Compartmentalization of Intracellular Membrane Glycocomponents Is Revealed by Fracture-Label

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ABSTRACT We used thin-section fracture-label to determine the distribution of wheat-germ agglutinin binding sites in intracellular membranes of secretory and nonsecretory rat tissues as well as in human leukocytes. In all cases, analysis of the distribution of wheat germ agglutinin led to the definition of two endomembrane compartments: one, characterized by absence of the label, includes the membranes of mitochondria and peroxisomes as well as those of the endoplasmic reticulum and nuclear envelope; the other, strongly labeled, comprises the membrane of lysosomes, phagocytic vacuoles, and secretory granules, as well as the plasma membrane. The Golgi apparatus was weakly labeled in all studied tissues.

The similarity between the biogenetic pathways for secretory and membrane proteins led Sabatini et al. (1) to propose that the former may be ontogenetically related to ancestral membrane proteins. While many of the molecular mechanisms that mediate cotranslational insertion and glycosylation of both secretory and membrane proteins are now established (see references 2 and 3 for reviews), the processes responsible for sorting and channelling these molecules to their specific locations remain unclear (4). Autoradiographic and cytochemical approaches have been used to illustrate the intracellular trajectories of secretory proteins: from the endoplasmic reticulum (where mannose-rich oligossacharides are implanted on nascent polypeptide chains) to the Golgi apparatus where, after partial removal of mannosyl residue, terminal glycosylation takes place (see references 2, 3, and 5 for reviews). These processes abut the accumulation in vesicles, either for future export or as constituents of the lysosomal apparatus. More obscure are the trajectories and sites of accumulation of membrane proteins upon completion of terminal glycosylation processes (4). Conventional biochemical approaches to the study of the traffic of secretory and membrane glycoproteins generally require the isolation of membrane fractions and, therefore, cannot easily resolve ambiguities related to the cross-contamination that may occur during isolation procedures. Autoradiographic techniques can successfully follow the rates and determine the sites of incorporation of sugar residues in secretory glycoproteins (6, 7), but their limited resolution makes it difficult to define the intracellular topology of membrane components. Cytochemical labeling of ultrathin frozen sections, while technically demanding, is possible: here, cross-contamination is avoided but resolution, while much improved (8–10), is generally insufficient to define the sidedness of labeled membrane components.

Recently, we have devised simple techniques for the labeling, in situ, of freeze-fractured intracellular membranes (11–13). In these "fracture-label" methods, tissues or isolated cells are frozen, freeze-fractured in liquid nitrogen, and the membrane halves (exposed by the fracture process) labeled by conventional cytochemical techniques. As, during fracture, biological membranes are split along their bilayer interior, fracture-label allows also the study of the sidedness of membrane components. In pancreatic cells, fracture-label shows (14) that while concanavalin A labels the exoplasmic faces of the endoplasmic reticulum, nuclear envelope, secretory granule membranes and the plasmalemma, wheat germ agglutinin (WGA)[1]—a label of terminal glycoconjugates (sialic acid and *N*-acetyl glucosamine) (15)—fails to bind to endoplasmic reticulum or nuclear envelope membranes. These results negate the existence of significant reflux of fully glycosylated products to the endoplasmic reticulum (16). We have continued these studies and report here the distribution of WGA-binding glycoconjugates in Golgi and lysosomal membranes as well as in mitochondria and peroxisomes of a variety of secretory and nonsecretory tissues from the rat and in human leukocytes. In all cases, analysis of the distribution of WGA

[1] *Abbreviations used in this paper:* E face, exoplasmic fracture face; P face, protoplasmic fracture face; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin.

leads to the definition of two endomembrane compartments: one, characterized by absence of label, includes the membranes of mitochondria and peroxisomes as well as those of the endoplasmic reticulum and nuclear envelope; the other, strongly labeled, comprises the membranes of lysosomes, phagocytic vacuoles, and secretory granules, as well as the plasma membrane. The membranes of the Golgi apparatus were always weakly labeled. This result is analyzed in regard to the dynamics of glycosylation and packaging in secretory processes.

MATERIALS AND METHODS

Cells: Liver and ileum tissues, as well as adrenal and salivary glands, were excised from adult Sprague-Dawley rats. Cells were isolated by digestion with collagenase type IV or collagenase type II (5 mg/ml in Hanks' solution, Worthington Biochemical, Freehold, NJ) for 5–10 min at 37°C. Cells and tissues were washed twice in Hanks' solution, fixed in 1% or 2% glutaraldehyde in phosphate-buffered saline (PBS) pH 7.4 (2 h, 4°C), impregnated in 30% glycerol, and frozen as described elsewhere (11, 12). Human peripheral blood lymphocytes and monocytes isolated from fresh heparinized blood by Ficoll-Hypaque gradients (17) were washed twice in PBS and fixed in 1% glutaraldehyde (2 h, 25°C). Human neutrophils were isolated as described above, followed by Plasmagel (Roger-Bellon, Paris) sedimentation and lysis of erythrocytes with Tris-buffered ammonium chloride. Isolated neutrophils were washed twice in 0.1 M sodium cacodylate-HCl buffer and fixed in 1.5% glutaraldehyde (2 h, 25°C). Some samples were incubated for 30–40 min at 25°C in Karnovsky's diaminobenzidine saturated solution with H_2O_2 as substrate for the peroxidase reaction (18, 19). All cells were impregnated in glycerol and frozen.

Fracture-labeling: Frozen cells were immersed in liquid nitrogen and crushed with a glass pestle (10–13). The freeze-fractured cells were thawed (1% glutaraldehyde, 30% glycerol in PBS) and deglycerinated in 1 mM glycylglycine. Fractured cells were incubated in solutions of WGA (0.25–1 mg/ml in 0.1 M Sorensen phosphate buffer containing 4% polyvinylpyrrolidone, pH 7.4) for 1 h at 37°C. Controls were preincubated in 0.4 M *N*-acetyl-D-glucosamine for 15 min at 37°C, then treated with WGA in the presence of the sugar (1 h, 37°C). All samples were incubated in the presence of colloidal gold-ovomucoid complex at 25°C for 60 min (20). Fractured cells were also incubated and labeled with 1 mg/ml WGA-ferritin conjugates (E-Y Laboratory, San Mateo, CA) in PBS (1 h, 37°C). To locate thiamine pyrophosphatase activity, we processed salivary cells, fixed in 2% glutaraldehyde in cacodylate buffer, as described above and, after fracture, thawing, and deglycerination, treated for thiamine pyrophosphatase reaction (21) and labeled with WGA-colloidal gold-ovomucoid complexes.

Processing for Electron Microscopy: Fracture-labeled cells and tissue fragments were fixed in 1% osmium tetroxide in veronal acetate buffer, pH 7.6 (2 h, 4°C), stained en bloc with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections were examined unstained or poststained with uranyl acetate and lead citrate.

RESULTS

Lysosomal System

Human lymphocytes, monocytes, and neutrophils, as well as rat hepatocytes, were used here to search for examples of fracture-labeled lysosomes, phagocytic vacuoles, and secretory granules. WGA-binding sites, labeled by ovomucoid-coated colloidal gold, were always densely distributed over the exoplasmic fracture faces (E face) of lysosomes (Fig. 1, *a* and *b*) and phagocytic vacuoles (Fig. 1, *g–j*). In human neutrophils, labeling of E faces was intense in both peroxidase-positive azurophil granules (Figs. 1, *d* and *e*; see reference 22 for cytochemical characterization) and peroxidase-negative specific granules (Fig. 1, *c* and *e*). In rat hepatocytes, labeling of the E faces of lipoprotein secretory granules (23) was equally strong (Fig. 1 *f*). Protoplasmic faces (P face) could only be positively identified in "cracks" of the cell (see references 12, 14 for detailed explanation), where complementary views of the E face and its associated organelle were available (Fig. 1, *a* and *e*). The P faces of fracture-labeled membranes of lysosomes and granules were either weakly labeled (Fig. 1 *a*) or remained unlabeled (Fig. 1 *e*). Cross fractures of lysosomes, peroxidase-positive azurophyl granules, peroxidase-negative specific granules, or of lipoprotein secretory granules (Fig. 1 *g*) were not labeled. As expected (see reference 14), endoplasmic reticulum (Fig. 1 *f*), nuclear envelope and cross-fractured cytoplasm were not labeled. In controls (i.e., fractured cells preincubated and treated with the lectin in presence of *N*-acetyl-D-glucosamine), density of the label was reduced by 80–90%.

Mitochondria and Peroxisomes

WGA failed to label the membranes or cross-fractured matrix of freeze-fractured mitochondria (Fig. 2, *a*, *b*, *e*, and *g*) or peroxisomes (Fig. 2, *c*, *f*, and *g*; see Reference 24 for morphological characterization). Strong label of lysosomal membranes in the proximity of mitochondria attested that absence of label reflected a genuine absence of WGA-binding sites (Fig. 2 *g*). The outer face of lipid droplets was not labeled (Fig. 2 *d*).

Golgi Apparatus

Together with our previously published results (12–14), our observations cover the main membrane systems in a eucaryotic cell, except for the membrane systems in the Golgi apparatus. Functionally, its position is a bridge between the endoplasmic reticulum (not labeled by WGA; see reference 14) and the membranes of secretory vesicles, lysosomes, and the plasma membrane. We made an intensive search for unequivocal instances of fractured Golgi apparatus in cell types usually considered as nonsecretory (see reference 7) as well as in salivary gland cells. In every instance, label of Golgi membranes appeared weak or was absent (Fig. 3). Significant amounts of label could only be observed over fractured vesicles situated at the periphery or near the Golgi apparatus (Fig. 3, *b* and *g*). Because fractured Golgi frequently lack the elements for a unequivocal identification of cis- or trans-cisternae, we combined the reaction for thiamine pyrophosphatase activity (see reference 21), specific for trans-cisternae, with WGA fracture-label in salivary cells (Fig. 3, *e* and *f*). Over both regions, colloidal gold label was scant or absent. Since, in our experience, gold label may be less sensitive than ferritin conjugates to detect WGA-binding sites, we labeled fractured cells with WGA conjugated to ferritin. Again, fractured Golgi membranes exhibited little label. As observed with colloidal gold, labeling was somewhat stronger over peripheral areas of the Golgi apparatus (Fig. 3 *k*).

DISCUSSION

The membranes of lysosomes, phagocytic vacuoles as well as those of secretory granules are shown here to contain numerous WGA-binding sites. In contrast, the membranes of peroxisomes as well as those of mitochondria were not labeled. The membranes of the Golgi apparatus (except vesicles at its periphery) were not labeled or were sparsely labeled. These results complement our initial fracture-labeling studies (13, 14) that show the presence of WGA-binding sites in plasma and secretory granule membranes and their absence in endoplasmic reticulum and nuclear envelope membranes. Taken together, our results show that "fracture-label" can

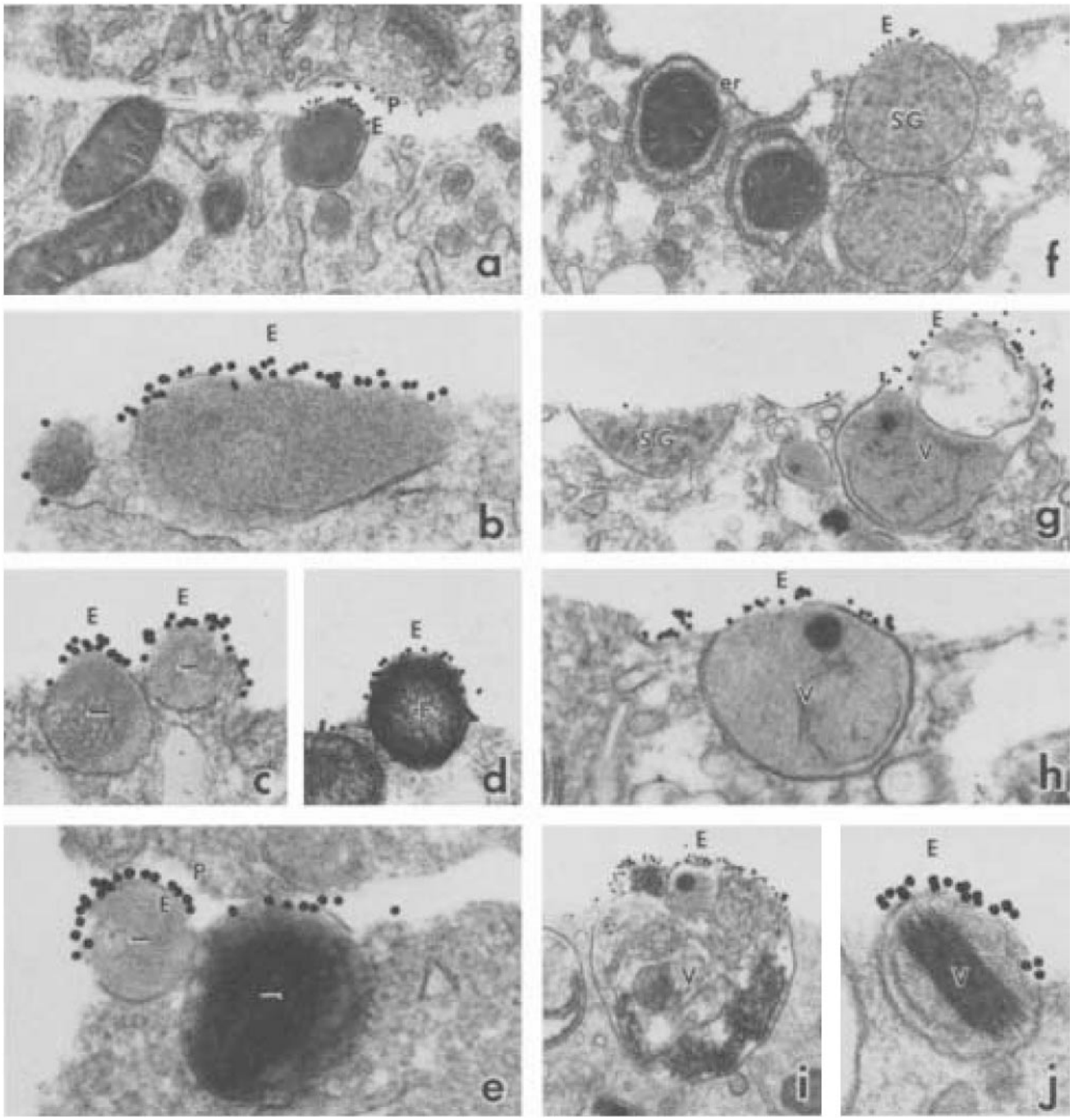

FIGURE 1 WGA fracture-labeling of lysosomes, phagocytic vacuoles, and secretory granules. E faces (*E*) are strongly labeled. Cracks (*a* and *e*) provide complementary views and show that P faces (*P*) display little or no label. (*a* and *b*) human lymphocyte, lysosome; (*c–e*) human neutrophils, peroxidase-positive azurophils (+), peroxidase-negative specific granules (−); (*i* and *j*) phagocytic vacuoles in a human monocyte and lymphocyte, respectively; (*f–h*) rat hepatocytes, lipoprotein secretory granules (*sg*); phagocytic vacuoles (*v*) are strongly labeled (*g* and *h*), but fractured endoplasmic reticulum (*er*) is not (*f*). (*a*) × 40,000; (*b*) × 95,000; (*c*) × 85,000; (*d*) × 60,000; (*e*) × 100,000; (*f*) × 30,000; (*g*) × 50,000; (*h*) × 60,000; (*i*) × 30,000; (*j*) × 110,000.

provide direct cytochemical evidence for the definition of two endomembrane compartments (25, 26): (*a*) the membranes of lysosomes, phagocytic vacuoles, and secretory granules contain branch sugars of complex carbohydrates (sialic acid, *N*-acetyl glucosamine) and appear ontogenetically derived from the Golgi apparatus; (*b*) the membranes of mitochondria and peroxisomes as well as those of the endoplasmic reticulum and the nuclear envelope (14) lack terminally glycosylated components and define the second endomembrane compartment. In the following paragraphs we analyse compartmentalization of endomembranes and we interpret the weak labeling of Golgi membranes as reflection of its intermediate "assembly line" (27) position between the endomembrane compartments defined above.

Membrane Compartmentalization as Defined by WGA Fracture-Label

Dense-labeling over the exoplasmic faces of the membranes of lysosomes, phagocytic vacuoles, and secretory granules is

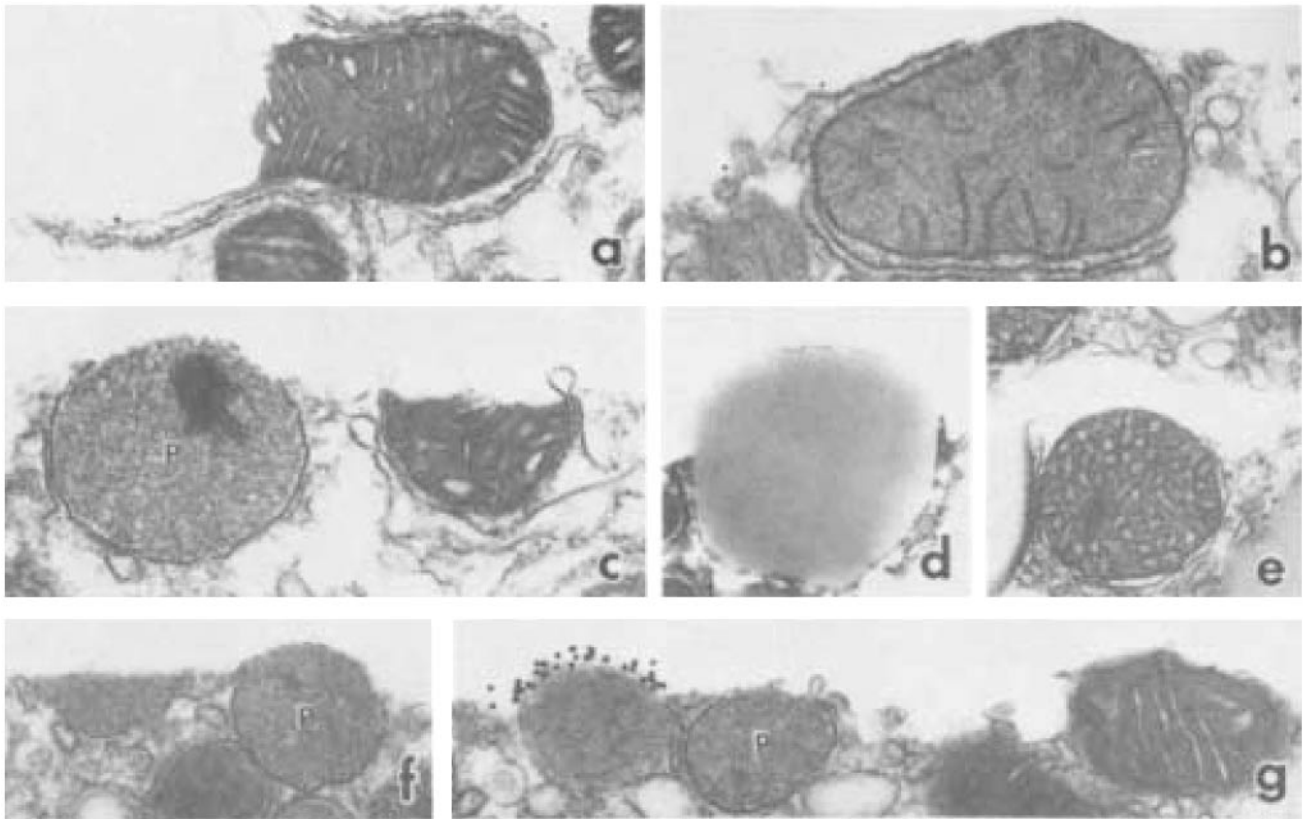

FIGURE 2 Absence of WGA-binding sites over fractured peroxisome (*P*) and mitochondrial membranes. (*a–d*, *f*, and *g*) Rat hepatocytes; (e) rat adrenal gland. (*g*) Strong label over the E face of a lysosome and no label over a fractured peroxisome and mitochondrion. (*d*) The outer face of a lipid droplet is not labeled. (*a*) × 35,000; (*b*) × 35,000; (*c*) × 50,000; (*d*) × 18,000; (e) × 35,000; (*f*) × 45,000; (*g*) × 50,000.

expected. As WGA primarily binds to sialic acid residues or to subterminal *N*-acetyl glucosamine (15), it is used here as a marker to detect the presence of terminally glycosylated membrane components. Labeling of "peroxidase positive azurophil granules", defined as primary lysosomes (19) and derived from the trans side of the Golgi apparatus (22), is of interest because it demonstrates that the sialoglycoconjugates in the membrane of a primary lysosome derive directly from the Golgi apparatus. In all instances, most of the label is found over E faces and represents sites exposed at the luminal (exoplasmic) side of the membrane, as expected from previous cytochemical and biochemical investigations (27–30). While labeling of E faces may, initially, appear difficult to explain, we have shown (11, 12, 31) that it is due to postfracture reorganization of membrane components into interrupted bilayered structures: the original orientation of amphipathic lipids can be reversed upon thawing and exposure to an aqueous solution. The weaker labeling of P faces of lysosomal membranes probably reflects the presence of transmembrane WGA-binding glycoproteins that partition preferentially with the protoplasmic half of the membrane (11, 32). The absence of label over fractured peroxisome membranes constitutes further indication that these organelles are ontogenetically unrelated to the Golgi apparatus. In mitochondria, absence of WGA label is also expected because their membrane proteins are synthesized either within the cytosol or inside the mitochondrial matrix. Both processes are unrelated to the Golgi apparatus and their terminal sialyltransferases (reviewed in reference 4).

The Golgi Apparatus: an Assembly Line Bridges Two Endomembrane Compartments

The membranes of the Golgi apparatus, even its trans elements as defined by thiamine pyrophosphatase activity, appeared to have few sites capable of binding WGA. WGA labeling of ultrathin frozen sections of liver tissue as well as isolated Golgi fractions indicates also the scarcity of binding sites on the cisternal side of Golgi membranes, except for the trans-cisternae, particularly their dilated rims (33; and C. de Lemos, G. Kreibich, E. Rodriguez-Boulan, and D. Sabatini, personal communication). At first, weak labeling of Golgi membranes by WGA seems a paradox because it is in this organelle that the terminal glycosyltransferases responsible for the implantation of sialic acid residues on membrane glycoproteins are located (29, 30, 34). Previous biochemical studies indicate the presence of sialoglycoproteins in Golgi-enriched membrane fractions (26, 30). Recent observations of frozen sections of kidney cultured cells infected or not by Semliki Forest virus show the presence of galactose residues (as defined by labeling with *Ricinis communis* agglutinin-colloidal gold) in the trans-cisternae as well as one or two cis-cisternae of the Golgi apparatus (10). We believe that these variations in the amount of labeling of Golgi membranes are to be expected as the density of WGA-binding glycoproteins will depend on the synthetic activity of the cell. This is reflected in the decrease of labeling of Golgi membranes observed in virally infected cells upon incubation with cycloheximide (10). In addition, it is expected that the intensity of labeling will also depend on

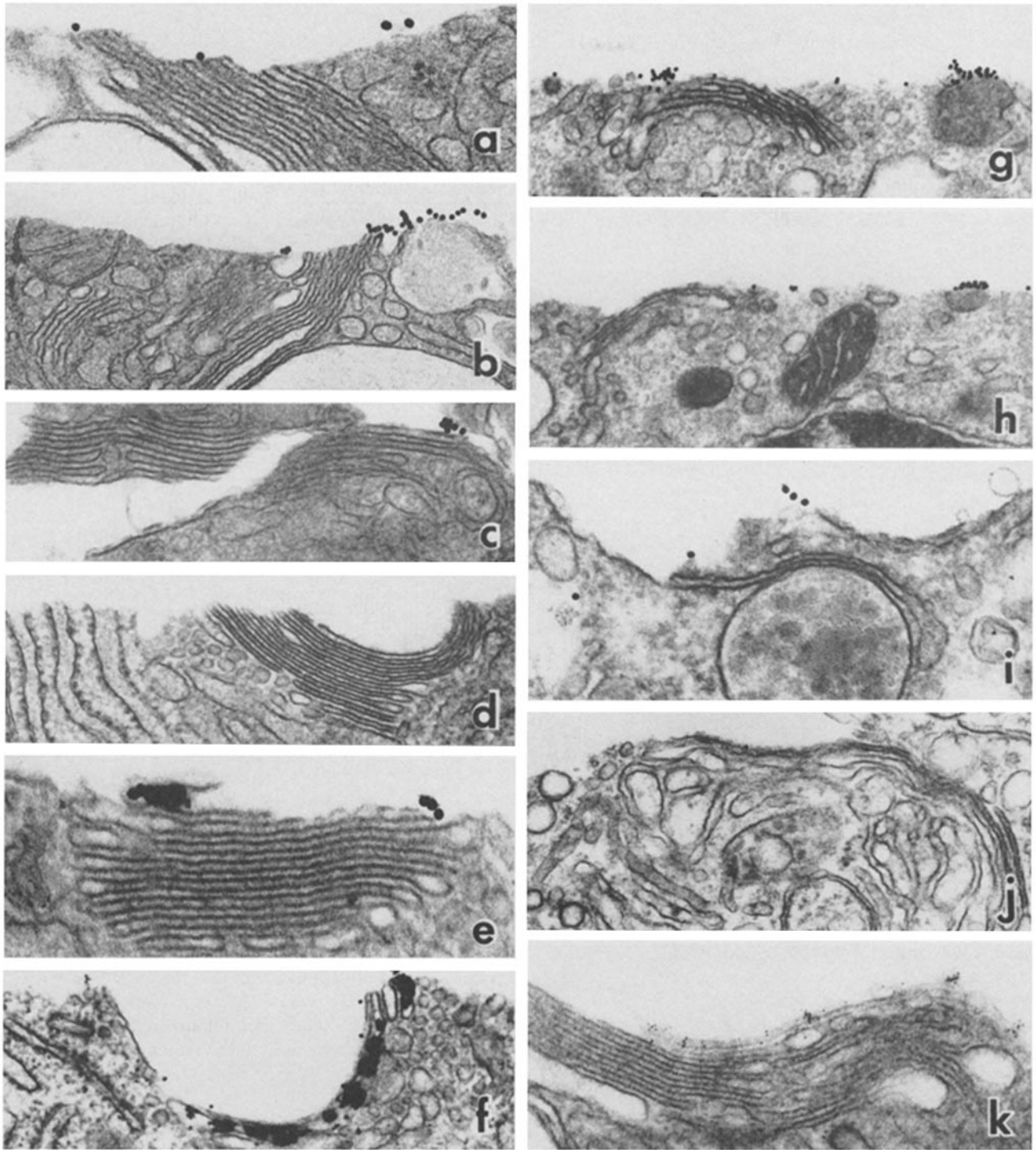

FIGURE 3 WGA fracture labeling of the Golgi apparatus. Label is absent from most Golgi membranes, except for vesicles at its periphery. Label by WGA-ferritin conjugates is also weak (*k*). Trans-cisternae reacted for thiamine pyrophosphatase show that little or no WGA label is associated with this region (*e* and *f*). (*a–f* and *k*) Rat salivary gland; (*g* and *h*) human lymphocytes; (*i*) rat hepatocyte; (*j*) rat ileum. (a) × 90,000; (*b*) × 60,000; (c) × 70,000; (*d*) × 55,000; (e) × 110,000; (*f*) × 45,000; (*g*) × 45,000; (*h*) × 40,000; (*i*) × 75,000; (*j*) × 45,000; (*k*) × 65,000.

the time spent within Golgi membranes by terminally glycosylated glycoproteins. Our results show that, in the cells and tissues we studied, labeling of Golgi membranes, even at the trans side (the site of terminal glycosylation: see references 29, 30, 34), can be much lower than that of membranes "downflow" from the Golgi apparatus (lysosomes, secretory granules, and the plasma membrane) (35). These results accord with biochemical studies that reveal the membranes of the Golgi apparatus to be relatively poor in protein (36, 37). The Golgi apparatus can, therefore, be viewed as an assembly line where, at a given time, the density of terminally glycosylated glycoproteins may be less than that found in the membranes of the lysosomal system or in the plasma membrane. In other words, while the final steps of glycosylation take place in the membranes of the Golgi apparatus, newly synthesized membrane glycoproteins may not necessarily accu-

mulate in the membranes of this organelle but, rather, are transferred to their final cellular destinations.

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