Is Cytochrome P-450 Transported from the Endoplasmic Reticulum to the Golgi Apparatus in Rat Hepatocytes?

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ABSTRACT The Golgi apparatus mediates intracellular transport of not only secretory and lysosomal proteins but also membrane proteins. As a typical marker membrane protein for endoplasmic reticulum (ER) of rat hepatocytes, we have selected phenobarbital (PB)-inducible cytochrome P-450 (P-450[PB]) and investigated whether P-450(PB) is transported to the Golgi apparatus or not by combining biochemical and quantitative ferritin immunoelectron microscopic techniques. We found that P-450(PB) was not detectable on the membrane of Golgi cisternae either when P-450 was maximally induced by phenobarbital treatment or when P-450 content in the microsomes rapidly decreased after cessation of the treatment. The P-450 detected biochemically in the Golgi subcellular fraction can be explained by the contamination of the microsomal vesicles derived from fragmented ER membranes to the Golgi fraction. We conclude that when the transfer vesicles are formed by budding on the transitional elements of ER, P-450 is completely excluded from such regions and is not transported to the Golgi apparatus, and only the membrane proteins destined for the Golgi apparatus, plasma membranes, or lysosomes are selectively collected and transported.

The existence of endoplasmic reticulum (ER)[1] marker enzymes such as P-450, NADPH-cytochrome *c* reductase, and glucose-6-phosphatase (G-6-Pase) in the Golgi apparatus remains controversial. A small amount of P-450 has been detected in the Golgi fraction by various authors (1–3). Jarasch et al. (4) studied this problem extensively and presented much evidence that P-450 exists not only in the Golgi apparatus but also in the plasma membranes. Howell et al. (5) reported that a significant amount of NADPH-cytochrome *c* reductase and G-6-Pase are present in isolated Golgi fractions when assayed immediately after their isolation. By immunoabsorption, Ito and Palade (6) concluded that NADPH-cytochrome *c* reductase is a bona fide Golgi membrane enzyme that probably distributed unevenly among the elements of Golgi complex. These observations have lead Rothman (7) to propose a distillation tower model of the Golgi apparatus: that is, the Golgi apparatus is a multistage distillation tower which may act sequentially to refine the protein export of the ER by removing escaped ER proteins.

In the previous papers (8, 9), however, we have presented qualitative immunoelectron microscopic evidence that suggests that P-450 does not exist either on the Golgi apparatus or transport vesicles.

Whether or not P-450 is transported to the Golgi apparatus accompanied by secretory proteins is so important for the consideration of sorting mechanisms that might work in the intracellular transport between the ER and the Golgi apparatus that we have investigated this problem in detail by combining biochemical and quantitative ferritin immunoelectron microscopic techniques.

We found that phenobarbital (PB)-inducible cytochrome P-450 (P-450[PB]) is not detectable on the membranes of Golgi cisternae either when P-450 is maximally induced in rat hepatocytes by PB treatment or when P-450 content in the microsomes rapidly decreased after cessation of the treatment. It is very probable, therefore, that P-450 is sorted out on the membranes of the transitional ER and is not incorporated into the limiting membranes of transport vesicles when they are formed by budding mechanisms, thus is not transported to the Golgi apparatus. A part of this paper has been reported at the 3rd International Congress of Cell Biology (10).

MATERIALS AND METHODS

Materials: PB was purchased from Sanko Seiyaku Kogyo Co., Ltd., Tokyo. ^{125}I-Labeled protein A (30 mCi/mg protein) was obtained from Amer-

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; G-6-Pase, glucose-6-phosphatase; PB, phenobarbital; P-450(PB), major form of cytochrome P-450 in hepatic microsomes of PB-treated rats; VLDL, very low density lipoprotein.

sham International, England. Bio-gel A 1.5 M was from Bio-Rad Laboratories, Richmond, CA. All other chemicals used were of the highest purity commercially available.

Preparation of Golgi and Microsome Fractions from Rat Livers: Male Sprague-Dawley rats (~150 g body weight) were fed at libitum on laboratory chow and were given an intraperitoneal injection of PB (80 mg/kg body weight) once a day for 4 d.

The animals were killed by a blow to the head. The livers were quickly removed and perfused with 0.25 M sucrose, weighed, and minced with a razor. The Golgi fraction was prepared according to Hino et al. (11).

Briefly, the minced liver was crushed with a wooden spatula through a stainless-steel mesh (100 mesh), and the disrupted cells were suspended in 2 vol of homogenizing medium (H-medium; 0.5 M sucrose containing 50 mM Tris-maleate buffer, pH 6.75 and 1% dextran). The suspension was centrifuged at 1,000 *g* for 5 min, and the supernatant plus the fluffy layer were further centrifuged at 5,000 *g* for 10 min. The loosely packed pellet was stirred with a glass rod, and the thick suspension (~1 ml) was loaded onto ~6 ml of 1.2 M sucrose and centrifuged at 90,000 *g* for 60 min in a Hitachi PRS 40 rotor (equivalent to Beckman SW 40 Ti rotor). The membranous materials on the 1.2 M sucrose layer were pooled, diluted ~5 times with H-medium, placed over a small amount of 1.2 M sucrose, and centrifuged at 5,000 *g* for 15 min. The washed membranes thus obtained were gently resuspended in H-medium and used as the Golgi fraction.

Total microsome fraction was prepared from the supernatant of the second centrifugation at 5,000 *g* for 10 min. After further centrifugation at 10,000 *g* for 20 min, the supernatant was centrifuged at 77,000 *g* for 80 min, and the pellets were used as total microsomes.

Biochemical Analyses: P-450 was determined by the method of Omura and Sato (12). NADPH-cytochrome *c* reductase was estimated as described by Omura and Takesue (13). G-6-Pase, galactosyltransferase, cytochrome oxidase, acid phosphatase, and cathepsin D activities were determined by the procedures of Leskes et al. (14), Fleischer et al. (15), Wharton and Tzagoloff (16), Pricer and Ashwell (17), and Yamamoto et al. (18), respectively.

These enzyme activities were assayed immediately after their isolation. Protein was determined by the method of Lowry et al. (19) using bovine serum albumin as the standard.

Purification of P-450(PB) and Preparation of the Antibodies: P-450(PB) was purified from liver microsomes of PB-treated rats according to the procedures of Elshourbagy and Guzelian (20) with some modifications as described previously (21). Antiserum to the cytochrome was elicited in rabbits, and the IgG fraction of the antisera was purified by repeated ammonium sulfate fractionation followed by DEAE column chromatography (21). The specific antibody was purified by affinity chromatography using Sepharose 4B conjugated with the purified cytochrome.

Characterization of the Specific Antibody by Immunoblotting: The immunological specificity of the antibody was tested by double immunodiffusion analysis as described previously (22) and by immunoblotting of P-450(PB) from microsomal and Golgi membranes according to Burnett (23). Briefly, proteins of the microsomal and Golgi fractions prepared as described above were separated by SDS PAGE and transferred electrophoretically to a nitrocellulose sheet. The sheet was incubated with the antibody specific for P-450(PB) (20 μg/ml) and then with ^{125}I-labeled protein A (1 μCi/ml) and visualized by autoradiography. For quantitative assay, the portion of nitrocellulose sheet corresponding to the radioactive band was cut out, and the radioactivity was measured with a Packard model 5780 autogamma spectrometer. As a control, purified P-450(PB) was analyzed simultaneously.

Preparation of Ferritin Antibody Conjugates and Immunoelectron Microscopy: Ferritin was purified from horse spleen according to the procedures of Granick (24) with a slight modification (25). Ferritin and the specific antibodies were coupled together by using glutaraldehyde as a coupling agent (26), and the ferritin antibody conjugates with the molar ratio of IgG to ferritin of ~1:1 were isolated by gel filtration on Bio-Gel A 1.5 M as described previously (27). The conjugates were concentrated to ~2.5 mg of ferritin and 0.5 mg IgG/ml. The control conjugates were prepared in the same way by coupling ferritin with IgG from nonimmunized rabbits.

The rat liver microsome and Golgi fractions were incubated for 30 min at 0–4°C with either antibody conjugates or control conjugates. The incubation with antibody conjugates was done at the saturation level of the antibody as described previously (27), and the corresponding concentration of control conjugates were used for the control experiments.

The number of ferritin particles on the membranes perpendicular to the section were counted, and the length of the membranes was measured by a Mutoh digitizer model G-2 (Mutoh Industrial Inc., Tokyo, Japan), connected to a Sord microcomputer (model 223, Mark III, Sord Inc., Tokyo, Japan), and the average number of ferritin particles per micrometer of microsomal and Golgi membranes was calculated as described previously (27, 28).

RESULTS

Enzymatic Characterization of the Rat Liver Golgi Fraction

Table I shows the enzyme activities of the Golgi fraction prepared from PB-treated rats according to Hino et al. (11). Galactosyltransferase activity of this fraction was about 30 times more enriched than that of whole homogenate. As reported by Hino et al. (11), this fraction contains ER marker enzymes such as G-6-Pase, NADPH-cytochrome *c* reductase, and P-450 at a level of about one quarter of those found in the microsomal fraction.

Our problem is to determine whether P-450 in the Golgi fraction is simply due to contamination of ER membrane to the Golgi fraction or it is a bona fide Golgi enzyme.

Immunochemical Specificity of the Antibody Against P-450(PB) and Content of P-450(PB) in the Golgi Fraction

In Ouchterlony immunodiffusion analysis, a single precipitation line was formed between the specific antibody and the purified P-450(PB). As described by Masaki et al. (21), P-450(PB) presumably corresponds to cytochrome P-450b of Levin and co-workers (29, 30). No precipitation lines were formed between the major form of P-450 in liver microsomes from 3-methylcholanthrene-treated rats (P-450[MC]) (data not shown).

The monospecificity of the antibody against P-450(PB) was also tested by the Western blotting analysis of the rat liver microsome and Golgi fractions from PB-treated rats. As shown in Fig. 1*A*, SDS PAGE of both the microsome and the Golgi fractions clearly shows a distinct band at 53 kD as

TABLE I. *Various Enzyme Activities in Rat Liver Golgi and Microsomal Fractions Prepared from PB-treated Rats**

Fraction	G-6-Pase‡	NADPH-cyt. c reductase‡	P-450§	Gal-transferase‖	Cytochrome oxidase‡	Acid phospha-tase‡	Cathepsin D¶
Whole homogenate	24.8 ± 2.4	21.6 ± 2.5	—	6.2 ± 1.1 (1)	351 ± 43.8	16.2 ± 1.2	1.33 ± 0.08
Total microsome	116 ± 33.5 (100%)	160 ± 4.0 (100%)	1.74 ± 0.17 (100%)	10.8 ± 0.1 (1.7×)	22.0 ± 5.3	14.9 ± 3.1	1.01 ± 0.12
Golgi	26.3 ± 6.5 (23%)	47.4 ± 3.3 (29.6%)	0.46 ± 0.13 (28.2%)	187 ± 61 (30.2×)	32.1 ± 1.0	58.6 ± 3.1	7.6 ± 0.95

* Enzyme activities are shown as specific activities (mean ± SD, $n = 3$).
‡ nmol substrate catalyzed/min per mg protein.
§ nmol/mg protein.
‖ nmol galactose transferred/h per mg protein.
¶ μg tyrosine solubilized/min per mg protein.

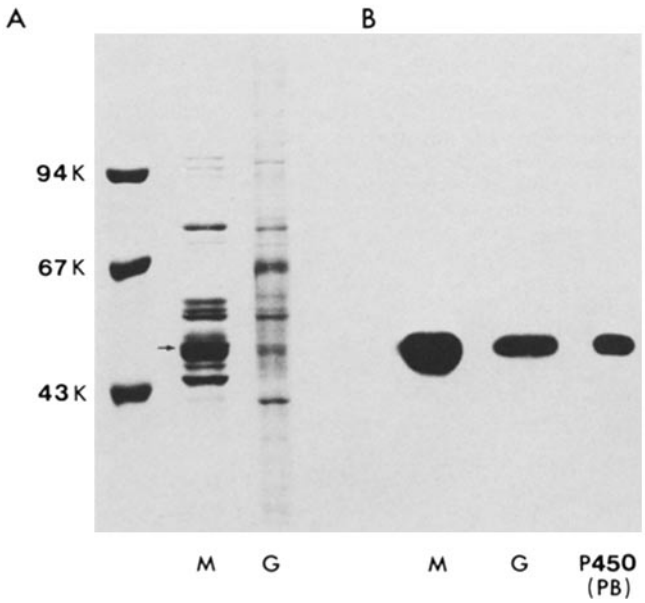


FIGURE 1 SDS PAGE and immunoblotting of the proteins from the microsomal (*M*) and the Golgi (*G*) fractions. The microsomal and the Golgi proteins (24 μg each) were separated on SDS PAGE and stained with Coomassie Brilliant Blue (*A*), or transferred electrophoretically to a nitrocellulose sheet and analyzed by autoradiography after labeling with ^{125}I-protein A (*B*). Purified P-450(*PB*) (~400 ng) was analyzed by immunoblotting simultaneously (*P-450[PB]*). An arrow indicates the position corresponding to 53 kD.

shown by a small arrow, which corresponds to P-450(PB) in molecular weight. As shown in Fig. 1 *B*, a single component ~53 kD was detected in the autoradiograph of the immunoblot, and no other membrane proteins were labeled. This result clearly indicates not only the monospecificity of the antibody but also the fact that no degradation products of P-450(PB) are detectable both in the microsome and the Golgi fractions.

The amount of P-450(PB) in each fraction was estimated by measuring the radioactivity as described in Materials and Methods. The P-450(PB) content of the Golgi fraction was 24% of that of the microsomal fraction. This ratio was equal to that of the total P-450 content of the two fractions.

Ferritin Immunoelectron Microscopy of Liver Microsome Fraction Prepared from PB-treated Rats

Fig. 2 shows an electron micrograph of rat liver microsome fraction incubated with ferritin antibody conjugates against P-450 (PB). Since the microsome fraction was prepared from PB-treated rats, the surfaces of most of the microsomal vesicles are completely covered by ferritin particles except for the regions studded with ribosomes.

It is to be noted that there are a few smooth vesicles that are hardly labeled at all as shown by star marks in Fig. 2. Such unlabeled vesicles are composed of ~1% of all the microsomal vesicles.

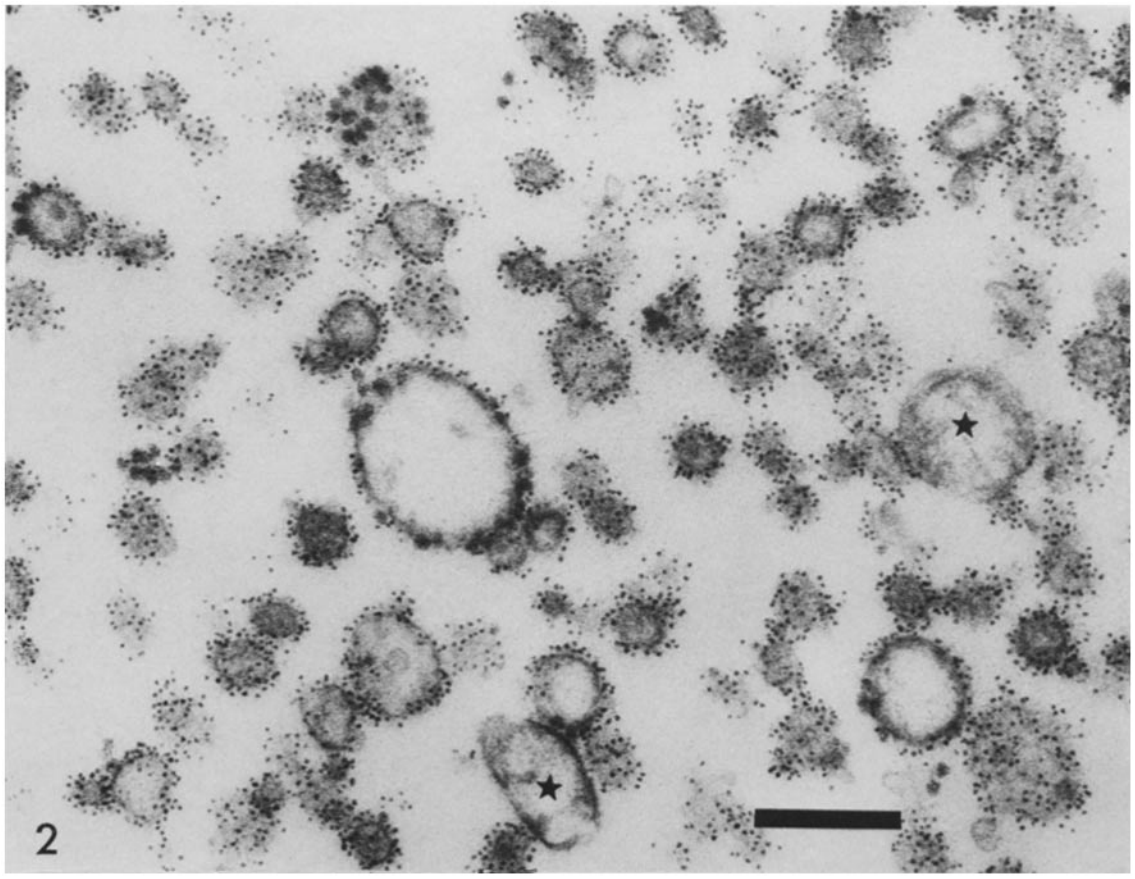
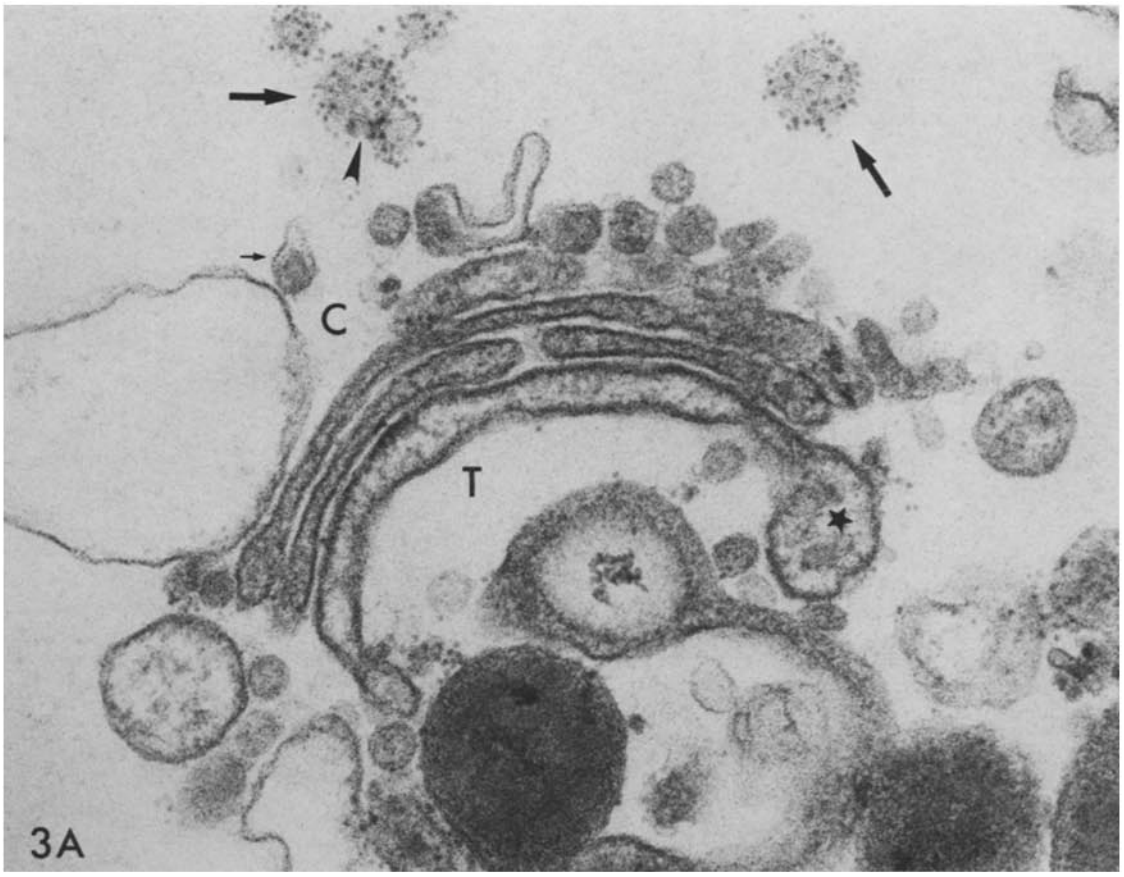


FIGURE 2 Electron micrograph of the total microsome fraction incubated with ferritin-antibody conjugates against P-450(PB). Total microsomes of liver were prepared from rats previously treated with PB. Note that the small vesicles presumably derived from ER membranes are wholly covered by ferritin particles, whereas some of the vesicles that are presumably derived from other than ER membranes (*stars*) are hardly labeled at all. Bar, 0.2 μm. × 96,000.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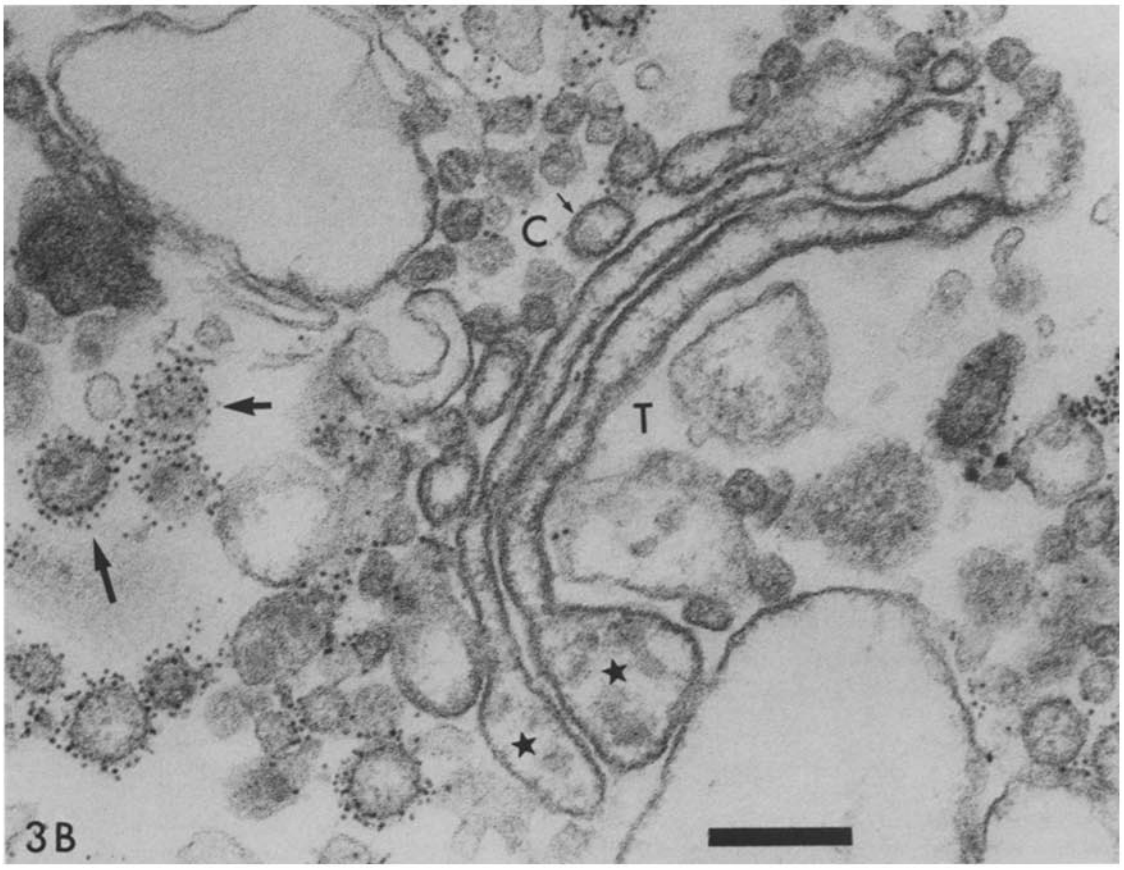
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Ferritin Immunoelectron Microscopy of Liver Golgi Fraction Prepared from PB-treated Rats

Fig. 3, *A* and *B* show electron micrographs of the Golgi fraction prepared according to the procedure of Hino et al. (11). In this procedure rat livers were crushed with a wooden spatula through a stainless steel mesh, and no strong shearing force was applied throughout the preparation procedure. Most of the Golgi apparatus thus prepared preserved well their original structural organization as shown in Fig. 3. They are composed of stacks of three or four cisternae, usually showing a convex *cis* or forming face (*C*) and a concave *trans* or maturing face (*T*). The outermost *cis*-Golgi cisterna is occasionally fenestrated and associated with small vesicles. They sometimes contain very low density lipoprotein (VLDL) particles and presumably correspond to the transport vesicles found between transitional ER and the Golgi apparatus. Occasionally secretory vesicles containing a number of VLDL particles are associated with the *trans* Golgi cisternae. As shown in Fig. 3, such complex structure of the Golgi apparatus is preserved even after incubation with ferritin antibody conjugates and repeated washing procedures, if carefully treated. Since these profiles of the isolated rat liver Golgi apparatus were quite similar to those in vivo as described previously (9), it is not likely that one or more of the Golgi cisternae, especially the outermost one, were lost during preparation of the Golgi apparatus.

This fraction also contains Golgi elements dissociated in various degrees from the Golgi apparatus such as single Golgi lamellae, transport vesicles, and secretory vesicles. In addition, it also contains a small number of lysosomes, rough and smooth microsomal vesicles, and mitochondria.

It is quite evident from Fig. 3 that not only the Golgi cisternal membrane of the *cis* side but also that of the *trans* side are hardly labeled with ferritin particles. Furthermore, neither small vesicles on the *cis* side, which occasionally contain a VLDL particle (small arrows) and therefore presumably correspond to transport vesicles, nor the secretory vesicles on the *trans* side were labeled (not shown). Dissociated Golgi cisternae, usually buckled and sometimes containing VLDL particles, are also hardly labeled and at control level.

In marked contrast to these Golgi apparati, this fraction contains a few small vesicles heavily labeled with ferritin particles (large arrows), occasionally simultaneously studded with ribosomes (Fig. 3*A*, arrowhead).

We counted ferritin particles on these various membranes and calculated the average particle density of the membranes (Table II). In this table, the membranes in the Golgi fraction are classified into the *cis*-most and *trans*-most membranes of the undissociated Golgi apparatus and those of the other vesicles, and the particle densities on these various membranes were shown, together with that of the total microsomal fraction. Golgi cisternae other than the *cis*-most and the *trans*-most ones were excluded from the calculation, because the ferritin antibody conjugates could not penetrate into the intercisternal spaces of the Golgi apparatus. The average particle density of all the membrane structures in the Golgi fraction was ~25% of that of the total microsome fraction, and the particle density of both the *cis*-most and the *trans*-most Golgi cisternae were at the control level; hardly any ferritin particles were found there when the fraction was incubated either with ferritin–antibody conjugates or control conjugates.

TABLE II. *Density of Ferritin Particles on Membranes in Golgi and Micosomal Fractions**

Fraction	Conjugates: Anti-P-450	Conjugates: Non-immune IgG	Specific binding‡	Ratio in specific binding of microsome fraction
				%
Total microsome fraction	76.7	1.1	75.6	100
Total Golgi fraction	19.4	1.1	18.3	24.2
Golgi cisternae: *cis*-most	1.0	0.9	0.1	0.1
Golgi cisternae: *trans*-most	0.8	0.7	0.1	0.1
Other vesicles in the Golgi fraction	19.9	1.2	18.7	24.7

* No. of ferritin particles bound per micrometer of membrane. In this Table two hundred vesicles each in the Golgi and microsome fractions were analyzed.

‡ Specific binding was calculated by subtracting the particle density of the control (incubated with nonimmune IgG) from the experimental specimen (incubated with anti-P-450 conjugates).

The distribution of ferritin particles on the small vesicles other than those composing the Golgi apparatus was more precisely analyzed by comparing the distribution of the number of profiles in relation to the ferritin load on the vesicles of the Golgi fraction with that of the microsome fractions (Fig. 4). In the Golgi fraction, there are evidently two groups of vesicles; one of which is heavily loaded with ferritin (group *B* in Fig. 4) and another is hardly loaded (group *A* in Fig. 4).

Table III indicates that the surface area of the vesicles heavily loaded with ferritin was composed of ~22% of that of the total membrane profiles in the Golgi fraction. The particle density of the former (78.0) was exactly similar to that of the total microsomes (75.6). These results strongly suggest that the vesicles heavily studded with ferritin particles are exclusively derived from the ER membranes, and P-450(PB) detectable in the Golgi fraction, therefore, can be explained by these contaminated ER membranes.

Biochemical and Immunoelectron Microscopical Analyses of P-450 after Cessation of PB Treatment

After cessation of PB treatment, the P-450 content of both the microsome and Golgi fraction decreases rapidly to the control level in 1 wk as shown in Fig. 5. We investigated whether or not P-450 is transported to the Golgi apparatus in this regression phase.

FIGURE 3 Rat liver Golgi fractions incubated with ferritin antibody conjugates against P-450(PB). Undissociated Golgi apparatus were prepared from PB-treated rats by Hino's procedures (11). *C* and *T* show *cis* and *trans* sides of the Golgi apparatus. Large arrows indicate microsomal vesicles heavily loaded with ferritin particles, and small arrows indicate small transport vesicles containing a VLDL particle. Peripheral dilated rims of the Golgi saccules containing VLDL particles are indicated by asterisks. Arrowhead indicates a ferritin-labeled vesicle with attached ribosomes. Bar, 0.2 μm. × 96,000.

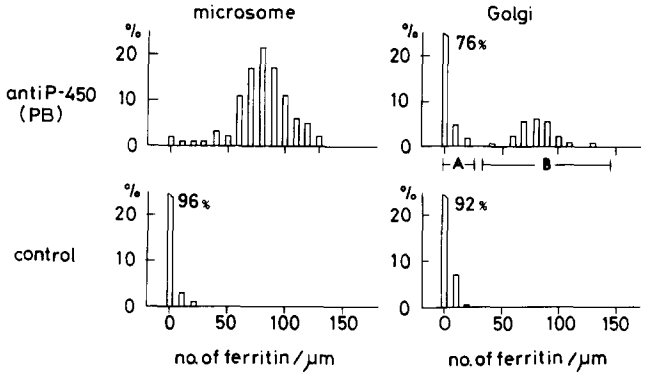

FIGURE 4 Distribution of number of profiles in relation to the ferritin load on the microsomes and on the vesicles in the Golgi fraction from PB-treated rat livers. Golgi cisternae were excluded. The vesicles that were loaded with <25 ferritin particles/μm membrane were named as group *A*, and the others were named as group *B*.

TABLE III. *Density of Ferritin Particles on Group A and B Vesicles in Golgi Fraction**

Group	Surface area‡	Conjugates: Anti-P-450(PB)	Conjugates: Control	Specific binding
	% of total membrane profiles			
A	62.8	1.0	1.2	0
B	22.2	79.2		78.0

* No. of ferritin particles bound per micrometer of membranes. In this Table the total surface area of the vesicles analyzed was 260 μm, and about 500 vesicles were analyzed.

‡ Approximately 15% of the total membrane profiles in the Golgi fraction was Golgi cisternal membranes. No ferritin particles bind to these cisternal membranes (Table II).

As shown in Fig. 6 and Table IV, the cisternal membranes of the Golgi apparatus were not stained with ferritin throughout this regression phase.

DISCUSSION

It has been well established (31) that secretory proteins synthesized on the membrane-bound ribosomes are co-translationally segregated into the intracisternal space of ER and transported to the transitional portion of the ER (transitional ER), where secretory proteins are packaged in transport vesicles. These vesicles presumably fuse with the outermost cisternae of the Golgi apparatus, and thus the secretory proteins are transported in the intracisternal space of the Golgi apparatus. It is further suggested that the membrane vesicles that serve to ferry secretory protein pinch off from the Golgi cisternae and recycle back to the transitional ER, thus serving as shuttles between the two compartments (31–33).

Biochemical analyses of the ER and Golgi membranes indicated that the lipid (34, 35) or the protein (3, 36) of the two membranes in the pancreas and in the liver are different. It was suggested that there is no mixing among either the lipid or the protein components of the ER and Golgi membranes. Existence of marker enzymes specific for the two membranes also imposes severe restrictions on the exchange of molecular components between the ER and the Golgi membranes at the time the two membranes establish continuity (31, 37).

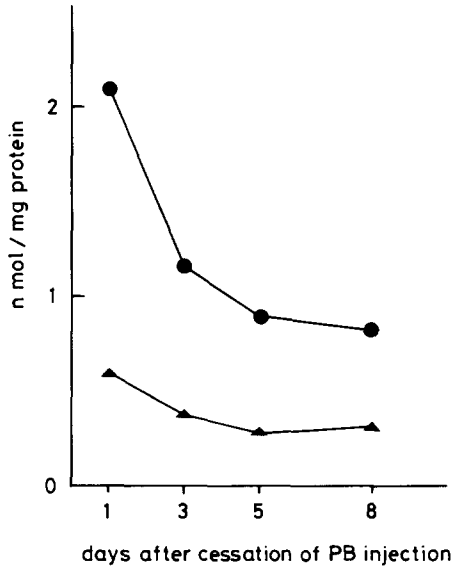

FIGURE 5 Changes in the total content of P-450 in rat liver microsome (●) and Golgi (▲) fraction after cessation of PB treatment. The means of three experiments are plotted. The P-450 content at 8 d after cessation of PB treatment is at the level of control rats (not treated with PB). The maximum deviation was <20% of the average values.

If ER marker enzymes such as P-450, NADPH-cytochrome *c* reductase, and G-6-Pase, however, do exist in significant amounts in the Golgi membranes as described in the Introduction, the restriction imposed on the interaction between the ER and the Golgi membrane may be not so stringent. In fact, it is suggested by Rothman (7) that these ER marker proteins are transported together with secretory proteins but are removed from the rims of *cis*-Golgi cisternae by budding vesicles that then fuse with ER. The Golgi apparatus, especially the *cis* compartment, thus functions as a multistage fractional distillator by removing escaped ER proteins.

To investigate precisely whether or not the ER membrane proteins are transported to the Golgi apparatus, P-450(PB) appears to be the best ER marker enzyme, because it is certainly the most abundant membrane protein of the ER when maximally induced by PB treatment; the amount of P-450(PB) of rat liver smooth microsomes arrives at the level of ~1.0 nmol/mg microsomal protein (21). Since the membrane proteins represent 85–90% of the total microsomal proteins (38), it is estimated that ~6% of all the ER membrane proteins is P-450(PB). This membrane protein presumably exists mainly on the cytoplasmic domain of the ER membranes (39, 40), and it is suggested that ~10% of all the cytoplasmic surface of the ER membrane is occupied by P-450(PB) under such conditions.

After cessation of the PB treatment, both the content of total P-450 and of P-450(PB) in the microsome fraction decreases rapidly to the control level within several days (21, 41). If P-450(PB) is transported to the Golgi apparatus accompanied by secretory proteins or transported further to the lysosomes to be degraded, P-450(PB) should be detectable at least on the *cis* side of the Golgi membranes.

A biochemical approach to this problem by assaying P-450 in the Golgi fraction is not favorable, because it is not possible to prepare the Golgi membranes without contamination of the ER membranes (microsomes). We therefore applied quan-

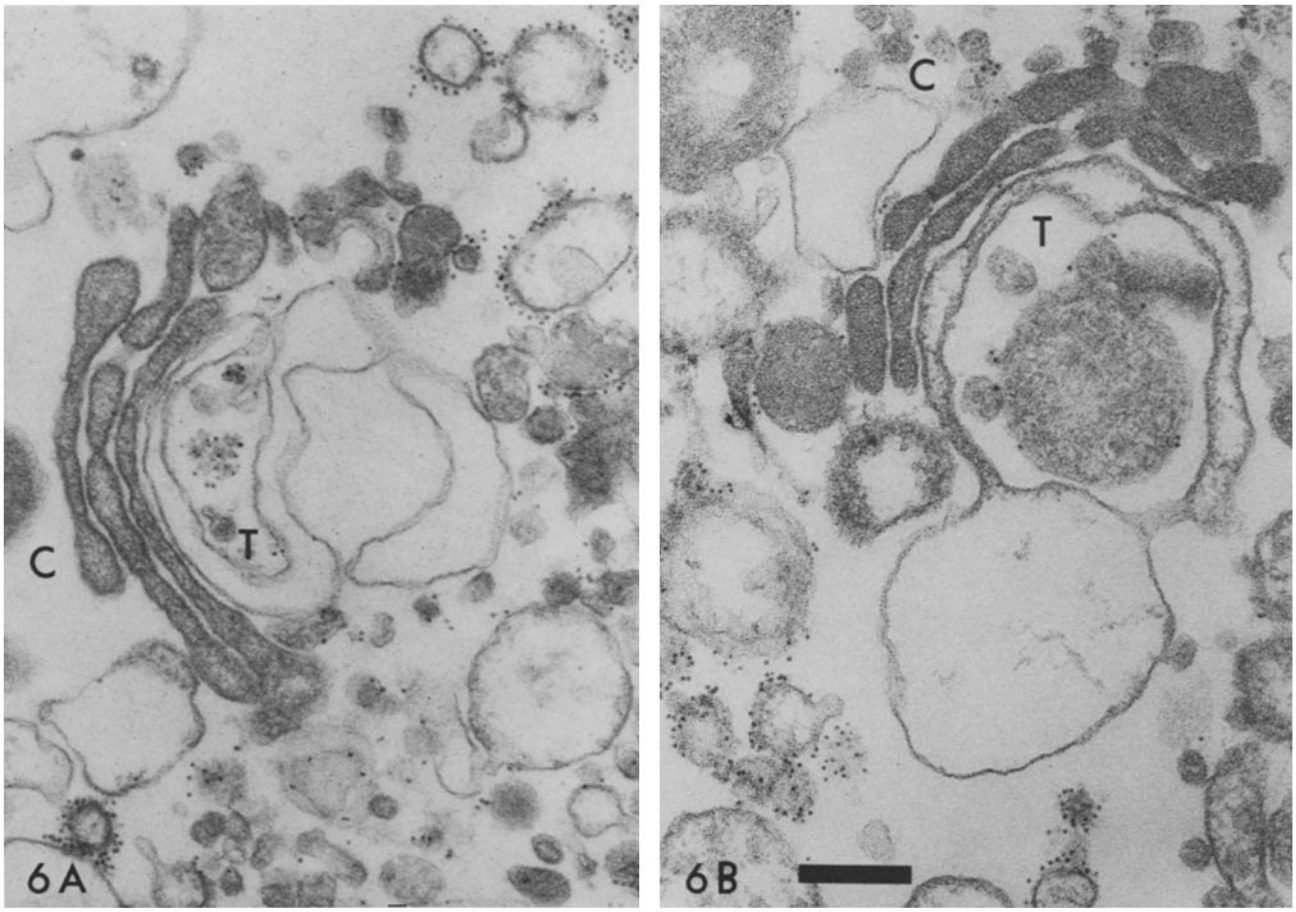

FIGURE 6 Rat liver Golgi fraction prepared at day 5 after cessation of PB treatment and incubated with ferritin antibody conjugates against P-450(PB). *C* and *T* indicates *cis* and *trans* sides of the Golgi apparatus, respectively. Bar, 0.2 μm. × 75,000.

TABLE IV. *Binding of Ferritin Anti-P-450 Antibody Conjugates to Microsomes and Golgi Cisternae after Cessation of Treatment with PB**

Time after PB treatment	Microsomes	Golgi cisternae	
		cis	*trans*
d			
1	75.6	0.1	0.1
3	70.2	0.6	0
5	66.8	0.3	0
8	37.8‡	0	0

* No. of ferritin particles bound per micrometer of membrane. The total number of microsomal vesicles and Golgi apparatus analyzed at each time point were ~100.

‡ This value was equal to the particle density of the liver microsomes from control rats (not treated with PB).

titative ferritin immunoelectron microscopic techniques which have been devised in our laboratory (8) and successfully applied for quantitative estimation of the distribution of several plasma membrane proteins such as asialoglycoprotein receptor, 5′-nucleotidase, and (Na^+, K^+)ATPase in hepatocytes (28, 42, 43).

We have shown that no significant amount of P-450 was detectable on the Golgi cisternal membranes either when P-450 was maximally induced by PB treatment or during the rapid decrease in the amount of P-450 after cessation of the PB treatment. The Golgi fraction, however, did contain small vesicles heavily labeled with ferritin particles. The particle density of these vesicles, however, was exactly similar to that of the microsomal vesicles in the microsome fraction (Fig. 4), and we suggested that these heavily labeled vesicles were derived from ER membrane, because the ratio in the length of heavily labeled vesicles to the total membrane of the Golgi fraction was ~22% (Table III), which was equal to the ratio in the content of P-450(PB) in the Golgi fraction to that of the microsomal fraction as shown by immunoblotting analysis. It is very probable, therefore, that P-450(PB) detected in the Golgi fraction can be exclusively attributed to the contamination of the fragmented ER membranes to the Golgi fraction.

Table II indicates that the particle density of the microsomes (~80/μm) was much higher than that of the Golgi cisternae, which was equal to the control level. This particle density on the microsomes is presumably underestimated; by PB treatment specific contents of P-450(PB) in liver microsomes increased 10–20 times (21, 44, 45), whereas the increase in the particle density was only ~2 times. After cessation of PB treatment, the P-450(PB) content decreased rapidly and markedly (21), whereas the number of ferritin particles on the microsomal membranes decreased more slowly to the level of only one half of the maximal level (Table IV).

At least two factors should be considered. One is the decrease in the apparent particle density due to the overlapping of ferritin particles on the cross-sectional profiles. Since experimental estimation of this effect on the microsomal vesicles is quite difficult, we simply estimated the overlapping effect

by computer simulation, assuming that the diameter of the ferritin core is 5 nm and the thickness of the ultrathin section is 60 nm. The latter value was estimated by the fold method proposed by Small (46). According to our calculation (Yamamoto, A., E. Otsuki, and Y. Tashiro, manuscript in preparation), the correction factor was 1.4 at the particle density of 80 particles/μm.

Another factor is the decrease in the apparent particle density due to the steric hindrance by the size of the ferritin antibody conjugates. When maximally induced by PB, ~10% of the cytoplasmic face proteins of the ER membrane is presumably composed of P-450(PB) as discussed above. The average intermolecular distance of P-450 on these membranes will be much less than the diameter of the ferritin particles (~10 nm). Since P-450 molecules presumably exist in large clusters as reported previously from this laboratory (8, 27), the number of ferritin antibody conjugates that can bind to P-450 molecules will be much less than the expected number of P-450 molecules on the microsomal vesicles. The contribution of the latter factor appears to be much bigger than that of the former.

It is strongly suggested, therefore, that the difference in the surface density of P-450(PB) between the ER and Golgi membranes is much higher than 80-fold. We conclude, therefore, that when the transport vesicles are formed on the transitional elements of ER by a budding mechanism, ER proteins such as P-450 are efficiently sorted out from such regions and only the membrane proteins destined for either the Golgi apparatus, plasma membranes, or lysosomes are clustered and transported. Instead of the multistep distillation hypothesis, we suggest that the sorting of Golgi–lysosome–plasma membrane proteins from ER membrane proteins is carried out in a single step when the transport vesicles are formed by a budding mechanism on the transitional ER.

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