Immunolocalization of UDP-glucose:glycoprotein glucosyltransferase indicates involvement of pre-Golgi intermediates in protein quality control

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The UDP-glucose:glycoprotein glucosyltransferase (GT) is a protein folding sensor and glycosyltransferase that constitutes an important component of the protein quality control machinery. With the use of quantitative immunogold electron microscopy, we established the subcellular distribution of GT in rat liver and pancreas and *Drosophila melanogaster* salivary gland as well as cell lines and correlated it with that of glucosidase II, calreticulin, and pre-Golgi intermediate markers. Labeling for GT, as well as for glucosidase II and calreticulin, was found in the endoplasmic reticulum (ER), including nuclear envelope and pre-Golgi intermediates located between ER and Golgi apparatus, and in the cell periphery. In the rough ER, labeling for GT was inhomogeneous, with variously sized labeled and unlabeled cisternal regions alternating, indicative of a meshwork of quality control checkpoints. Notably, labeling intensity for GT was highest in pre-Golgi intermediates, corresponding to twice that of rough ER, whereas the Golgi apparatus exhibited no specific labeling. These results suggest that protein quality control is not restricted to the ER and that the pre-Golgi intermediates, by virtue of the presence of GT, glucosidase II, and calreticulin, are involved in this fundamental cellular process.

Newly synthesized secretory and membrane proteins are subjected to quality control (1) by a machinery consisting of chaperones (2, 3), lectins (4–6), glucosidase II (gls II) (7–9), UDP-glucose:glycoprotein glucosyltransferase (GT) (10–12), and endomannosidase (13). In the endoplasmic reticulum (ER), chaperones help proteins co- and posttranslationally to mature (14, 15). Furthermore, the lectins calnexin and calreticulin bind to glycoproteins carrying monoglucosylated $Glc_1Man_9GlcNAc_2$ oligosaccharides generated by gls II (16, 17) and GT (18, 19), and the dissociation of such complexes can be brought about by gls II, which is located in the ER and pre-Golgi intermediates‖ (13, 20), and by endomannosidase in the pre-Golgi intermediate/Golgi apparatus (13). If correctly folded, glycoproteins may exit the ER. In contrast, glycoproteins displaying nonnative conformations are retarded, reglucosylated by GT (12, 18), and will enter a further round of the calnexin/calreticulin–chaperone–gls II cycle (9, 21).

GT is a soluble, calcium-dependent 170-kDa enzyme (22) that has been detected in mammalian, plant, fungal, and protozoan cells (11, 12). It is unique in that it serves a twofold function as a protein folding sensor and as glycosyltransferase (10–12). GT recognizes two elements exposed in malfolded conformers, namely the innermost GlcNAc unit of the oligosaccharide and not yet fully characterized protein domains (10). By reglucosylating the oligosaccharide, GT tags the protein for further processing by the folding machinery (10, 11, 22–24).

Although many of the biochemical and molecular aspects of GT have been elaborated, to date its detailed *in situ* subcellular distribution is unknown. The close relationship between organelle organization and cell function makes information about the subcellular distribution of GT crucial for further understanding of protein quality control under physiological conditions. To analyze these aspects of GT, we have carried out high-resolution quantitative immunogold labeling on ultrathin cryosections with antibodies against GT, and double immunogold labeling combined with antibodies against gls II, p58, sec23p, and calreticulin in rat and *Drosophila melanogaster* tissues and cell lines synthesizing and secreting large amounts of proteins. We observed a nonhomogeneous immunolabeling for GT in the rough ER. Although positive, the smooth ER of hepatocytes exhibited only 11% of rough ER labeling intensity. Notably, GT was enriched in the pre-Golgi intermediates with a labeling intensity twice that of the rough ER. Thus, the presence of GT as well as of gls II and calreticulin in the pre-Golgi intermediates suggests their involvement in protein quality control.

Materials and Methods

Antibodies. Rat liver GT was purified to apparent homogeneity (22). About 300 μg of native GT in complete Freund's adjuvant was intradermally injected in rabbits, followed by two subcutaneous booster injections of the same amount of GT in incomplete adjuvant at 30-day intervals. Thirty days later the animals were bled. Polyclonal rat antiserum against *D. melanogaster* GT was prepared as described (11) and affinity-purified. Polyclonal antibodies against gls II and calreticulin (kindly provided by H. D. Söling, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany), rat p58 (affinity-purified and kindly provided by J. Saraste, Univ. of Bergen, Bergen, Norway), and yeast sec23p (kindly provided by R. Schekman, Univ. of California, Berkeley) were used. Mouse monoclonal antibody against Golgi mannosidase II was from Babco (Richmond, CA), and affinity-purified Fab fragments of goat anti-rabbit IgG and goat anti-mouse IgG (unlabeled and rhodamine red-X-conjugated) were from Jackson ImmunoResearch. An Alexa 488 labeling kit from Molecular

Abbreviations: ER, endoplasmic reticulum; gls II, glucosidase II; GT, UDP-glucose:glycoprotein glucosyltransferase.

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‖The term "pre-Golgi intermediate" is used synonymously with intermediate compartment (31, 32), ERGIC-53 (49, 50), and VTCs (51) located either between ER and the Golgi apparatus or in the cell periphery.

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Probes was used to prepare Alexa 488-conjugated Fab fragments of goat anti-rabbit IgG. Affinity-purified goat anti-rat IgG and goat anti-rabbit IgG (Jackson ImmunoResearch) and staphylococcal protein A (Amersham Pharmacia) were complexed with 6-nm, 8-nm, 10-nm, and 12-nm gold particles according to standard procedures (25, 26).

Immunofluorescence Staining and Confocal Laser Scanning Microscopy. Rat liver-derived clone 9 and BRL3A cells (American Type Culture Collection) were grown on glass coverslips and fed with fresh medium 16 h before fixation in freshly prepared 3% formaldehyde (Fluka) in Hanks' buffered salt solution–Hepes (10–20 mM, pH 7.0). Coverslips were rinsed briefly with fixative (37°C) and fixed in fresh fixative for 10 min at 37°C, followed by 0.5% formaldehyde fixative for 30 min at ambient temperature. After two rinses in PBS (10 mM phosphate buffer, pH 7.2/0.15 M NaCl), coverslips were transferred to 50 mM NH_4Cl in PBS for 30 min at 4°C, rinsed twice in PBS, and immediately processed for immunofluorescence as described (13). Immunofluorescence was recorded with a Leica confocal laser scanning microscope with the use of the 100× objective ($n = 1.4$). In double immunofluorescence overlays, effects of pixel shift were excluded. The z axis resolution of this equipment was, at maximum, 300 nm per voxel, and the x, y settings were between 50 and 250 nm per voxel.

Immunoelectron Microscopy. Male adult Wistar rats (about 250 g body weight) were anesthetized and perfused via the left cardiac ventricle with oxygenated Hanks' balanced salt solution–Hepes (10–20 mM, pH 7.0) containing 3% polyvinylpyrrolidone (30 kDa; Fluka) and 70 mM $NaNO_2$ (Merck) for 2 min at 37°C, followed by the same solution containing 4% formaldehyde for 15 min at 37°C. Afterward, pieces of liver and pancreas were immersion-fixed for 1.5 h and processed for ultracryotomy (27, 28). Another protocol consisted of 4% formaldehyde perfusion fixation (15 min) and immersion fixation (15 min), followed by 2% formaldehyde immersion fixation for 1.5 h and stepwise sucrose infiltration in the presence of 1% formaldehyde (B.G., K. Tokuyasu, and J.R., unpublished work). In addition, cultured rat liver hepatocytes, clone 9, and BRL3A cells were formaldehyde-fixed as described above. Salivary glands from *D. melanogaster* third-instar larvae and Schneider S2 cells (kindly provided by W. Gehring, Biozentrum, Univ. of Basel, Basel) were fixed in 3% formaldehyde/0.1% glutaraldehyde in Ringer's solution for 2 h at ambient temperature.

Grids with attached ultrathin cryosections were conditioned on droplets of PBS containing 1% BSA, 0.01% Triton X-100, and 0.01% Tween 20 for 10 min; incubated on droplets of primary antibodies diluted in conditioning buffer for 2 h at ambient temperature or overnight at 4°C; rinsed on droplets of PBS; incubated with 8-nm or 10-nm protein A-gold or gold-labeled goat anti-rat IgG for 1 h; rinsed; fixed with 2% glutaraldehyde in PBS for 10–20 min; and embedded and stained according to the method of Tokuyasu (27, 28). For double labeling of rat tissues and cell lines, the sequential protein A-gold method was applied (26). For GT and sec23p double labeling, ultrathin cryosections from *Drosophila* salivary glands were simultaneously incubated in the respective primary antibodies, rinsed, and simultaneously incubated with 6-nm gold-labeled goat anti-rat IgG and 12-nm gold-labeled goat anti-rabbit IgG.

In controls, sections incubated with protein A-gold or gold-labeled goat anti-rabbit IgG and goat anti-rat IgG alone showed only occasional gold particles.

Quantification of Immunolabeling. Micrographs from immunogold-labeled rat liver and pancreas cryosections from three different incubations were taken at an original magnifications of ×10,000 (nuclear envelope, endoplasmic reticulum, mitochondria, nucleoplasm, zymogen granules) and ×20,000 (pre-Golgi intermediates, Golgi apparatus), and the number of gold particles per square micrometer of various cellular compartments was calculated according to standard protocols (29). The density of immunogold labeling for GT in *Drosophila* salivary glands over the ER and pre-Golgi intermediates was obtained by measuring the distance between gold particles.

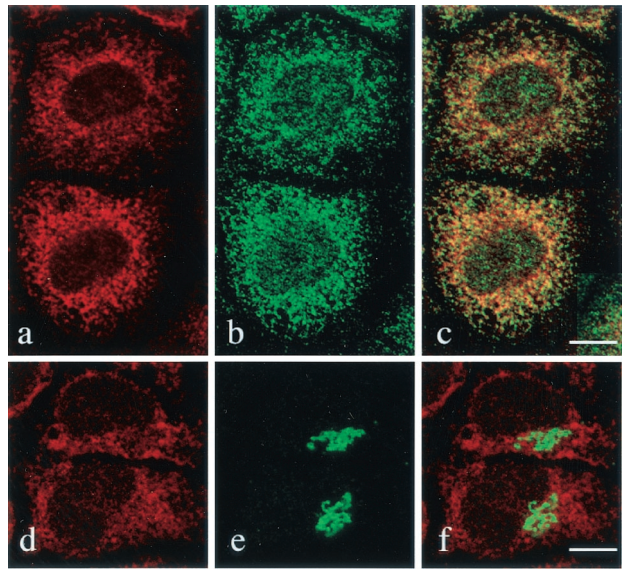

Fig. 1. Immunofluorescence localization of GT, gls II, and Golgi mannosidase II in clone 9 hepatocytes. In a single optical section, a similar staining pattern is observed for both GT (*a*) and gls II (*b*), with the overlay shown in *c*. The immunofluorescence of GT (*d*) and that of Golgi mannosidase II (*e*) are mutually exclusive (*f*). (Bars, 10 μm.)

Results

By confocal laser scanning immunofluorescence, GT immunostaining in clone 9 rat liver cells appeared throughout the cytoplasm (Fig. 1*a*), as reported for *Drosophila*-derived Kc cells (11). Immunofluorescence for gls II (Fig. 1*b*) exhibited a pattern similar to that of GT (Fig. 1*c*). In contrast, immunofluorescence for GT (Fig. 1*d*) and that for Golgi mannosidase II (Fig. 1*e*) were mutually exclusive (Fig. 1*f*).

Studies on rat tissues and cells were performed on ultrathin cryosections from only formaldehyde-fixed material because glutaraldehyde was deleterious for GT detection, notwithstanding low pH antigen retrieval (30). It should be emphasized that after initial fixation tissues and cells were continuously exposed to formaldehyde until the antibody incubation of the cryosections commenced. Gold particle labeling for GT was observed over nuclear envelope and rough ER in rat liver (75.4 ± 3.4 gold particles per μm^2 and 181.8 ± 2.1 gold particles per μm^2, respectively) and pancreas as well as the hepatocyte lines and was consistently absent over the Golgi apparatus, nucleus, mitochondria, and zymogen granules (Figs. 2 *a*, *b*, and *f*, and 3 *a–c*, and Table 1). The same labeling pattern was found in *Drosophila* salivary glands (Fig. 4) and Schneider 2 cells (not shown). In liver, smooth ER exhibited specific labeling for GT (Fig. 2*b*), albeit at only 11% (20.9 ± 1.6 gold particles per μm^2) of the labeling intensity of rough ER (181.8 ± 2.1 gold particles per μm^2). The rough ER of all studied cell types consistently exhibited a nonhomogeneous labeling for GT with variously sized labeled and unlabeled cisternal regions alternating (Fig. 2). Immunolabeling for GT was also present in the pre-Golgi intermediates (Fig. 3). Notably, pre-Golgi intermediate immu-

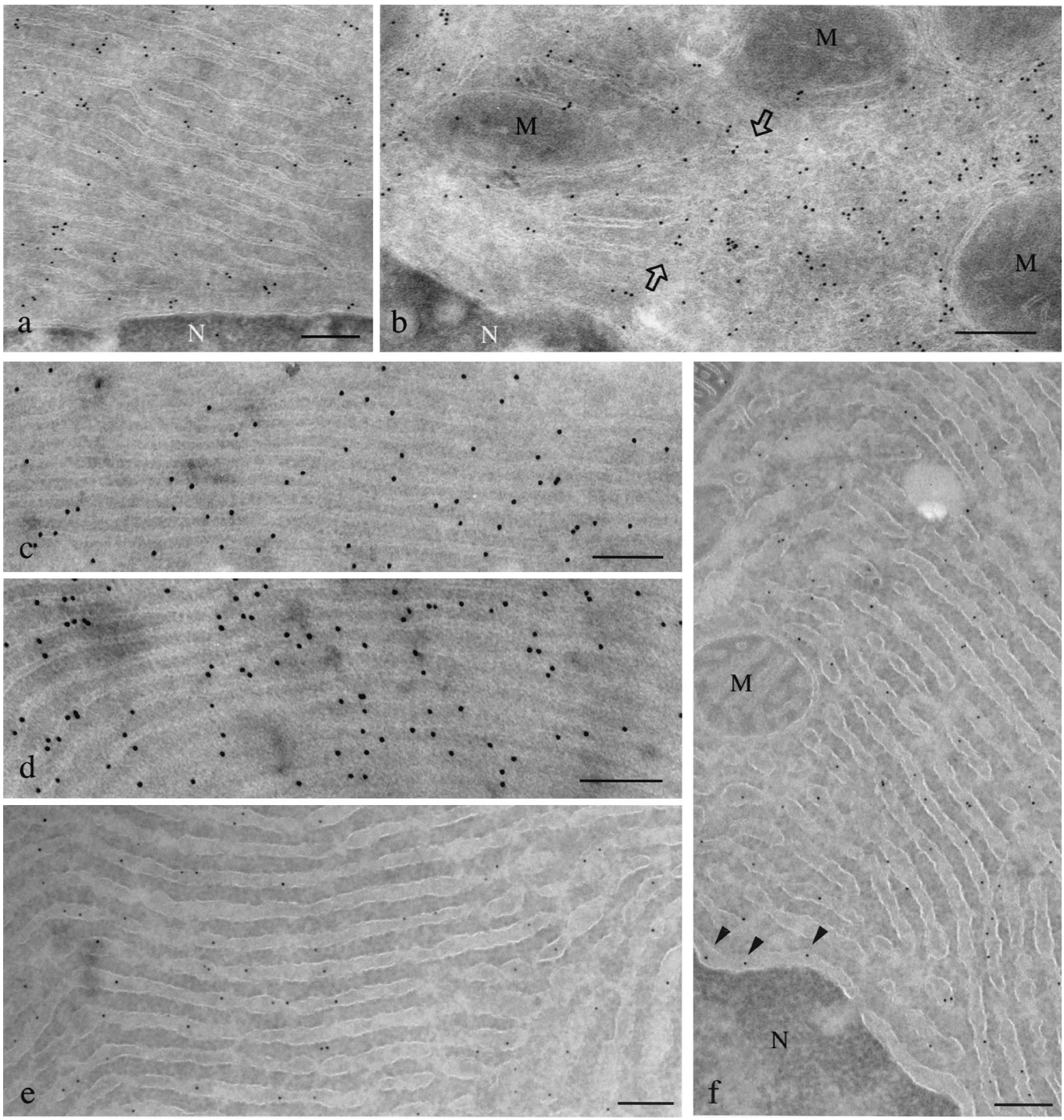

Fig. 2. Immunogold labeling of GT in ultrathin cryosections of liver (*a* and *b*), primary hepatocyte cultures (*c* and *d*), and pancreas (*e* and *f*). Gold particle labeling is present over the rough ER and, in addition, over the smooth ER in hepatocytes of liver (*b*). The zone of transition from rough to smooth ER is indicated by open arrows in *b*. Labeling of nuclear envelope is also evident (arrowheads in *f*). A nonuniform labeling can be consistently observed over the rough ER, with ER cisternae free of immunogold labeling over long distances (*a, c–f*). N, nucleus; M, mitochondria. (Bars, 0.25 μm.)

nolabeling was twice that of rough ER in pancreatic acinar cells (Table 1). This level of immunolabeling certainly represents an underestimation, inasmuch as the measured pre-Golgi intermediate surface area also comprised the cytosolic compartment, whereas the data for the rough ER were based solely on the surface area of the ER cisternal lumen. A 1.4-fold higher labeling density for GT in the pre-Golgi intermediate versus rough ER was also found in *Drosophila* salivary glands (mean distance between gold particles in rough ER, 2.1 $\pm$ 0.08 μm; transitional ER, 2.0 $\pm$ 0.07; and pre-Golgi intermediates, 1.5 $\pm$ 0.03 μm). For the identification of the pre-Golgi intermediates, immunostaining for p58 (31, 32) and for sec23p in *Drosophila* (33) was combined with GT immunolabeling. Double labeling for GT (small gold particles) and p58 (large gold particles) is shown in Fig. 3 *c* and *f*, and double labeling for GT (small gold particles) and sec23p (large gold particles) is shown in Fig. 4*b*. Fig. 3*b*

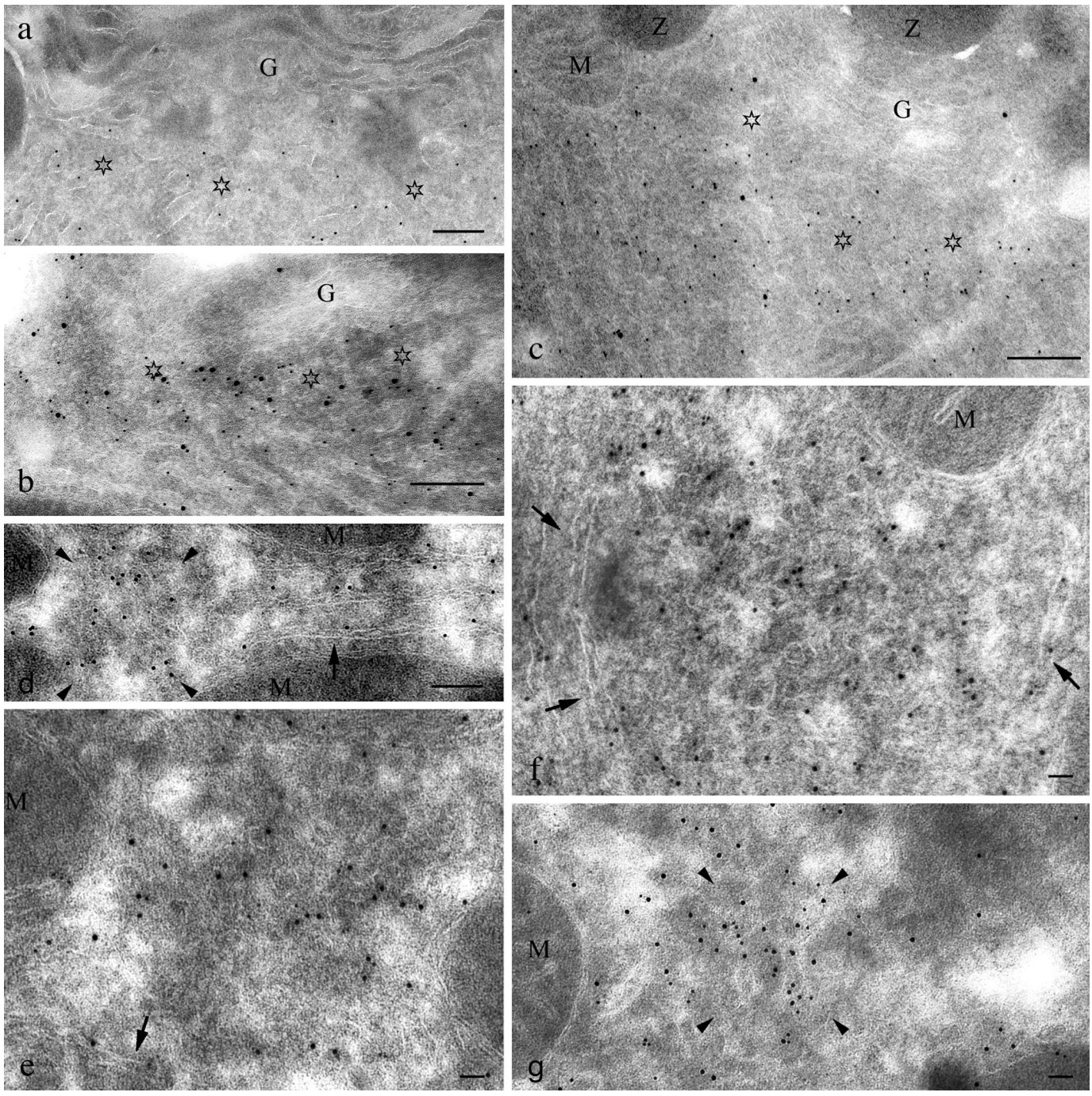

Fig. 3. Immunogold labeling of GT and double labeling of GT and gls II, p58, or calreticulin in ultrathin cryosections of pancreas (*a–c*) and liver (*d–g*). Vesicular tubular structures of pre-Golgi intermediates (asterisks) in pancreas exhibit intense labeling for GT, whereas the Golgi apparatus (G) is not labeled (*a*). As shown by double labeling in *b*, both GT (small gold particles) and gls II (large gold particles) are enriched in the pre-Golgi intermediate (asterisks) when compared with the labeled adjacent rough ER (see also Table 1 for quantification of GT labeling). In *c*, double labeling for GT (small gold particles) and p58 (large gold particles) in a pre-Golgi intermediate (asterisks) is shown. In liver hepatocytes, peripheral pre-Golgi intermediates (marked by arrowheads in *d* and *g*) and rough ER cisternae (arrows in *d–f*) exhibit labeling for GT. Such peripheral pre-Golgi intermediates are positive for both GT (small gold particles) and gls II (large gold particles), GT (small gold particles) and p58 (large gold particles), and GT (small gold particles) and calreticulin (large gold particles), as shown in *e*, *f*, and *g*, respectively. Z, zymogen granules; M, mitochondria. (Bars, 0.25 μm.)

demonstrates double labeling for GT (small gold particles) and gls II (large gold particles) in pre-Golgi intermediates of pancreatic acinar cells, which was also observed in liver and hepatocyte cell lines (not shown; see also refs. 13 and 20). Vesicular tubular clusters as part of peripheral ER export complexes (34) were positive for p58 (13, 32) and GT (Fig. 3*d*). By immunogold double labeling, such clusters were labeled for both GT and p58 (Fig. 3 *c* and *f*), GT and gls II (Fig. 3 *b* and *e*), and GT and calreticulin (Fig. 3*g*).

Discussion

The protein folding sensor GT represents a key element of the protein quality control machinery because it not only recognizes glycoproteins with nonnative conformation, but also

Table 1. Labeling density for glucosyltransferase in rat pancreas

Location	Gold particles/μm^2
Nuclear envelope*	74.3 ± 5.6
Rough ER†	172.0 ± 14.2
Pre-Golgi intermediates‡	342.8 ± 20.3
Golgi apparatus§	3.7 ± 0.1
Zymogen granules¶	3.6 ± 1.0
Mitochondria‖	4.2 ± 0.8
Nucleoplasm**	1.0 ± 0.6

*38 fields, total of 758 gold particles.
†30 fields, total of 879 gold particles.
‡30 fields, total of 980 gold particles.
§30 fields, total of 35 gold particles.
¶48 fields, total of 451 gold particles.
‖60 fields, total of 263 gold particles.
**39 fields, total of 191 gold particles.

converts them into a substrate for calnexin and calreticulin by reglucosylation (12). This conversion into a substrate represents an import event in protein quality control (1), as it prevents further transport of structurally imperfect glycoproteins and hence impaired cellular function because of malfunctioning or nonfunctioning glycoproteins. Our present results provide insight into the *in situ* subcellular organization of the protein quality control machinery. By high-resolution quantitative electron microscopic immunogold labeling, we show that GT is present not only in the rough ER but also in the smooth ER and unequivocally exists in pre-Golgi intermediates of rat and *Drosophila* tissues and cell lines. Thus, this aspect of protein quality control appears to be highly conserved between insect and mammalian cells. In more general terms, it also demonstrates that classical ER residential proteins like GT and gls II (14, 15, 22, and present study) are indeed present beyond COP II budding profiles of transitional ER. With the use of double immunogold labeling, we demonstrated the presence of gls II and calreticulin in pre-Golgi intermediates (see also refs. 13 and 20), both of which are functionally close associates of GT. The presence of three elements of the quality control of glycoprotein folding (GT, gls II, and calreticulin) in the ER and pre-Golgi intermediates is consistent with the fact that all of them are soluble proteins displaying ER retrieval sequences at their C termini. In the case of gls II, this sequence is not present in the catalytic but in a tightly associated β-subunit. Remarkably, when the immunogold labeling for GT was quantified, labeling intensity was 2-fold higher in the pre-Golgi intermediates as compared with rough ER. This higher labeling intensity indicates that GT has not just leaked out of the rough ER, but that the pre-Golgi intermediates represent a major cellular location for GT. Because the present immunoelectron microscopic study analyzed the subcellular distribution of immunoreactive protein, the functional interpretation of the data naturally is limited, although there is no reason to assume that GT, gls II, and calreticulin would be functional in the ER and not in the pre-Golgi intermediates. Under the assumption that GT-driven calreticulin association and gls II-driven calreticulin dissociation of glycoproteins occur in pre-Golgi intermediates, the presence of these proteins in this structure indicates its involvement in the control of protein folding. Taking into account the high dynamics of traffic at the ER–Golgi interface (35, 36), retrograde transport (37) or back-up to the ER of glycoproteins not achieving a native conformation may ultimately occur, eventually followed by retrotranslocation and ubiquitin-proteasome-mediated degradation in the cytosol (38–41). However, pre-Golgi intermediates not only are sites where concentration of mature proteins occurs (42), but also seem to be sites of accumulation of misfolded proteins (43–45). The nonhomogeneous labeling for GT by immunofluorescence and immunogold labeling deserves comment. Taking into account evidence for heterogeneity of the ER (46, 47), the GT labeling pattern in the ER could be indicative of a microdomain-like meshwork of multiple checkpoints engaged in the control of protein quality. The existence of a protein network in the ER composed of calnexin, calnexin-substrate complexes, other chaperones, and additional proteins was reported by Tatu and Helenius (48) and was proposed to function as a proteinaceous matrix to restrict the exit of early folding and assembly intermediates of influenza virus from the ER. On the other hand, the pre-Golgi intermediates, because of the enrichment of GT and the presence of gls II and calreticulin, could represent decisive quality checkpoints for anterograde protein transport under physiological conditions. In summary, our present study on GT and previous ones on gls

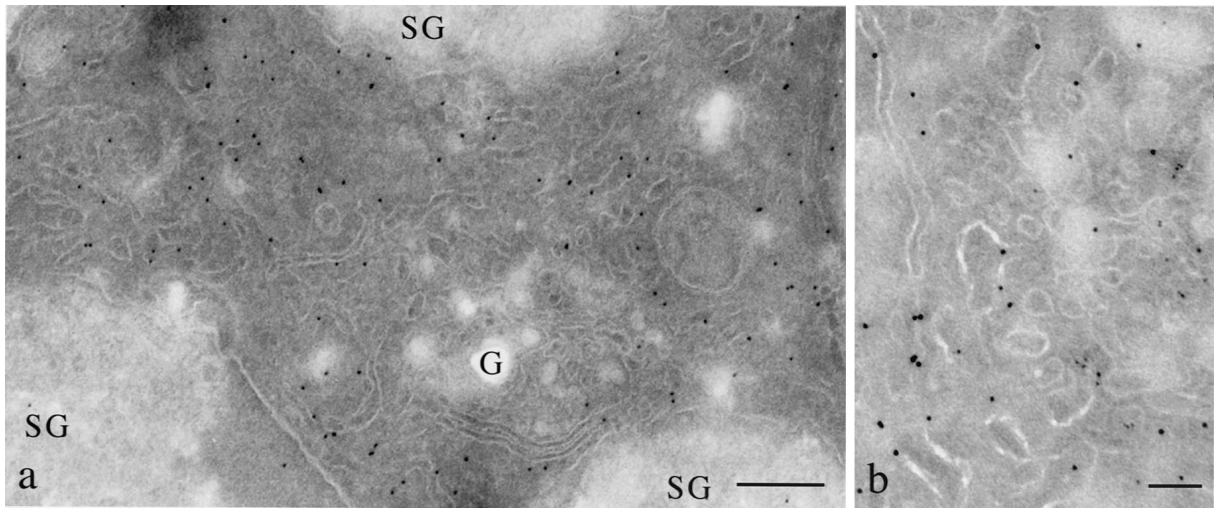

Fig. 4. Immunogold labeling of GT in ultrathin cryosections of *Drosophila* salivary gland. Gold particle labeling exists over the ER and pre-Golgi intermediates, and the Golgi cisternal stack (G) and secretory granules (SG) are unlabeled (*a*). At higher magnification, double labeling for GT (small gold particles) and sec23p (large gold particles) is present in and around vesicular tubular elements of a pre-Golgi intermediate (*b*). Adjacent ER (on the left) shows only GT labeling. (Bars, 0.25 μm.)

II (13, 20), calreticulin, and endomannosidase (13) indicate that protein quality control in the secretory pathway is not limited to the ER and points to the involvement of additional parts of the secretory pathway in this fundamental cellular process.

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