Viral Membrane Proteins Acquire Galactose in Trans Golgi Cisternae during Intracellular Transport

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ABSTRACT   Frozen, thin sections of baby hamster kidney (BHK) cells were incubated with either concanavalin A (Con A) or *Ricinus communis* agglutinin I (RCA) to localize specific oligosaccharide moieties in endoplasmic reticulum (ER) and Golgi membranes. These lectins were then visualized using an anti-lectin antibody followed by protein A conjugated to colloidal gold. All Golgi cisternae and all ER membranes were uniformly labeled by Con A. In contrast, RCA gave a uniform labeling of only half to three-quarters of those cisternae on the *trans* side of the Golgi stack; one or two *cis* Golgi cisternae and all ER membranes were essentially unlabeled. This pattern of lectin labeling was not affected by infection of the cells with Semliki Forest virus (SFV). Infected cells transport only viral spike glycoproteins from their site of synthesis in the ER to the cell surface via the stacks of Golgi cisternae where many of the simple oligosaccharides on the spike proteins are converted to complex ones (Green, J., G. Griffiths, D. Louvard, P. Quinn, and G. Warren. 1981. *J. Mol. Biol.* 152:663–698). It is these complex oligosaccharides that were shown, by immunoblotting experiments, to be specifically recognized by RCA. Loss of spike proteins from Golgi cisternae after cycloheximide treatment (Green et al.) was accompanied by a 50% decrease in the level of RCA binding. Hence, about half of the RCA bound to Golgi membranes in thin sections was bound to spike proteins bearing complex oligosaccharides and these were restricted to the *trans* part of the Golgi stack. Our results strongly suggest that complex oligosaccharides are constructed in *trans* Golgi cisternae and that the overall movement of spike proteins is from the *cis* to the *trans* side of the Golgi stack.

Exported proteins frequently undergo a series of precise, structural changes as they move from the rough ER, through the Golgi complex, to the cell surface. The best-studied changes in structure are those that occur in the oligosaccharides linked to the polypeptide through asparagine (17). A high-mannose oligosaccharide is transferred from dolichol pyrophosphate (18, 25) to the nascent polypeptide as the protein is assembled in the ER (35, 42). Terminal glucose residues are progressively lost (18, 21) and, 20–30 min after transfer, the oligosaccharide is trimmed of some of the mannose residues (19, 42), and terminal sugars (N-acetylglucosamine, galactose, sialic acid, and fucose) are added to give the complex oligosaccharide. Autoradiographic and cell fractionation studies have shown that the trimming and conversion to complex oligosaccharides occurs in the Golgi complex (4, 17, 24, 38, 39, 41) but the

precise location (if any) of these events within this organelle is still unclear.

One promising approach to locate the site of an oligosaccharide modification would be to utilize the sugar specificity of plant lectins. Fluorescently labeled lectins have already been introduced as markers for the ER and Golgi complex (48), but, with one notable exception (52), these studies were carried out using light microscopy and no attempt was made to confirm the membrane specificity of these lectins at the electron microscopic level.

Here we have used lectins to label thin, frozen sections of BHK cells. This technique allows us to determine, precisely, the intracellular membrane to which these lectins bind and to quantitate the binding. We have tried to determine whether these lectins allow us to locate, with more precision, the oligo-

saccharide changes that occur in the Golgi complex, and we have also tried to discriminate between those oligosaccharides bound to viral proteins in transit and those bound to endogenous membrane components on the intracellular transport pathway.

MATERIALS AND METHODS

Cells

BHK-21 cells were grown and infected with SFV as described previously (13). The cells were shown not to secrete significant amounts of protein by labeling experiments with [^{14}C]leucine. After an 8-h incubation, <1% of the total acid-precipitable label was found in the medium. The Ric^R-14 line of BHK cells, selected by their resistance to RCA (28), were provided by Dr. Colin Hughes, National Institute for Medical Research (N.I.M.R.), London. They were grown in the same way as BHK cells and tested occasionally for their continued resistance to RCA.

Lectins and Antisera

WGA, Con A and RCA 120, purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), showed a single band on SDS PAGE. One mg of each lectin was mixed with an equal amount of purified porcine stomach mucin (32) (to prevent the lectins from binding to cell surfaces after injection) and emulsified with complete Freund's adjuvant. The antigens were injected into rabbits in at least 10 intradermal sites along the back. After 6 wk, a further 0.5 mg of lectin-mucin complex, emulsified in incomplete Freund's, was injected into the subscapular cavity and subcutaneously in the neck region. 8 wk later the same amount, in PBS, was injected intramuscularly in the posterior legs, followed on each of the next 2 d by an intravenous injection of the same amount into the marginal ear vein. The rabbits were first bled 10 d after the last intravenous injection. The antibodies were assayed by an immunodiffusion technique in the presence of an appropriate sugar to prevent binding of lectins to the oligosaccharides on the antibodies. A single precipitin line was obtained for each of the three lectins and there was no cross-reactivity. No antibodies could be detected against mucin. The titer of antibody was very high since a precipitin line was obtained using a 1 mg/ml solution of lectin and a 100-fold dilution of antiserum.

Colloidal Gold—Protein A

This was prepared by the method of Faulk and Taylor (10) and separated into different size classes on sucrose gradients as described by Slot & Geuze (40). Protein A was purchased from Pharmacia Inc. (Piscataway, NJ) or Sigma Chemical Co. (St. Louis, MO).

Preparation and Labeling of Thin Frozen Sections

FIXATION

BHK cells were released from the dish by treatment, on ice, with 50 µg/ml proteinase K (SERVA Feinbiochemica Gmbh & Co., Heidelberg, Federal Republic of Germany). By repeated, gentle pipetting, the cells were all released within 5 min. After centrifugation for 1-2 min. at 1,000-2,000 g, the cell pellet was dispersed briefly in 0.5-1% glutaraldehyde (Merck & Co., Inc., Rahway, NJ) in 100 mM piperazine N, N'-bis (2-ethane sulfonic acid) (PIPES) buffer, pH 7.0, containing 5% (wt/vol) sucrose. The suspension was then sedimented and resuspended in the same fixative for 30 min at room temperature. The cells were sedimented and washed three times in 100 mM PIPES buffer pH 7.0 containing 10% (wt/vol) sucrose. In the presence of azide (0.02%, wt/vol) these fixed cells could then be stored at 4°C for up to 1 wk.

INFUSION, FREEZING AND LABELING

All subsequent stages in the technique, including antibody labeling, were carried out essentially as described by Tokuyasu (44, 45, 46). Small (1-2 mm) pieces of the cell pellets were immersed in 2.3 M sucrose in 0.1 M PIPES buffer pH 7.0 at room temperature and left for 15 min. The use of 2.3 M sucrose, suggested by Geuze et al. (11), instead of the 0.6-1.6 M used by Tokuyasu (44), improved the plasticity of the frozen blocks and greatly facilitated sectioning. The pieces of pellet were mounted on the copper specimen holder, frozen in liquid nitrogen, and sectioned in the Sorvall MT2B ultramicrotome (DuPont Instruments—Sorvall Biomedical Div., Dupont Co., Newtown, CT) equipped

with an FTC cryochamber. Sections were cut at −100°C to −110°C, picked up with a 2-mm platinum loop of 2.3 M sucrose in PIPES buffer, and transferred to 100-mesh, hexagonal-lattice, copper or nickel grids. These grids, having formvar films, were carbon-coated and then rendered hydrophilic using a glow-discharge apparatus. The films on hexagonal grids are much stronger than those on the normal rectangular lattice grids so that the 100-mesh size could be used routinely (Dr. J. W. Slot, personal communication). The labeling procedure was carried out as described previously (11, 14, 45) with an additional lectin step before the antibody labeling. The lectins and the anti-lectin antisera were diluted in PBS, and the dilutions were determined empirically to give the highest ratio of specific labeling to background over the cytoplasm and the nucleus. Optimal concentrations of the lectins were found to be 5 µg/ml for Con A and 50 µg/ml for both WGA and RCA. For the antisera, the dilutions were 1:60 for anti-Con A, 1:40 for anti-RCA and 1:12 for anti-WGA. The grids were incubated with the lectins and anti-lectin antisera for 15 min at room temperature. Between these steps the grids were washed for 12-15 min on drops of PBS.

Double-labeling of thin sections using two sizes of colloidal gold was carried out as described by Geuze et al. (12). In a typical experiment the grid was treated sequentially with RCA, anti-RCA, protein A-gold (5-nm diameter) for 15 min., free protein A (0.1 mg/ml in PBS) for 10 min., anti-spike antibodies (0.2 mg/ml in PBS) for 30 min, and protein A-gold (12 nm diameter) for 15 min. Between the steps the grids were washed as described above. The complementary experiment was also always carried out to eliminate possible technical artefacts, i.e., 12-nm gold was used before 5-nm gold.

CONTRASTING AND EMBEDDING

A modification of the procedure of Tokuyasu (45) was used which has greatly improved the contrast of the frozen section. Following a 5-min staining with uranyl acetate oxalate, pH 7.4 (45, 46), the grids were rinsed three times for 1 min in small drops of distilled water and then stained with 2% uranyl acetate for 5 min. The grids were then transferred directly, without washing, onto drops of a 1.5% solution of methyl cellulose (Tylose MH-300; Fluka) on ice. The grids were transferred quickly from one drop to the next (three drops in total) for a total time of 15-20 s and then embedded using a 3-mm loop as described by Tokuyasu (45). Enough excess liquid was removed with filter paper to give a gold-blue interference color after air drying.

The precise type of contrast obtained with this modification varies from section to section. Usually, a mixture of positive and negative staining was obtained. The nucleus, for example, was always stained positively but membranes were either positively (e.g. Figs. 2 and 3) or negatively stained (e.g. Fig. 10). Similarly, ribosomes may be quite distinct (e.g. Figs. 1 and 3) or hardly visible (e.g. Fig. 2). Though the reasons for this are not entirely clear, there are three factors that seem to modify the contrast. The first factor is the amount of "destaining" that occurs; frozen sections, unlike Epon sections, take up heavy metal stains in seconds but will "destain" equally rapidly on a water droplet. If one does not "destain" at all but merely dries the section, one gets a classical negatively stained image that is heavily contrasted, making it difficult to see gold particles and impossible to see ferritin. Hence we have tried to control the "destaining" by putting the grids directly onto methyl cellulose, without washing. There is always a few seconds variability between the grids in the time it takes for the excess methyl cellulose to be taken off and for the film to dry. This obviously affects the rate of destaining and therefore the contrast. The second factor that appears to play a role is the section thickness. In general, the thinner the sections the more likely one is to get a positively stained image. The third parameter is the actual thickness of the methyl cellulose film, which is often variable (as seen by its interference color) over any one grid. The thicker the film the more likely one is to get negative contrast. It is often possible to see, on the same section, areas that are positively stained (where the methyl cellulose film is thinner) and adjacent areas (where the film is thicker, usually near grid bars) that are heavily negatively stained. Therefore, in order to get a positively stained image with good structural preservation, one must compromise between a methyl cellulose film that is thick enough to support the structures and, on the other hand, thin enough to facilitate positive or partially positive staining so that the gold particles are easily visible.

CONTROLS

INHIBITORS: The lectins were pre-incubated for 10 min with sugar(s) known to prevent the binding of the lectin to oligosaccharides (26). Con A was pretreated with 200 mM α-methylmannoside whereas RCA was pretreated with 300 mM D-galactose. For WGA a mixture of 200 mM N-acetyl D glucosamine and 200 mM N-acetylneuraminic acid (sialic acid) was used to completely prevent binding. N-acetylglucosamine alone did not always abolish WGA binding.

ANTIBODIES: The lectins bound to the thin sections were revealed by specific anti-lectin antibodies followed by protein A gold. However, non-immune IgG will also bind to these lectins via the oligosaccharide side chains present on

many of these molecules. To demonstrate the specificity of the reaction it was, therefore, necessary to block the remaining oligosaccharide binding sites on the lectin with mucin (Sigma Chemical Co.), a highly glycosylated protein, and then to use the specific anti-lectin antibody. The grids were incubated with the lectin and then for 5 min with 0.5 mg/ml mucin. Subsequent incubation in the presence of mucin with rabbit IgG followed by protein A gold gave no significant labeling. Labeling was obtained only after treatment with the specific anti-lectin antibodies. Having established the specificity of the reaction, we carried out most of the subsequent studies without mucin, because the specificity of the approach lies in the lectin and not in the means used to detect it.

RIC^R-14 BHK CELLS: These cells have <10% of the normal β-N-acetylglucosaminyl transferase activity and are unable to construct complex oligosaccharides from the partially trimmed, high-mannose oligosaccharides (28). Because these oligosaccharides lack galactose, they should not bind RCA and this provides a control showing that RCA in normal BHK cells is only binding to complex oligosaccharides containing galactose.

Quantitation of Lectin Labeling on Frozen Sections

SFV-infected BHK cells were prepared for immune labeling as described above before or after treatment with 10 μg/ml cycloheximide for 90 min. Frozen sections of both preparations were labeled, in parallel, in an identical fashion, with each of the three lectins. The quantitation, which gives relative values only, was carried out on a series of electron micrographs and is essentially the same as that performed in a previous study (13). For both treatments, and for all three lectins, 25–30 random micrographs were taken of the perinuclear region of cells showing acceptable fine-structural preservation, at an initial magnification of 17,000 on the Philips 400 EM. These were then photographically enlarged 2.5-fold. The "average" width of the rough ER cisternae, from membrane to membrane, was estimated by measurement on 20 different micrographs; this was 37 ± 8.6 nm. On all micrographs, a random process was used (using a random numbers table) to select parts of the ER or nuclear envelope that were between 2 and 5 cm long. Multiplying this length by the mean width gave the surface area of micrograph accounted for by ER. In true morphometric terms, this is directly proportional to ER volume density (S$_V$) (49). The gold particles within this surface area and within one membrane width from the membrane were counted. The surface area of micrograph (also a parameter directly proportional to Golgi volume density) accounted for by identifiable Golgi cisternae was estimated using a lattice-grid system (49). This comprised 15–25 Golgi stacks per treatment. As for ER, the gold particles within this area were then counted.

Binding of SFV Spike Proteins by Con A and RCA

Virions of SFV, prepared from infected BHK cells or infected Ric^R-14 BHK cells (13), were fractionated by SDS PAGE. The proteins were then transferred to nitrocellulose by "Western Blotting" using the method of Towbin et al. (47) as modified by Burnette (8). Two percent (wt/vol) bovine haemoglobin (Sigma Chemical Co., grade IV), instead of BSA, was used throughout the procedure. The nitrocellulose paper with bound viral proteins was first incubated for 90 min with lectin (5 μg/ml Con A, 50 μg/ml RCA) followed by 30-min incubation with the anti-lectin antibody (100-fold dilution of anti-Con A and anti-RCA) and radio-iodinated protein A (New England Nuclear). The bound lectin was visualised using pre-flashed Kodak XR-5 film and a fluorescent screen at −30°C (23).

RESULTS

Lectin Labeling of BHK Cells

GOLGI COMPLEX: Con A uniformly labeled all of the cisternae in any Golgi stack. There was no evidence for a gradient of labeling across the stack nor for a local concentration of labeling at, for example, the dilated rims of the cisternae (Figs. 1 and 2).

RCA, on the other hand, labeled the half to three-quarters of the Golgi cisternae on one side of the stack. The precise number labeled depended on the number visible in a particular section of the stack. When four cisternae were visible, no more than three, and usually two, were labeled. When six cisternae were visible, three, and no more than four, were labeled (Figs. 4–7). The labeled cisternae bound at least 20 times as much RCA as the cisternae on the other side of the stack, which had

a level of labeling even lower than the background level over the nucleus. Thus, there was an abrupt transition in RCA labeling across the Golgi stack. Within the labeled region, however, RCA binding appeared to be uniform.

WGA, of all the lectins, gave the most variable results. Usually, it gave a pattern of labeling similar to that of RCA but, on occasion, the entire stack of cisternae was labeled. Even under these circumstances, however, the ER was still unlabeled (data not shown).

For all three lectins, the gold label was located within one membrane width of a Golgi cisternal membrane but it was not possible to determine the sidedness of the binding. Not only were the membranes of any cisternae closely opposed but the flattened cisternae were themselves packed closely together in the stack.

OTHER MEMBRANES: The ER membrane, including the nuclear envelope, was uniformly labeled by Con A (Figs. 1 and 2) but not by RCA (Fig. 3). This confirms previous studies using isolated membrane fractions (6, 16). In clear transverse sections, the great majority of the gold particles were found on the luminal side of the membrane where the oligosaccharides are known to reside (6, 10). The rest of the gold particles were found within one membrane width of the ER membrane. The outer membrane of the nuclear envelope, which is continuous with ER, was also labeled by Con A. The inner nuclear membrane appeared to be labeled.

The plasma membrane was labeled heavily by both lectins (Figs. 1, 3, and 7) as has been previously observed (29). The density of the labeling was usually higher than that seen in ER and Golgi membranes, and almost all the label was on the outer side of the membrane.

The secondary lysosomes in BHK cells are characterized by their dense, membrane-bounded profiles and by the appearance of lipid droplets and many small vesicles in their interior. They were always heavily labeled by RCA (Figs. 4 and 12), WGA, and Con A (data not shown), the label being associated not only with the lysosomal membrane but also with many of the internal components. Because these results show that RCA labels the PM and lysosomes in addition to Golgi membranes, caution should be exercised when this lectin is used as a marker for the Golgi complex (48).

Mitochondria lack the oligosaccharides that can specifically bind Con A and RCA and thus they can serve as controls for the nonspecific binding of lectins to membranes. The labeling of the mitochondria by any of the lectins was rarely higher than the low level of labeling of the cytoplasm and the nuclear interior (eg. Fig. 1).

SFV-Infected BHK Cells

BHK cells, infected with SFV, synthesize only viral proteins. The viral spike proteins, each comprising two spanning membrane glycoproteins, are assembled in the ER membrane and are transported to the plasma membrane via the Golgi complex (13). Pulse chase, fractionation, and immunocytochemical studies have shown that the terminal sugars, including galactose, are added in the Golgi complex. Hence, this defined system was used to relate the patterns of lectin labeling to the changes in oligosaccharide structure that are known to occur during intracellular transport.

LECTIN LABELING AND CYTOCHEMISTRY: The pattern of lectin labeling was not changed by infection with SFV (Figs. 10–14) with the exception of two additional features. The first was the appearance of virus budding profiles at the cell surface

that could be labeled by RCA (Fig. 13), Con A, and WGA (data not shown), and the second feature was the appearance of "capsid structures" in the cell cytoplasm, which fortuitously allowed us to distinguish between *cis* and *trans* Golgi in thin frozen sections. These structures are of unknown function (1) but were always seen in the cytoplasm of SFV-infected cells after ~6 h of infection. They are very prominent rod-shaped periodic structures (Fig. 17, *inset*) that are usually membrane-bounded and covered with nucleocapsids, and that can extend for up to several microns in the cytoplasm. In cross-section they appear as vesicles with capsids around their outer surface

(Figs. 11, 14 and 18), and their membranes were always labeled by RCA (Figs. 11 and 14), Con A and WGA (data not shown) as well as antibodies to the spike proteins (Fig. 21). They were often found very close to one side of the Golgi stack. In this location they frequently had very few bound nucleocapsids (Figs. 17 and 19). Cytochemical studies with thiamine pyrophosphatase and acid phosphatase, two well-known markers for *trans* Golgi cisternae (9, 30), showed that the great majority of these capsid structures in the vicinity of the Golgi complex were on that side of the Golgi stack which reacted for these enzymes (Figs. 18 and 19). Labeling with RCA (Figs. 10, 11
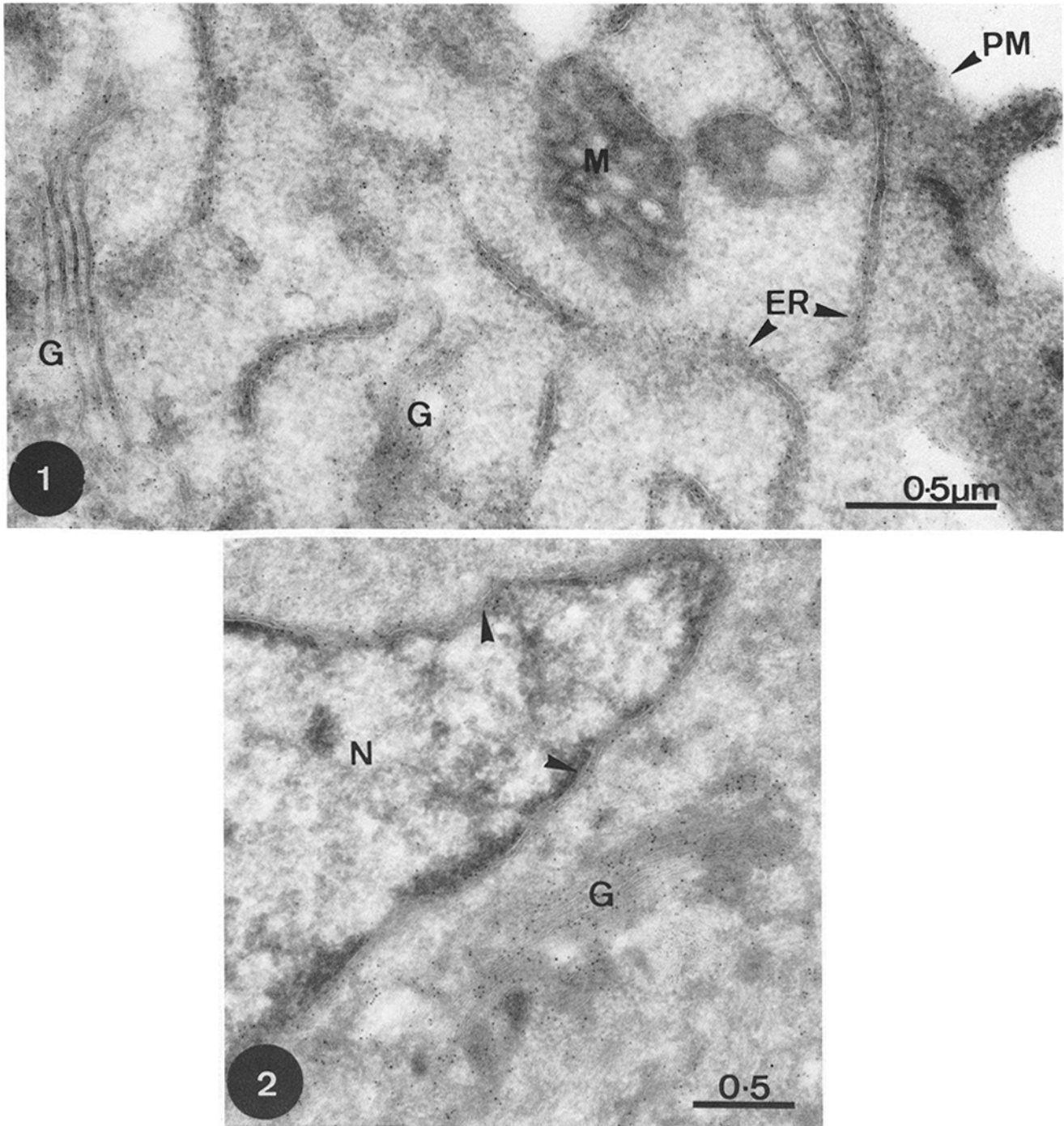

FIGURE 1 and 2   BHK cells labeled with Con A. The rough *ER*, including the nuclear envelope (arrowheads in Fig. 2), the stack of Golgi cisternae (*G*) and the plasma membrane (*PM*) were all specifically labeled. The nuclear matrix (*N*), the cytoplasm, and mitochondria (*M*) were not labeled. Fig. 1, × 50,000; Fig. 2, × 33,000. Magnification markers in all figures are given in microns.

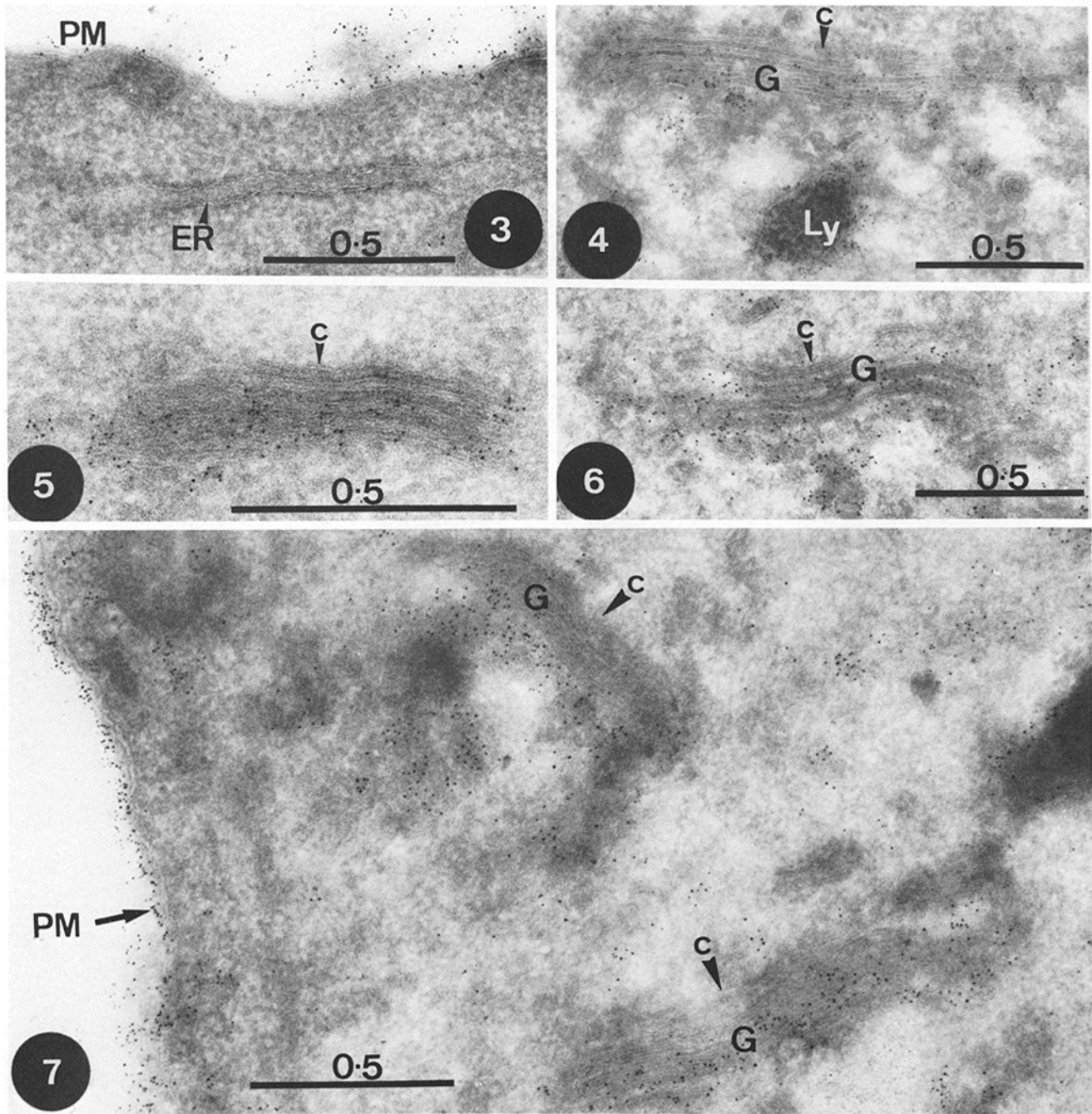FIGURE 3–7 BHK cells labeled with RCA. The outside surface of the plasma membrane (*PM*) was heavily labeled, whereas the rough *ER* was unlabeled. × 60,000. Figs. 4–7: BHK cells labeled with RCA. In all cases the Golgi stacks were preferentially labeled on one side. The unlabeled side is marked as the *cis* side (*c*) (see text). In Fig. 4, a secondary lysosome (*Ly*) near the *trans* side of the Golgi complex was labeled. In Fig. 7, the outer surface of plasma membrane (*PM*) was heavily labeled. Fig. 4, × 55,000; Fig. 5, × 91,000; Fig. 6, × 55,000; Fig. 7, × 64,000.

and 14) showed that these structures were also adjacent to the lectin-labeled side of the Golgi stack. This provides indirect evidence that the labeling by RCA was restricted to the *trans* side of the Golgi stack in BHK cells. In early rat spermatids where the polarity of the Golgi complex is easily discerned, we have obtained direct evidence that RCA labels only *trans* Golgi cisternae (data not shown).

Infected BHK cells were also used to verify the partial labeling of the Golgi stack by RCA. One could always argue that spike proteins bearing appropriate oligosaccharides were present in *cis* cisternae but, for unknown technical reasons,

were inaccessible to the lectin. Double-labeling experiments proved that this was not the case. Sections were first labeled with RCA, anti-RCA and protein A conjugated to 5-nm gold. They were then labeled with anti-spike protein antibodies and protein A conjugated to 12-nm gold. As shown in Fig. 20, the spike proteins were present and hence accessible throughout the Golgi stack (confirming our previous results, reference 13), whereas RCA labeling was limited to one side of the stack. The pattern of antigen labeling was not affected when each antibody was visualized with the other size of gold (Fig. 21) although it was clear that the level of labeling was always
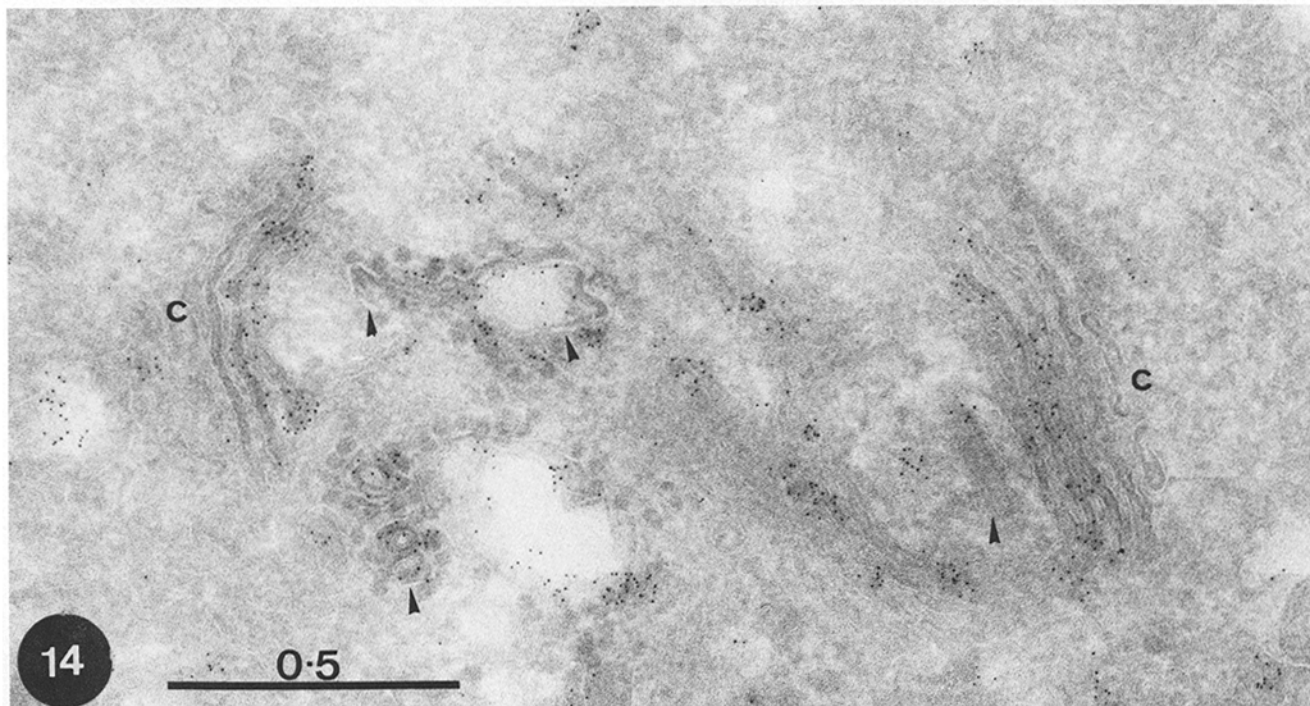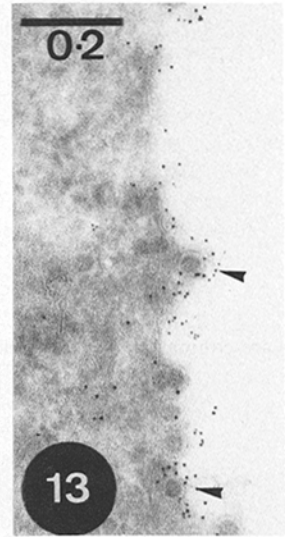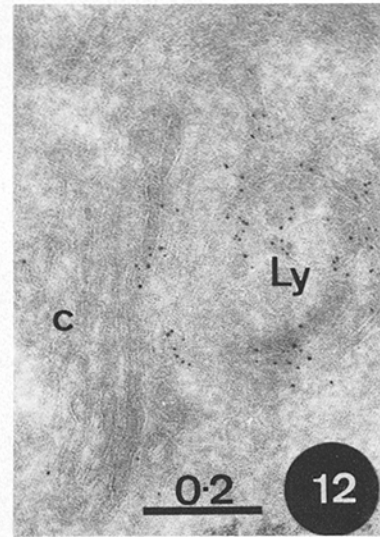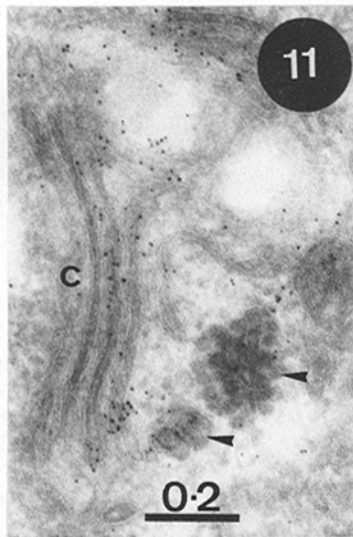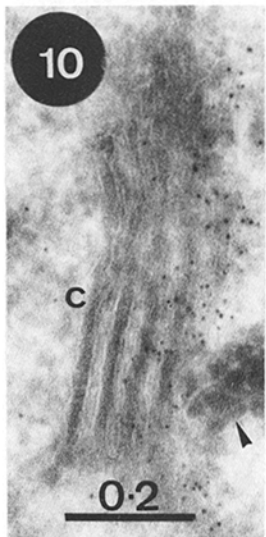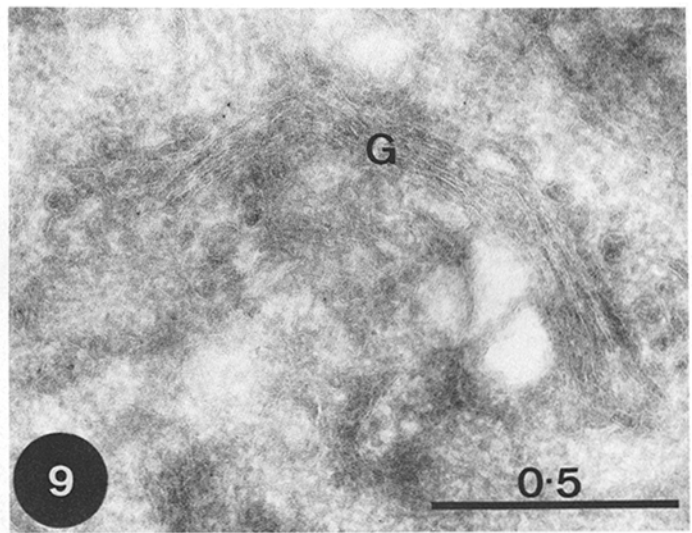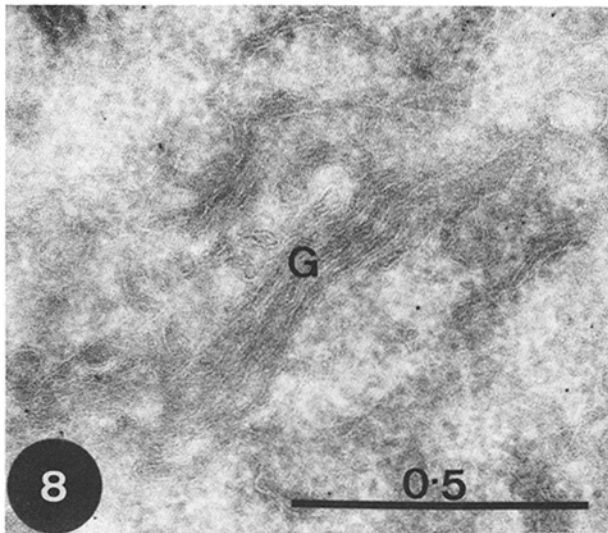
significantly lower when gold of the larger size was used. This observation has previously been documented (12).

BINDING OF LECTINS TO VIRAL GLYCOPROTEINS CONTAINING COMPLEX AND SIMPLE OLIGOSACCHARIDES: To determine which type of oligosaccharide the lectins were binding to in thin, frozen sections of infected cells, viral glycoproteins from virions were fractionated by SDS PAGE and then electroeluted onto nitrocellulose paper. The paper was incubated with RCA or Con A, and the bound lectin was visualized using an appropriate anti-lectin antibody, radioiodinated protein A and autoradiography. Normal SFV contains the spanning glycoproteins E1, E2, and some residual p62. They contain both complex and simple oligosaccharides (27, 33, 34) and were labeled by both Con A and RCA (Fig. 22, lanes 1 and 3). SFV grown in the Ric^R-14 cell line contains simple oligosaccharides but no complex ones. These glycoproteins bound Con A (Fig. 22, lane 2) but not RCA (Fig. 22, lane 4). This shows that RCA only recognizes the viral glycoproteins once they have acquired complex oligosaccharides, whereas con A recognizes both simple and complex oligosaccharides on the viral glycoproteins. Similar experiments using WGA gave inconclusive results.

EFFECT OF CYCLOHEXIMIDE: BHK cells, 4 h after infection, were treated with 10 µg/ml cycloheximide for 90 min before freeze-sectioning and lectin labeling. The amount of lectin bound to ER and Golgi membranes was then quantitated on micrographs, and the results were compared to those obtained without cycloheximide treatment. The method used to quantitate binding accurately records relative changes in any given compartment. Because cycloheximide stops synthesis but not intracellular transport of newly synthesized proteins (20), we are comparing a system transporting large amounts of viral membrane proteins with one having essentially no protein in transit (13). The results show that the level of lectin binding dropped significantly after treatment with cycloheximide (Table I and cf. Figs. 11 and 12). The level of lectin labeling fell by 25% for ER membranes and 50–60% for Golgi membranes. A significant percentage of the oligosaccharides revealed by the lectins must have been present on the transported viral membrane proteins.

Controls

A number of controls were carried out to show that the lectins were binding to specific oligosaccharides in the thin, frozen sections. These are described in detail in Materials and Methods, and the results can be summarized briefly.

(a) The binding of a particular lectin to a section could be inhibited by a sugar known to bind specifically to the lectin (eg. Figs. 8 and 9).

(b) The Ric^R-14 line of BHK only has ~10% of the normal level of N-acetylglucosaminyltransferase (28), an enzyme that

is necessary for the subsequent construction of complex oligosaccharides from simple ones (18). The Golgi stacks in these cells were not significantly labeled by RCA (Fig. 17). Usually, the plasma membrane was also devoid of label (Fig. 15); the occasional labeling seen on the plasma membranes was probably due to the small amount of complex oligosaccharides that can still be made (Fig. 16).

DISCUSSION

The membrane specificity of the lectins used in this study reflected, in general, their sugar specificity. Con A binds to the mannose residues in both simple and complex oligosaccharides (31), and at least one of these classes is bound to membrane proteins throughout intracellular transport. Con A should and did bind to all membranes on the transport pathway. RCA binds to galactose (22), which is added to proteins somewhere in the Golgi complex (50). RCA should and did bind to Golgi but not ER membranes. WGA binds, though weakly, to both simple and complex oligosaccharides (51) and might have been expected to bind to both ER and Golgi membranes. In fact, it labeled the Golgi membranes but not the ER. This result emphasizes the difficulty in interpreting the interaction of a lectin with a glycoprotein at a molecular level. One cannot assume that a lectin will bind to a glycoprotein simply because a particular sugar is found in the oligosaccharide. To avoid these problems of interpretation, we defined the specificity of the lectins for our restricted purposes by using SFV membrane proteins containing defined classes of oligosaccharides. In immunoblotting experiments, Con A bound to both simple and complex oligosaccharides, whereas RCA only bound the latter. This result for RCA is particularly important because it preferentially binds to terminal galactose residues (2, 53). By binding to SFV membrane proteins containing complex oligosaccharides, we were able to confirm previous work (M. Pesonen, unpublished results) showing that many of these galactose residues are terminal.

Because virally infected cells only transport these defined viral membrane proteins, and infection did not change the pattern of lectin labeling, we were able to draw the following conclusions.

GALACTOSE IS ADDED TO TRANSPORTED PROTEINS IN TRANS GOLGI CISTERNAE: RCA divided the Golgi stack in BHK cells into two distinct parts: one-half to three-quarters of the cisternae on the trans side were heavily and fairly uniformly labeled by RCA; the other quarter to one-half of the cisternae on the cis side were essentially unlabeled. RCA bound isolated viral membrane proteins only after they had acquired complex oligosaccharides, and >50% of the RCA was bound to transported viral membrane proteins in thin, frozen sections of infected cells (see below). Taken together, these data show that spike proteins bearing oligosaccharides containing galac-

FIGURES 8–14 Labeling in control preparations of BHK cells. Fig. 8 is a section treated with RCA in the presence of 300 mM galactose. Fig. 9 is a section treated with Con A in the presence of 200 mM α-methylmannoside. The Golgi stacks (G) in both figures are not labeled. Fig. 8, × 78,000; Fig. 9, × 66,000. Figs. 10–14: SFV-infected BHK cells labeled with RCA. In Figs. 10–12 and 14, a clear polarity of labeling of the Golgi stacks was evident with one to three unlabeled cisternae on one side (labeled cis − (c)). In Figs. 10, 11, and 14, capsid structures, which were usually labeled are indicated by arrowheads. These structures were adjacent to the labeled side of the Golgi stack. Fig. 12 shows an equivalent preparation after treatment with cycloheximide. The labeling on one side of the Golgi stack was reduced considerably (cf. Figs. 10, 11, and 14). The unlabeled side is again marked as cis (c). A structure tentatively identified as a lysosome (Ly), adjacent to the labeled side of the Golgi stack, was also labeled by RCA. Fig. 13 shows typical heavy RCA-labeling of budding virions at the cell surface (arrowheads). Fig. 10, × 88,000; Fig. 11, × 62,000; Fig. 12, × 80,000; Fig. 13, × 66,000; Fig. 14, × 78,000.
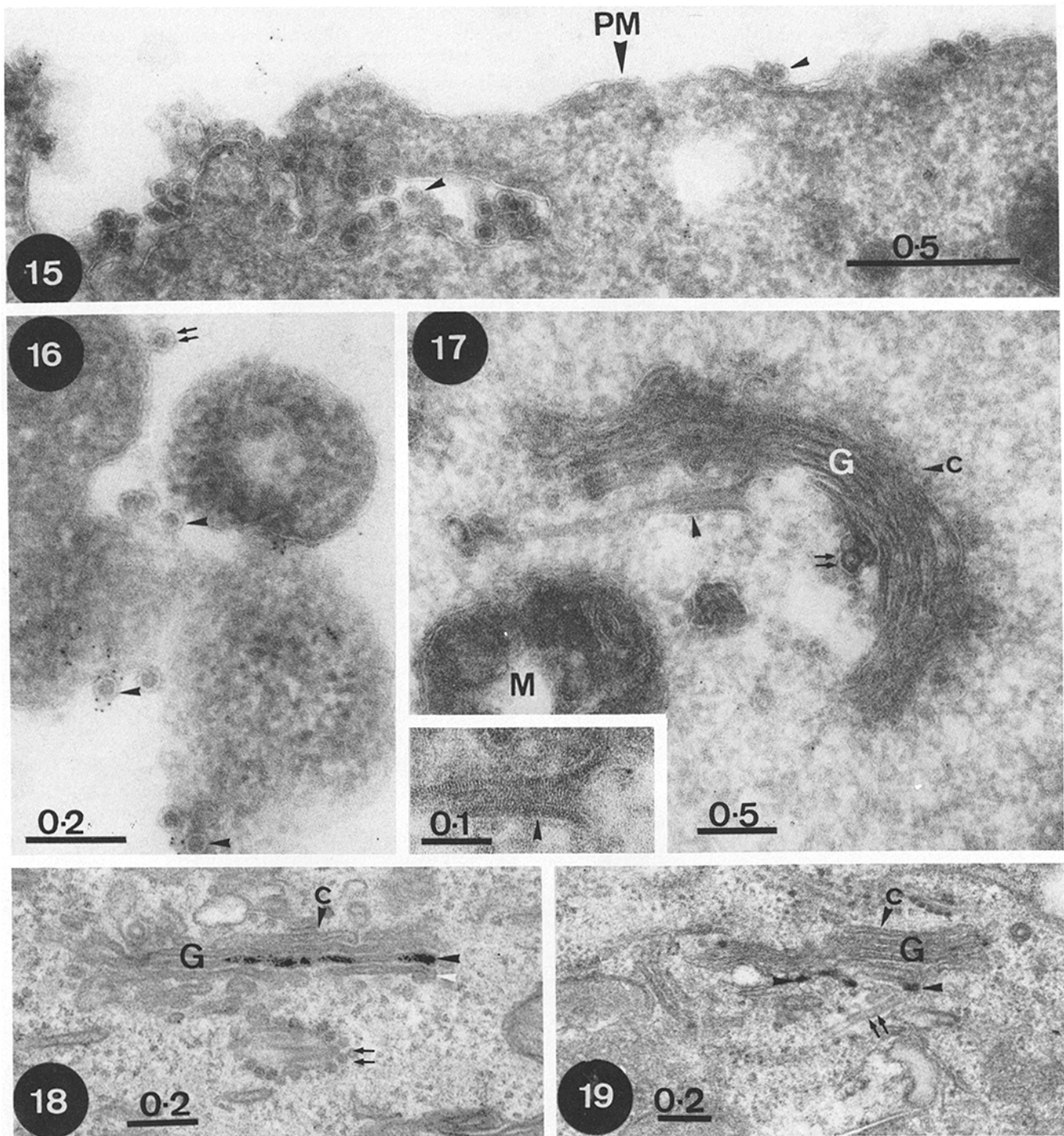
FIGURES 15–19   SFV-infected Ric^R-14 BHK cells labeled with RCA. In Fig. 15, the plasma membrane (*PM*) and all the SFV virions (arrowheads) were free of label. In Fig. 16 an example is shown where one, possibly two, virions (arrowheads) were specifically labeled, whereas five were unlabeled. The double arrow indicates an unlabeled virion in the process of budding from the plasma membrane. In Fig. 17, the Golgi stack (*G*) was free of label as was the structure, indicated by the arrowhead, contiguous to, and possibly continuous with, the Golgi cisterna on one side of the stack. This is a "naked" capsid-structure, i.e., the structure to which nucleocapsids are normally bound (cf. Figs. 10, 11, and 14). A photographic enlargement of this structure is shown (inset) to indicate its periodic nature. Optical diffraction of this structure shows the repeating unit to be ~50 Å apart. The double arrows in Fig. 17 indicate a transverse section of the capsid structure. The opposite face of the Golgi stack is indicated as *cis* (*c*). *M*, mitochondrion. Fig. 15, × 57,000; Fig. 16, × 82,000; Fig. 17, × 65,000; *Inset* Fig. 17, × 114,000. Fig. 18: Epon section of an SFV-infected BHK cell following incubation for TPPase. One cisterna on one side of the Golgi stack (*G*) reacted specifically (black arrowhead). Adjacent to this side of the stack is the cross-sectioned profile of a capsid structure (double arrows) with bound capsids on its periphery. The white arrowhead indicates the nonreactive cisterna which, by comparison with Fig. 19, should react for acid phosphatase. The opposite side of the stack is marked as *cis* (*c*). × 58,000. Fig. 19: Epon section of an SFV-infected BHK cell following incubation for acid phosphatase. Reaction product was specifically localised in one cisterna (arrowhead) on one side of the Golgi (*G*) stack. Adjacent to this is a "naked" capsid structure (double arrows). The opposite side of the stack is indicated as *cis* (*c*). × 43,000.
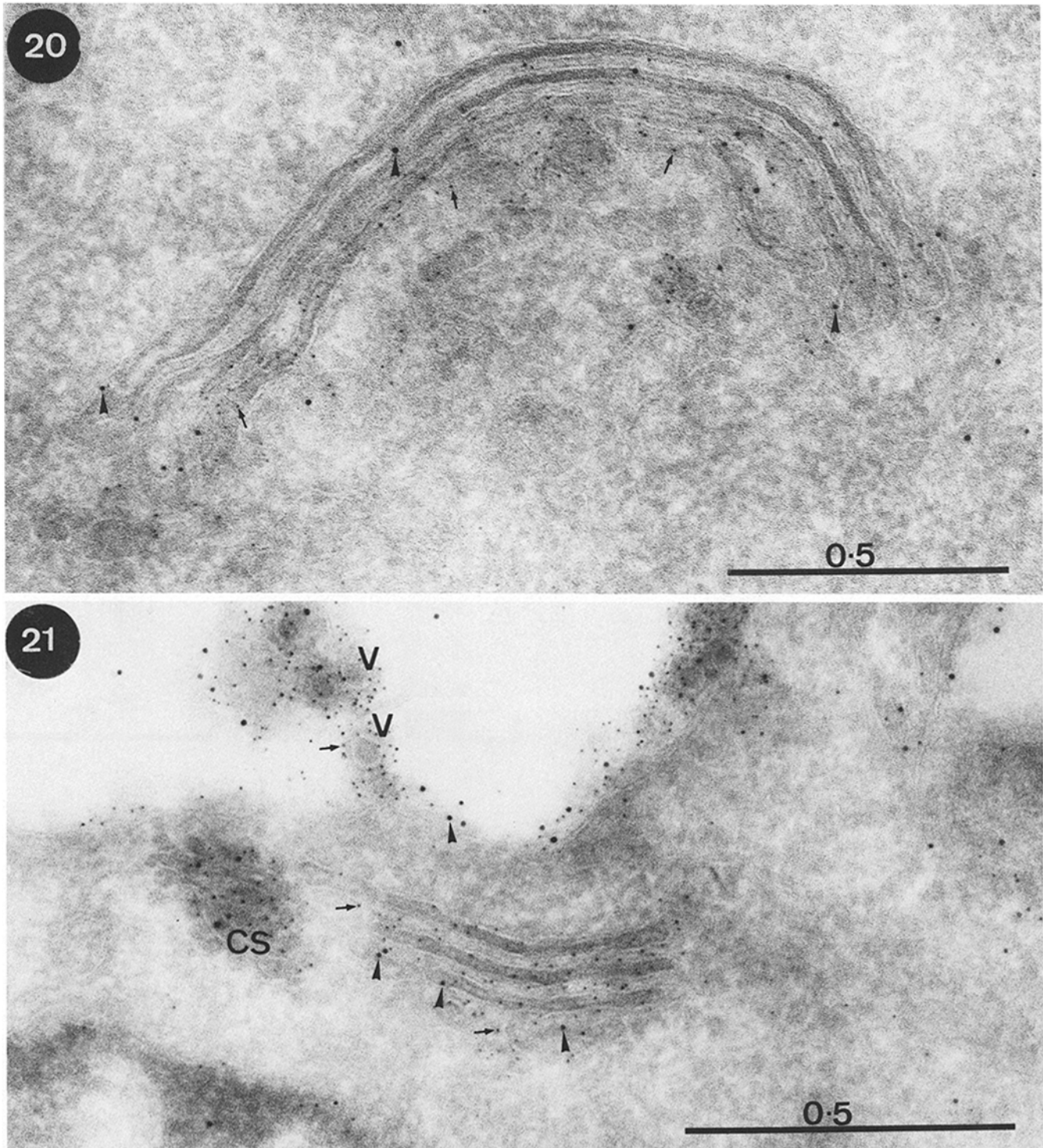
FIGURES 20 and 21   Double-labeled frozen sections using two different sizes of gold. In Fig. 20, RCA is visualized using 5-nm gold (small arrows); this labeling is restricted to one or two cisternae on one side of the stack. The antispike antibody is visualized using 12-nm gold (arrowheads). Whereas the frequency of this labeling was much reduced compared to that of the 5-nm gold, it is clear that it is fairly evenly distributed throughout the stack. In Fig. 21 the labeling pattern is reversed; here the anti-SFV antibody is visualized using the 5-nm gold (small arrows) whereas the RCA is visualized using the 12-nm gold (arrowheads). In this case the latter is restricted to one cisterna on the bottom side of the stack whereas the 5-nm (virus) labeling is uniform throughout. On the cell surface two virions (V) are indicated which in this case are almost exclusively labeled with the anti-virus antibody. CS, capsid structure. Fig. 20, × 95,000; Fig. 21, × 112,000.

tose are restricted to the *trans* side of the Golgi stack.

Galactose is added to the growing complex oligosaccharide by galactosyltransferase, and our data provide indirect evidence that this enzyme is restricted to *trans* Golgi cisternae. Earlier studies, with a few exceptions (5, 50), gave essentially the same result (3, 15, 37, 43) but with one important difference. The earlier studies, using various fractionation techniques, always suggested a gradient of galactosyl transferase activity that
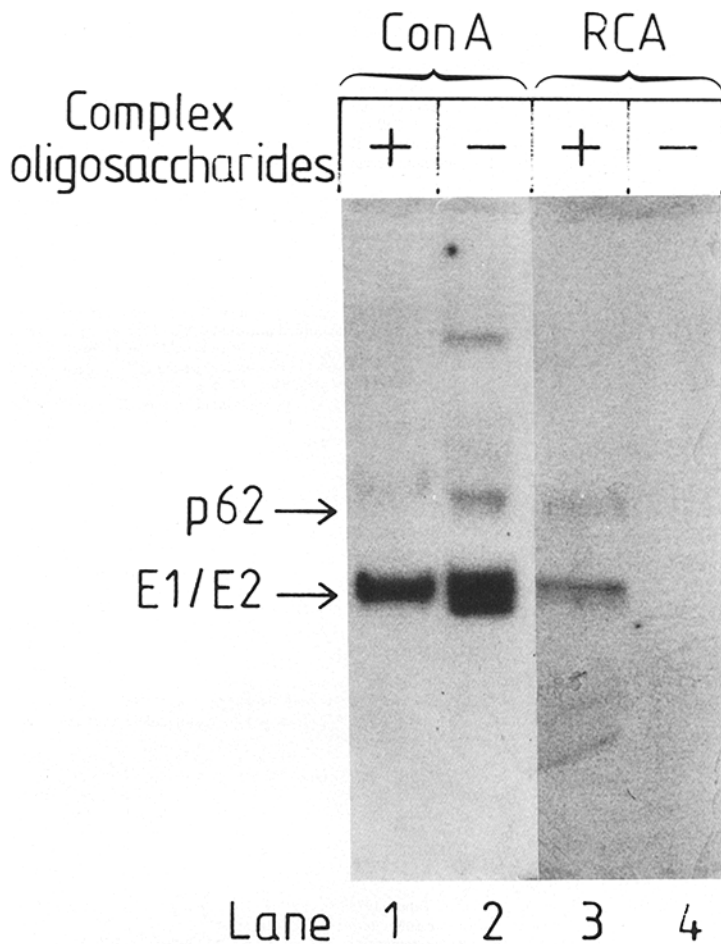
FIGURE 22 Binding of RCA and Con A to viral glycoproteins on nitrocellulose filters. Samples of SFV were fractionated by SDS PAGE, transferred to paper and then oligosaccharides located by lectins using a modified "Western Blotting" technique described in Materials and Methods. Though p62 is only a minor protein contaminant of the completed virus, the sensitivity of this technique distorts the apparent amount there. Lanes *1* and *3*: SFV prepared from infected BHK cells and containing both simple and complex oligosaccharides. Lanes *2* and *4*: SFV prepared from infected Ric^{R}14 BHK cells and containing simple but not complex oligosaccharides. The high molecular weight protein in lane *2*, labeled by *Con A*, is a glycoprotein contaminant of the partially purified virus from infected Ric^R-14 BHK cells.

TABLE I

Effect of Cycloheximide on Lectin Binding to ER and Golgi Membranes

			Gold particles/μm^2	
Organelle	Lectin	Control	+Cyclohex-imide	% De-crease
ER	Con A	864 (±58)	650 (±38)	25
Golgi complex	Con A	399 (±21)	157 (±13)	61
	WGA	463 (±48)	214 (±25)	54
	RCA	197 (±16)	95 (±12)	52

Background over nucleus = 11.4 (±2.9) gold particles/μm^2 (averaged from all micrographs containing nuclei). Experimental details given in text and Materials and Methods.

increased from *cis* to *trans* Golgi. In other words, the enzyme was present in all Golgi elements but in different amounts. This interpretation exceeded the resolution of the fractionation techniques used. Our data, obtained with a high-resolution, immunocytochemical technique, did not reveal a gradient of any kind. There was an abrupt transition in RCA labeling approximately in the middle of the Golgi stack, and the labeling of the *trans* half was fairly uniform. At least 90% of the galactose-bearing oligosaccharides were present in this half of the stack. Very recently, Roth and Berger (36) have used antibodies to galactosyltransferase to localize this enzyme in the Golgi stack. Their elegant studies show that it is restricted

to *trans* Golgi cisternae and is found in those cisternae that stain for TPPase. This result confirms the foregoing discussion but poses a problem because galactosyltransferase is restricted to the penultimate cisterna on the *trans* side, whereas RCA reveals galactose-bearing oligosaccharides in half to three-quarters of the cisternae on the *trans* side. If, as seems likely (see below), the overall movement of the spike proteins is from the *cis* to the *trans* side of the Golgi stack, it would seem that, having acquired galactose in the penultimate cisterna, they can move back to cisternae in the middle of the stack but not to those on the *cis* side. Further study of this apparent discrepancy may shed light on the functioning of the Golgi stack.

THE VIRAL MEMBRANE PROTEINS PASS FROM *CIS* TO *TRANS* GOLGI: SFV membrane proteins pass through the Golgi stacks during intracellular transport and are present in all cisternae of the stack (13). RCA labels *trans* Golgi cisternae, and approximately half of this lectin is bound to viral membrane proteins that have acquired complex oligosccharides. If the movement of these proteins was from *trans* to *cis* Golgi, it would be difficult to explain why the proteins bearing complex oligosaccharides would suddenly lose their ability to bind RCA as they passed to the *cis* cisternae, but subsequently regain the ability to bind at the plasma membrane. It is therefore reasonable to conclude that they move from *cis* to *trans* Golgi, the direction generally assumed for other transported proteins (9).

MANY OF THE OLIGOSACCHARIDES ARE ON TRANSPORTED PROTEINS: Cycloheximide treatment of infected

BHK cells significantly lowers the amount of lectin bound to sections of Golgi and ER membranes. Because the only discernible effects of cycloheximide are the cessation of protein synthesis and the loss of newly synthesized membrane proteins from the ER and Golgi complex (13), it is reasonable to conclude that the loss of lectin binding sites was caused by the loss of viral membrane proteins from those intracellular membranes.

More than half the lectin binding sites were lost from Golgi membranes. The percentage loss was very similar for all three lectins, and this is consistent with the loss of a single species of macromolecule such as the viral spike protein. Furthermore, the loss of Con A-binding sites amounted to ~250 sites/μm^2 of Golgi area density measured on micrographs. Roughly similar results were obtained for RCA and WGA if one corrects for the partial labeling of the Golgi stacks. It is comforting that these values compare very well with those obtained using antibodies to the spike proteins; in equivalent experiments the antibodies revealed about 350 sites/μm^2 (13).

~25% of the Con A-binding sites were lost from ER membranes after cycloheximide treatment. Caution must, however, be exercised because there are two potential sources of error that might serve to minimize the loss of sites. First, cycloheximide stops protein synthesis, but only completed viral membrane proteins can leave the ER. Nascent proteins on membrane-bound ribosomes will remain, and many will have high mannose oligosaccharides to which Con A will bind. Second, there are excess high mannose oligosaccharides on dolichol pyrophosphate, and these may bind Con A. Though the error caused by these two are difficult to estimate, it is clear that at least 25% of the Con A binding sites in ER membranes are present on transported proteins.

The remaining lectin binding sites probably represent oligosaccharides bound to endogenous ER and Golgi components. In rough microsomal membranes from rat liver, for example, there are 15–20 glycoproteins that bind to Con A; two of these are ribophorins I and II (7). For Golgi membranes, we can exclude the possibility that the remaining lectin-binding sites are nonspecific. ~95 sites/μm^2 remain after cycloheximide treatment, and this is significantly higher than the background levels (<10 sites/μm^2) present in the Ric^R-14 line of BHK that cannot construct complex oligosaccharides. The difference probably represents endogenous Golgi components though these have yet to be identified.

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