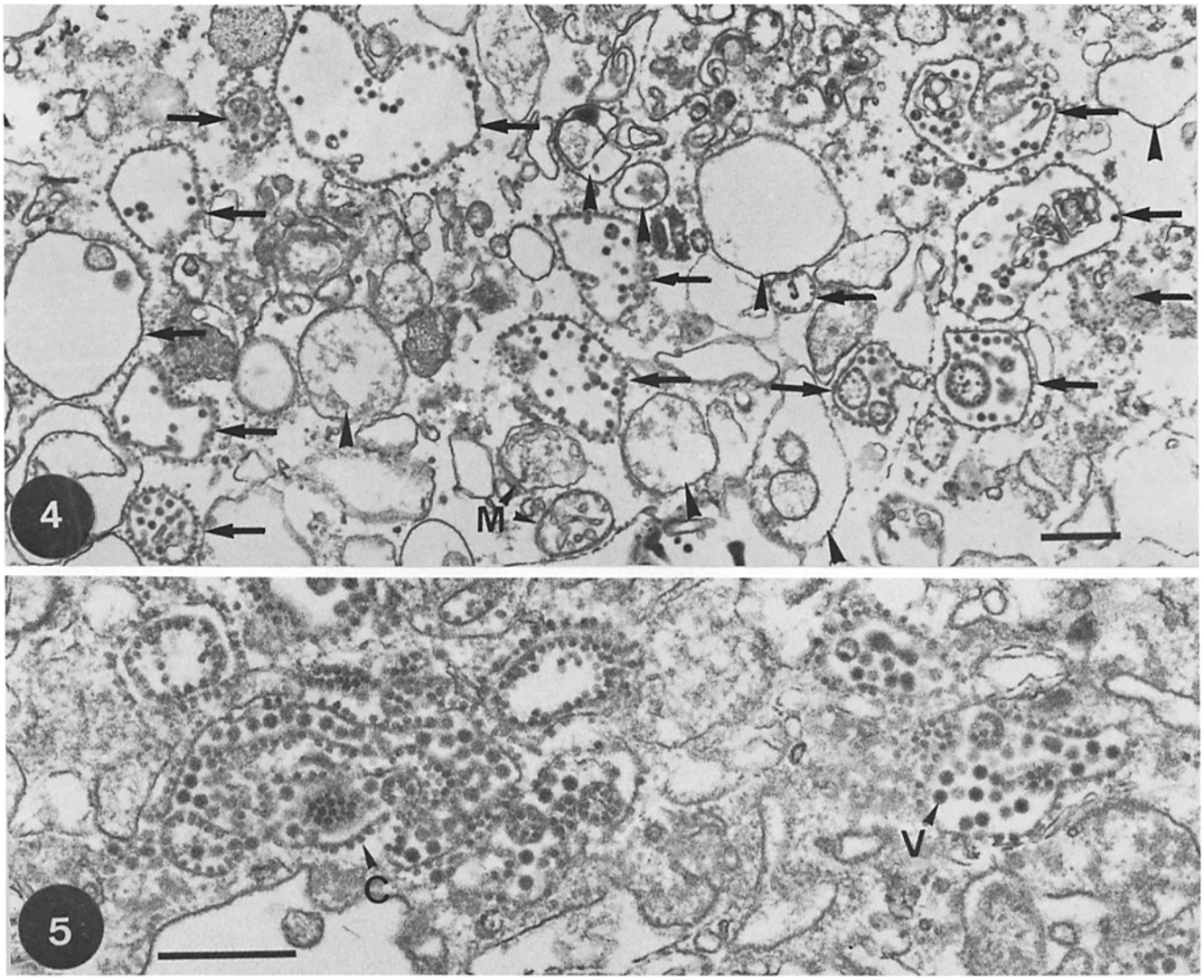


FIGURE 3 Membranes containing [^{3}H]mannose-labeled viral protein prepared from monensin treated cells refractionated on sucrose gradients. Samples were re-run under conditions permitting the interaction between viral membrane proteins and nucleocapsids (○) or under conditions known to disrupt this interaction (●).



FIGURES 4 and 5 Fig. 4: A section through a pellet of the high density membrane fraction obtained from monensin-treated, SFV-infected cells. The arrows indicate ICBM structures which are identical to those seen in vivo (cf. Fig. 5). The arrowheads indicate structures which may be ICBMs from which the nucleocapsids have been lost during fractionation. *M*, Mitochondria. Bar, 0.5 μm. × 23,000. Fig. 5: Part of the perinuclear cytoplasm of a BHK cell infected with SFV and treated with 10 μM monensin, showing typical ICBM structures (arrows). Most of these have nucleocapsids (*C*) on their cytoplasmic surface and budded virions on the luminal side (*V*). Bar, 0.4 μm. × 50,000.

occurs at about the same time as the proteins are being transferred to the Golgi complex, and may therefore occur in transitional elements or *cis* Golgi cisternae (26). At present this activity cannot be assayed in vitro, and it was therefore investigated by the use of short time-period incorporation of ^{3}H-labeled palmitic acid into viral proteins in vivo. Infected cells, preincubated with or without monensin, were labeled for 2.5 min with [^{3}H]palmitic acid and then fractionated. Protein from each fraction was collected and run on polyacrylamide gels which were then sliced and assayed for ^{3}H label. The radioactivity in viral protein in each fraction is shown in Fig. 7. Even after only 2.5 min, most of the palmitic acid–labeled viral protein was found in the ICBM fraction.

GALACTOSYL TRANSFERASE: The biochemical function first attributed to the Golgi complex was transfer of terminal sugars to the trimmed oligosaccharide of newly synthesized proteins (8, 20). The enzymes involved, especially galactosyl transferase, have been widely used as biochemical markers for the Golgi complex during isolation.

Infected cells were labeled with [^{3}H]mannose in the presence or absence of monensin. Postnuclear supernatants were centrifuged on sucrose gradients and the fractions obtained were assayed for ^{3}H-labeled protein and galactosyl transferase activity. The results showed that in the absence of monensin, viral protein and galactosyl transferase distributed through the gradient in the same manner (Fig. 8 *a*). After monensin treatment, the membranes containing the most labeled viral protein equilibrated at a higher density, while the density of those membranes containing galactosyl transferase was unchanged (Fig. 8 *b*). Clearly, the ICBMs can be distinguished from that part of the Golgi stack containing galactosyl transferase activity. This strongly suggests that at least this terminal transferase activity is absent from the *medial* Golgi membranes.

$\alpha_{1\text{-}2}$-MANNOSIDASE ACTIVITY: Trimming of the core sugars found on newly synthesized proteins must occur before the proteins can accept sugars from the terminal transferases (see

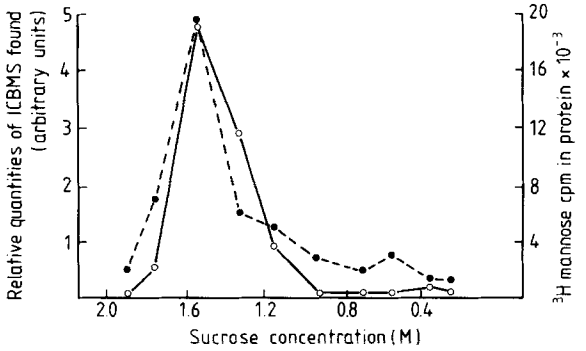


FIGURE 6 Density gradient distribution of ICBMs. Cells were treated for 4 h with 10 μM monensin, labeled and fractionated as described for Fig. 2. Fractions were assayed for ^{3}H-labeled protein (○). Membranes from each fraction were collected by centrifugation after dilution of the sucrose to 0.25 M, fixed, and examined by electron microscopy. The volume density of ICBMs in each fraction was determined. Total protein in each fraction was measured as an estimate of pellet volume. The product of these gives, in arbitrary units, the number of ICBMs in each fraction (●).

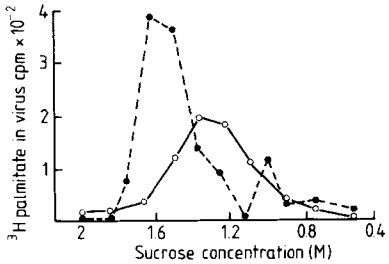


FIGURE 7 Density gradient fractionation of cells labeled for 2.5 min with [^{3}H]palmitic acid. Monensin treated cells (●) were incubated for 4 h with 10 μM monensin before labeling. Control cells (○) received an equal amount of ethanol without monensin. Protein was collected from the gradient fractions and run on polyacrylamide gels. These were sliced and assayed for ^{3}H radioactivity in viral protein.

reference 16). It has therefore been suggested that the trimming enzymes, including the α_{1-2}-mannosidase, are localized in a compartment of the Golgi complex separate from the terminal transferases, and earlier on the transport pathway (24). Infected cells, preincubated with or without monensin, were fractionated on sucrose gradients. Membrane proteins were concentrated from each fraction by phase separation into Triton X-114, and assayed for galactosyl transferase and α_{1-2}-mannosidase activities. The results showed no separation of the two activities, whether or not the cells were pretreated with monensin (Fig. 9).

Labeling with [^{3}H]mannose would have interfered with the mannosidase assay and it was therefore necessary to confirm in some other way that the monensin treatment had been effective in causing the viral glycoproteins from treated cells to equilibrate at a higher density than those from untreated cells. Samples from each gradient fraction were therefore run on polyacrylamide gels and then immunoblotted (4), and the amount of the viral glycoprotein E1 in each fraction was estimated. The density of the fraction containing the most E1 is marked with an arrow (Fig. 9).

DISCUSSION

We have subfractionated the Golgi stack of cisternae by exploiting the effect of monensin on SFV-infected BHK cells. By causing the *medial* cisternae to bind nucleocapsids, we increased their density, allowing them to be discriminated from other cisternae in the stack. Using a variety of criteria, we have shown that the membranes sedimenting to higher density are identical to the ICBMs observed in vivo. Conditions known to disrupt the interaction between nucleocapsids and viral spike proteins caused the membranes to regain the density of Golgi

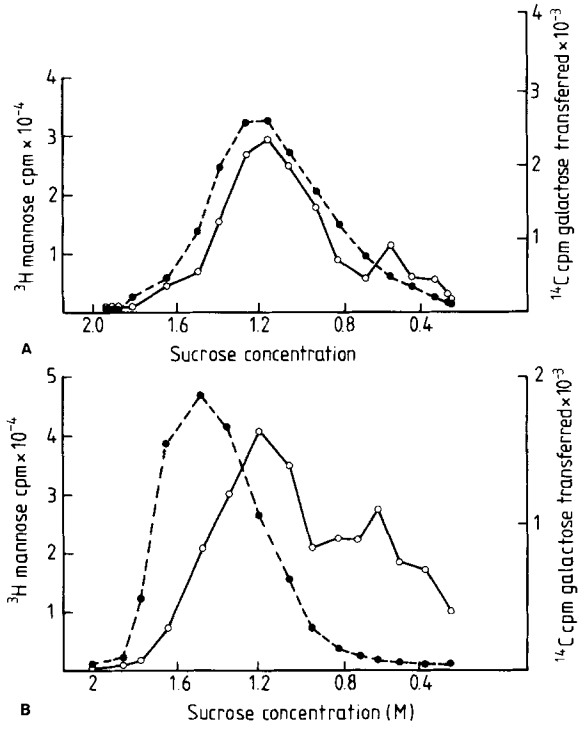


FIGURE 8 Distribution of galactosyl transferase activity. Cells were labeled with [^{3}H]mannose as described in Fig. 2 and fractionated on sucrose gradients. Fractions were assayed for [^{3}H]mannose-labeled protein (●) and galactosyl transferase activity (○). (*A*) Control cells. (*B*) Cells maintained in 10 μM monensin for the 4 h before harvest.

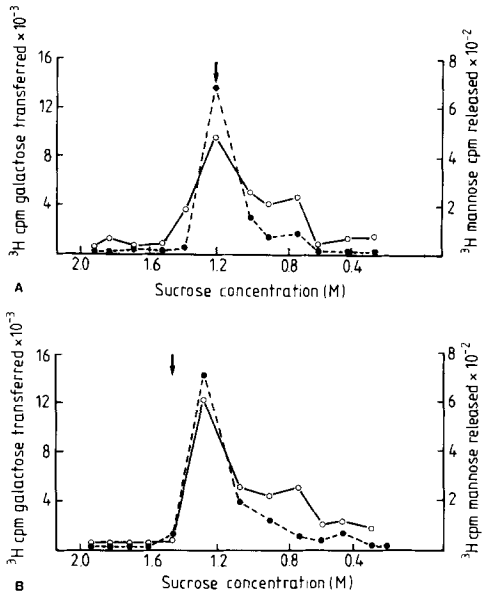


FIGURE 9 Distribution of α_{1-2}-mannosidase activity. Cells were fractionated on sucrose gradients without (*A*) and with (*B*) 4 h pretreatment with monensin. Membrane proteins from each fraction were concentrated by partitioning into Triton X-114 and assayed for galactosyl transferase (○) and α_{1-2} mannosidase (●) activities. The arrows mark the fractions containing the highest concentration of E_1, determined by immunoblotting (4).

membranes from untreated cells. Capsid-binding is thus both necessary and sufficient to explain the density changes and is consistent with changing the properties of only the *medial* cisternae, which alone bound significant numbers of nucleocapsids (13).

Addition of fatty acids to newly synthesized proteins is an earlier event than construction of complex oligosaccharides (26) and occurs in the presence of monensin (18). We have shown that monensin blocks transport from the *medial* to the *trans* cisternae (13); and fatty acid addition should therefore occur either before or when the proteins reach the *medial* cisternae. In this paper we have shown that after a 2.5-min. pulse with [^{3}H]palmitic acid most of the tritiated viral protein is found in the *medial* compartment. This suggests that fatty acid addition occurs either in this compartment or in the *cis* compartment, followed by rapid transfer of the protein to the *medial* compartment.

Though all the evidence in the previous paper suggested that monensin blocked transport from *medial* to *trans* cisternae (13), we were unable to excluce the possibility that monensin was having effects other than inhibition of transport. Inhibition of an enzyme involved at an early step in oligosaccharide processing would invalidate the use of the stage reached in that processing as a marker for the point reached in the transport pathway. If this occurred, the ICBMs could have originated from the fusion of post-Golgi vesicles as oligosaccharide processing is not required for transport (11, 23). Previous work (10) has shown that the viral proteins take 10–15 min to pass through the Golgi stack of four to six cisternae. Since fatty acid addition is an earlier event than construction of complex oligosaccharides, and since viral proteins labeled with a 2.5-min pulse of palmitic acid are found mostly in ICBMs, it is reasonable to conclude that the ICBMs must be derived from a *medial* rather than a post-Golgi compartment.

Both galactosyl transferase and α_{1-2}-mannosidase activities were unaffected by monensin treatment. This suggests that they are both absent from the *medial* cisternae. Since ICBMs in vivo contain viral proteins bearing only simple oligosaccharides (13), it is reasonable to conclude that the enzymes involved in trimming and complex oligosaccharide construction are restricted to the *trans* cisternae. This is in agreement with recent work by Roth and Berger (22) on the localization of galactosyl transferase but at first sight disagrees with work (5, 21) showing a separation on sucrose density gradients of trimming enzyme (mannosidase) and terminal transferase (galactosyl transferase) activities. These authors have concluded that mannosidase activity is localized in *cis* Golgi cisternae on the basis of such a separation and the fact that trimming is an earlier biochemical event than complex oligosaccharide construction (16). However, no immunocytochemical evidence for a *cis* location was presented. While both activities appear to be localized in the *trans* compartment as defined by the monensin block, it seems likely that they are in distinct compartments which can be resolved under some circumstances. We have simply not been able to show such a separation in our system.

The use of monensin to induce a change in density of a subcompartment of the Golgi complex of SFV-infected cells is both limited in site of action and complicated by possible side effects. It is possible, however, that the same principle could be applied in other ways to different compartments of the intracellular transport pathway. An example would be the use of temperature-sensitive virus mutants, some of which have proteins that are not transported beyond specific points when shifted to the nonpermissive temperature. The viral membrane proteins therefore accumulate in a specific compartment of the transport pathway, and may then, or when returned to the permissive temperature, bind viral nucleocapsids (25). These mutants should not only be free of ill-defined side effects, but offer the potential for perturbing other specific compartments of the transport pathway.

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