5' Nucleotidase Is Sorted to the Apical Domain of Hepatocytes via an Indirect Route

Michael J. Schell,* Michele Maurice,‡ Bruno Stieger,§ and Ann L. Hubbard*

*Department of Cell Biology and Anatomy, Johns Hopkins School of Medicine, Baltimore, Maryland 21205;
‡Laboratoire de Biologie Cellulaire, INSERM U327, 16, Rue Henri Huchard, 75018 Paris, France; and
§Department of Clinical Pharmacology, University Hospital, Zurich, Switzerland

Abstract. In hepatocytes, all newly synthesized plasma membrane (PM) proteins so far studied arrive first at the basolateral domain; apically destined proteins are subsequently endocytosed and sorted to the apical domain via transcytosis. A mechanism for the sorting of newly synthesized glycophosphatidylinositol (GPI)-linked proteins has been proposed whereby they associate in lipid microdomains in the *trans*-Golgi network and then arrive at the apical domain directly. Such a mechanism poses a potential exception to the hepatocyte rule. We have used pulse–chase techniques in conjunction with subcellular fractionation to compare the trafficking of 5' nucleotidase (5NT), an endogenous GPI-anchored protein of hepatocytes, with two transmembrane proteins. Using a one-step fractionation technique to separate a highly enriched fraction of Golgi-derived membranes from ER and PM, we find that both 5NT and the polymeric IgA receptor (pIgAR) traverse the ER and Golgi apparatus with high efficiency. Using a method that resolves PM vesicles derived from the apical and basolateral domains, we find that 5NT first appears at the basolateral domain as early as 30 min of chase. However the subsequent redistribution to the apical domain requires >3.5 h of chase to reach steady state. This rate of transcytosis is much slower than that observed for dipeptidylpeptidase IV, an apical protein anchored via a single transmembrane domain. We propose that the slow rate of transcytosis is related to the fact that GPI-linked proteins are excluded from clathrin-coated pits/vesicles, and instead must be endocytosed via a slower nonclathrin pathway.

P ROTEINS anchored in the outer leaflet of the plasma membrane (PM)[1] via a glycophosphatidylinositol (GPI) anchor (reviewed in Cross, 1990) are an interesting subclass of domain-specific molecules in polarized epithelial cells. These molecules exhibit a predominantly apical localization at steady state (Lisanti et al., 1988, 1990). In transfection studies where the normally transmembrane domain of a basolateral protein is replaced with a GPI anchor, the chimera is sorted to the apical domain (reviewed in Lisanti and Rodriguez-Boulan, 1990).

A large body of evidence has shown that the sorting of domain-specific PM proteins can be accomplished at two distinct sites in epithelial cells: the *trans*-Golgi network (TGN) or the PM. The degree to which each site is used depends on the cell type (reviewed in Mostov et al., 1992). At one extreme is the hepatocyte, which probably does not use a direct route from the TGN to the apical surface and instead sorts apical PM proteins via transcytosis from the basolateral domain (Bartles and Hubbard, 1988). At another ex-

treme is the well-studied MDCK cell line, which sorts almost all PM proteins via the direct route, at or just after exit from the TGN. A bidirectional transcytotic pathway is used to a lesser extent in these cells (Brandli et al., 1990; Wollner et al., 1992). The phenotype of the Caco2 intestinal cell line is intermediate, where the route used and the efficiency of transcytosis is protein specific (Matter et al., 1990).

The sorting of apically enriched lipids may be relevant to the sorting of GPI-anchored proteins (Lisanti and Rodriguez-Boulan, 1990). These pathways have been studied in MDCK (van Meer et al., 1987) and Caco2 (van't Hof and van Meer, 1990). In both cell types, various sphingolipids synthesized from a common fluorescent precursor (C6-NBD-ceramide) rapidly assume their characteristic steady-state distributions, suggesting that the chief site for sphingolipid sorting is the TGN (the direct route); the transcytosis of newly synthesized sphingolipids occurs only slowly, over a period of many hours. The lipids most highly enriched on the apical domain are the glycosphingolipids, though it should be noted that approximately one third of PM glycosphingolipids in polarized intestinal cells reside at the basolateral domain at steady state (Simons and van Meer, 1988). The site(s) of lipid sorting in hepatocytes is unknown.

A model for the sorting of apical proteins has been sug-

---

1. *Abbreviations used in this paper*: DPPIV, dipeptidylpeptidase IV; Endo H, endoglycosidase H; 5NT, 5' nucleotidase; GPI, glycophosphatidylinositol; pIgAR, polymeric IgA receptor; PM, plasma membrane; TGN, trans-Golgi network.

gested whereby they cluster in the Golgi complex with the newly synthesized glycosphingolipids destined for direct transport to the apical membrane (Simons and Van Meer, 1988). This model has been elaborated by Lisanti and Rodriguez-Boulan (1990), with particular emphasis being placed on GPI-anchored proteins. Direct evidence for such a route has been elusive. The most provocative indirect evidence is the recent isolation of sphingolipid-enriched membrane domains which also contain GPI-anchored proteins (Brown and Rose, 1992). At least one report has suggested that the direct route for sorting GPI-anchored proteins operates in hepatocytes (Ali and Evans, 1990).

In this report we have studied 5' nucleotidase (5NT), a GPI-anchored ectoenzyme with a predominantly bile canalicular localization in hepatocytes (Reviewed in Luzio et al., 1986, and Zimmerman, 1992). Using metabolic pulse–chase techniques in conjunction with subcellular fractionation and immunoprecipitation, we have investigated the posttranslational biosynthetic trafficking of this protein in vivo. We have followed the transit of 5NT in cohort with two transmembrane molecules, dipeptidylpeptidase IV (DPPIV) and the polymeric immunoglobulin receptor (pIgAR); the trafficking of both has been previously well studied in a variety of epithelia (Bartles et al., 1987; Casanova et al., 1991; Matter et al., 1990; Sztul et al., 1985a,b; Mostov and Deitcher, 1986). We find that both 5NT and pIgAR move with remarkable efficiency through intracellular hepatocyte compartments while en route to the PM. A majority of both populations of nascent molecules has traversed the Golgi complex by 30 min of chase; DPPIV appears to move more sluggishly, ∼15 min behind. Importantly, we find that 5NT uses an indirect (transcytotic) route to the apical domain. However, in contrast to its movement through the ER and Golgi apparatus, the transcytotic redistribution is slow compared with most other apical molecules we have studied, requiring >3.5 h to achieve a domain distribution similar to that observed at steady state. The necessity for a newly synthesized GPI-anchored protein to undergo transcytosis raises questions pertaining to the endocytic and transcytotic pathways used by GPI-anchored molecules.

Materials and Methods

Young adult male Sprague-Dawley rats (150–200 g) were obtained from Dominion Labs (Dublin, VA); [^{35}S]methionine (1,200 Ci/mM), ultrapure sucrose, and recombinant endo-β-N-acetylglucosaminidase (Endo H) were from ICN Radiochemicals (Irvine, CA); uridine diphospho-D-[6^{3}H]galactose was from Amersham Corp. (Arlington Heights, IL); L-methionine, antipain, benzamidine, leupeptin, PMSF, uridine-5'-diphosphogalactose, thymidine 5'-monophosphate p-nitrophenyl ester, glucose-6-phosphate, and bacillus cereus phospholipase C were from Sigma Chemical Co. (St. Louis, MO); Trasylol was from FBA Pharmaceuticals (West Haven, CT); n-octylglucoside was from Boehringer-Mannheim Biochemicals (Indianapolis, IN); the mAbs recognizing DPPIV (HA 301) and pIgAR (SC 455) have been described (Bartles et al., 1985b; Scott and Hubbard, 1992), as have the polyclonal antibodies (Bartles et al., 1985a; Hoppe et al., 1985). Antibodies to 5NT (5N4-2, E5, and H3) were the generous gift of Dr. Paul Luzio (University of Cambridge) (Siddle et al., 1981; Bailyes et al., 1984).

Labeling with 1-[^{35}S]Methionine

Rats were fasted for 20–24 h and then pulse labeled by intravenous injection of 5 mCi of [^{35}S]methionine as described (Bartles and Hubbard, 1990). 10 min later, a chase consisting of 50 mg of unlabeled L-methionine in PBS was administered intraperitoneally. The chase was supplemented every 30 min by additional injections of 10 mg methionine.

One-Step Golgi Flotation

After various times of chase, the rats were killed by decapitation and the livers excised and perfused with ice-cold 0.9% NaCl containing the following protease inhibitors: 1 μg/ml of antipain and leupeptin, 100 KIU/ml Trasylol, and 1 mM benzamidine. Immediately before homogenization of the liver in sucrose, PMSF was added to a final concentration of 1 mM. Inclusion of protease inhibitors in all fractionation buffers was necessary to prevent partial degradation of pIgAR (Sztul et al., 1983).

Livers were homogenized in 5.5 vols of STKM buffer (0.25 M sucrose, 50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl$_2$). An enriched Golgi fraction was obtained (Bergeron et al., 1982), as described (Futerman et al., 1990). Three or four tubes (Beckman SW 28) were required for each liver, and fractions from each were pooled. The pellet was resuspended at 10 ml homogenization buffer per tube. Fractions were designated a through e, as described in the legend for Table I.

Immunoprecipitation After Golgi Isolation

In preliminary experiments, we determined the portion of each fraction from the Golgi flotation that could be quantitatively immunoprecipitated, as assessed by immunoblots of the extracts after incubation with various mAb-coupled Sepharose beads. A ratio of extract to bead that provided >90% depletion was chosen for each molecule and fraction. Before solubilization, the portion of the homogenate not loaded on the gradient and all but small aliquots of fractions b and d from the Golgi flotation were diluted threefold in 0.9% NaCl, centrifuged at 130,000 g (model Ti 60; Beckman Instruments, Inc., Fullerton, CA) for 1 h, and the pellets resuspended in 0.25 M SKTM containing protease inhibitors to a final volume convenient for immunoprecipitation and detection. Homogenate was resuspended in 0.25 M SKTM at twice the original volume; fractions b and d were each resuspended in 10% of their original volumes. Membranes were extracted for 45 min after the addition of a 5× stock of detergent; the final concentration of the solubilization buffer was: 50 mM octyl-β-D-glucopyranoside, 0.5% (wt/vol) Triton X-100, 0.3 M NaCl, 25 mM NaPO$_4$, pH 7.4, containing 0.02% NaN$_3$ and protease inhibitors. The mixtures were centrifuged at 150,000 g for 60 min at 4°C. Aliquots of supernatants were added to 70 μl of the designated mAb-Sepharose bead slurry (1 mg of IgG per ml of slurry) and then brought to a final volume of 1 ml with 1× solubilization buffer; BSA was then added to each tube from a 30% stock to a final concentration of 5 mg/ml.

Mixtures were incubated on a rotating wheel at 4°C overnight (5NT and pIgAR) or for 4 h (DPPIV). Washing, elution, and sample preparation for electrophoresis on 7.5% gels were done as described (Bartles and Hubbard, 1990), except that octylglucoside was left out of the wash buffers. Gels were fixed, soaked in salicylate (Chamberlain, 1979), dried, and exposed to film for 3–6 wk. By using salicylate-enhanced gels instead of direct blotting and autoradiography, we achieved an approximately twofold increase in sensitivity at the expense of a tolerable decrease in resolution. Quantitation of radioactivity was done by laser densitometric scanning of the fluorograms (Biomed Instruments Inc., Fullerton, CA). The distribution of labeled molecule was calculated as percent of recovered, i.e., the amount of labeled molecule found in fractions b, d, and e was set to 100%.

Plasma Membrane Sheet Preparation and Domain Gradients

Hepatocyte plasma membrane sheets were prepared, vesiculated by sonication, and resolved on large scale continuous sucrose density gradients, density range 1.06–1.17 g/ml, as described (Bartles et al., 1987). The immunoprecipitation procedure was identical to that described in detail (Bartles and Hubbard, 1990), except that the concentration of octylglucoside was 50 mM during solubilization, the wash buffers did not contain octylglucoside, and two fewer washes were found to be sufficient to give exposures (4–8 wk) on the autoradiogram free of contamination by nonspecific proteins.

Enzymatic Deglycosylation with Endo H

Extracts of homogenate or plasma membranes were incubated with 140 μl of a monoclonal-Sepharose bead slurry as described above. After the beads were washed, bound proteins were eluted by the addition of 66 μl 1% SDS, 50 mM Tris, pH 6.8, and boiled for 3 min. Supernatants were separated from the Sepharose and then split into 33 μl portions. 17 μl of 0.15 M sodium citrate, pH 5.5, was added to each tube; half of the tubes then received 1 μl of a 100 mU/ml stock solution of Endo H in 50 mM Tris, pH 6.8. Sam-

ples were incubated overnight at 37°C and processed for electrophoresis by the addition of 12 μl of 5× gel sample buffer (0.22 M Tris-HCl, pH 8.9, 22.5 mM EDTA, 9% SDS, 1.8 M sucrose, 170 mM DTT).

Treatment with Phospholipase C

Plasma membranes (2 mg) were fractionated on the domain gradient as described above. Two apical fractions (5 and 6) and two basolateral fractions (10 and 11) were diluted with 0.9% NaCl and centrifuged for 1 h at 200,000 g. Pellets were resuspended in 20 mM Hepes, pH 7.4, and Phospholipase C was added to a concentration of 6 U/ml. Samples were incubated 1 h at 37°C and were then repelleted. The pellets and supernatants were run on 7.5% gels and analyzed by Western blotting using the 5NT polyclonal.

Miscellaneous Procedures

mAbs were purified on DEAE Sepharose and coupled to CNBr-activated Sepharose as described (Bartles et al., 1985); for 5NT, equal portions of the three mAbs provided by Dr. Luzio were mixed together before coupling. The assays of alkaline phosphodiesterase and of 5NT have been described (Hubbard et al., 1983). Glucose 6-phosphatase was measured as described (Aronson and Touster, 1974). Galactosyl transferase was measured as described (Verdon and Berger, 1983). Protein was measured using the Pierce BCA assay kit (Pierce Chemical Co., Rockford, IL).

Results

Appearance of Complex Glycosylated Forms

Fig. 1 A shows the kinetics of maturation for three PM molecules after 10 min of pulse-labeling with [^{35}S]methionine. At early times of chase, each molecule in the absence of endo H appeared as a sharp band that migrated to a size similar to that previously reported for the core glycosylated (high-mannose) forms in liver, i.e., 67 kD for 5NT (Wada et al., 1986; van den Bosch et al., 1986), 95 kD for DPPIV (Bartles et al., 1985a) and 105 kD for pIgAR (Sztul et al., 1983). Each core-glycosylated band was completely sensitive to endo H, which produced forms of 57, 80, and 95 kD, respectively; these correspond to the size of the remaining deglycosylated peptide plus a single GlcNac at each N-linked site. At later time points, increasing amounts of the mature-sized forms appeared. The maturation of proteins is shown graphically in Fig. 1 B, which depicts the percent of each protein that exists as a mature-sized form, as determined by densitometric scanning of fluorograms after immunoprecipitation of the metabolically labeled proteins from liver homogenates. Mature pIgAR exists as a 116/120 kD doublet (Sztul et al., 1985a) that was not well resolved on our fluorograms. The 2-h time point in Fig. 1 A depicts bands from a different experiment than that depicted in Fig. 1 B. Although pIgAR appears completely mature in Fig. 1 A, it did not reach 100% maturity in the pulse–chase experiment done for the Golgi fractionation depicted in Figs. 2 and 3. The difference might be due to variations in the efficiency of chase; alternately, a slightly degraded mature form may have appeared as immature in this experiment.

Despite attaining >90% mature size by 45 min chase, the 5NT band formed two slightly smaller bands upon digestion with endo H (Fig. 1 A). We believe that the mature 5NT band migrating to 72 kD on gels consists of two populations of molecules with differences in glycosylation. We estimate that 50–70% of the N-linked glycosylation sites remain in a partial high mannose form which migrates at ∼66 kD upon digestion with endo H. We could not determine if the remaining population is endo H insensitive, or merely less sensitive.
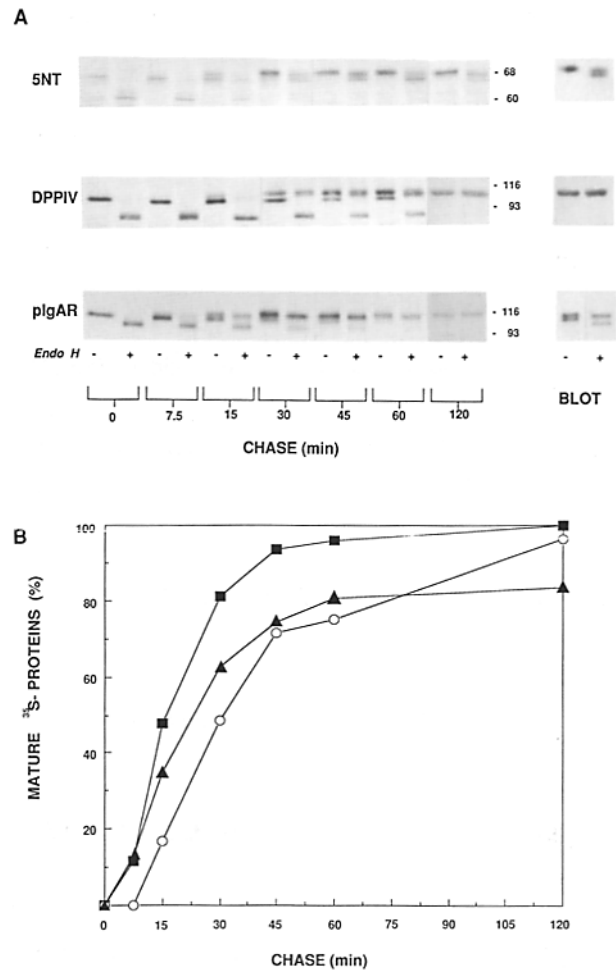

Figure 1. Kinetics of maturation of three PM proteins. Rats were pulse labeled for 10 min with L-[^{35}S]methionine and then chased with unlabeled L-methionine for the designated times. Proteins were immunoprecipitated from liver homogenate and treated with endoglycosidase or not. Dried gels were exposed to film for 3–6 wk and then quantitated by densitometry as described in Materials and Methods. Steady-state levels of endo H sensitivity were determined by immunoblot. (*A*) Autoradiograms of endo H sensitivity of molecules at various times of chase, and at steady state. Not all of the lanes show proteins from the same rats used in the Golgi flotation portion of the paper, and some experimental variation in the efficacy of chase was noted. (*B*) Graph of the percentage of the molecule migrating at its mature size. The data graphed are not that of *A*; rather they show data derived from homogenates of the same pulse-labeled rats used for Figs. 2 and 3. Each point represents the portion of the total density of the band that appeared mature sized, and is the mean of quadruplicate immunoprecipitations from a single rat. ■, 5NT; ○, DPPIV; and ▲, pIgAR.

sitive. The hybrid glycosylation persisted at the 2-h chase point and at steady state. Similar results have been found for 5NT in bovine liver (Harb et al., 1983) and in the H₄S hepatoma cell line (van den Bosch et al., 1986), although 5NT has been reported to be completely endo H resistant in primary cultures of hepatocytes (Baron and Luzio, 1987). We also find a hybrid glycosylation of 5NT in our enriched PM fraction; the endo H sensitivity appears similar in frac-
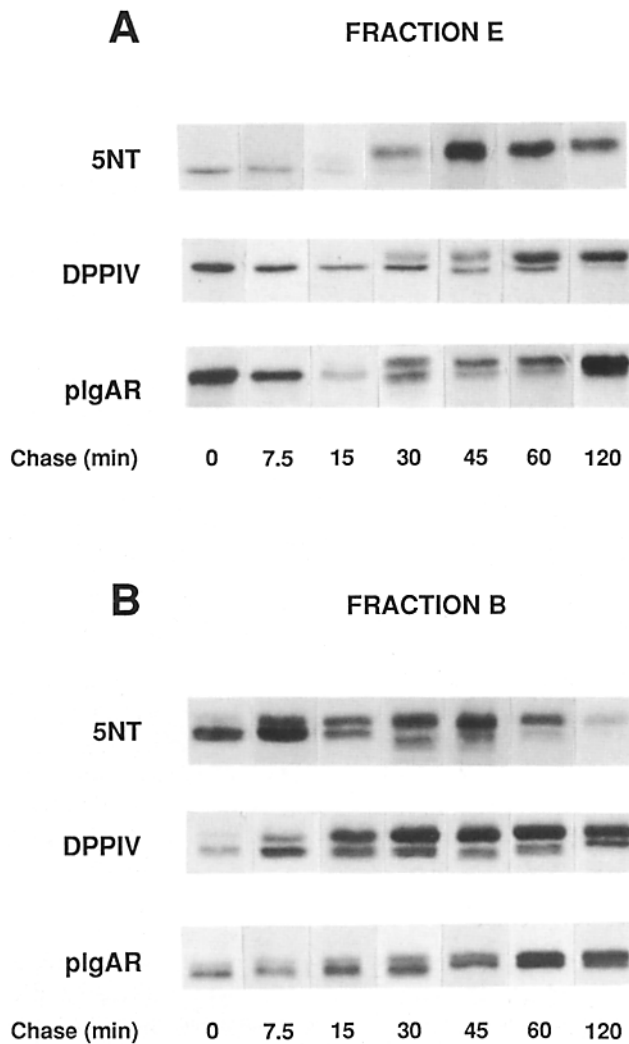
A   FRACTION E

	5NT						
DPPIV							
pIgAR							
Chase (min)	0	7.5	15	30	45	60	120

B   FRACTION B

	5NT						
DPPIV							
pIgAR							
Chase (min)	0	7.5	15	30	45	60	120

Figure 2. The time-dependent change of ^{35}S proteins in fractions *b* and *e* of the one-step Golgi flotation. After 10 min of pulse and the indicated times of chase, rat livers were fractionated according to Bergeron et al. (1982). Quantitative immunoprecipitation and fluorography were performed on homogenates and fractions *b*, *d*, and *e*. (*A*) Time course for three molecules immunoprecipitated from fraction *e* at various time points. Fraction *e* contains the bulk of markers for both ER and PM; the lower band is the core glycosylated form found at the ER, the upper band is the mature glycosylated form found at the PM. Fraction *d* is not shown; it usually had a percent mature similar to *e*; however the percent of labeled protein recovered in fraction *d* was high during the peak of labeling in *b*. (*B*) Time course for the appearance of three molecules in fraction *b*, a highly enriched fraction of Golgi membranes. For 5NT and DPPIV, the bands shown for *b* were immunoprecipitated from 18% of the total volume of that fraction, while the bands shown for *e* were immunoprecipitated from 1.1% of the total volume of that fraction. For pIgAR, which has a different steady-state distribution and a higher relative abundance, the bands shown for *b* were immunoprecipitated from 1.1% of the fraction, while those for *e* were immunoprecipitated from 0.2% of the fraction.

tions enriched in either domain (not shown). 5NT has five putative N-linked glycosylation sites (Misumi et al., 1990).

The percentages of mature pIgAR shown at late time points are influenced by its rapid release into the bile (Sztul et al., 1985*a*; Solari et al., 1989). In hepatocytes in vivo
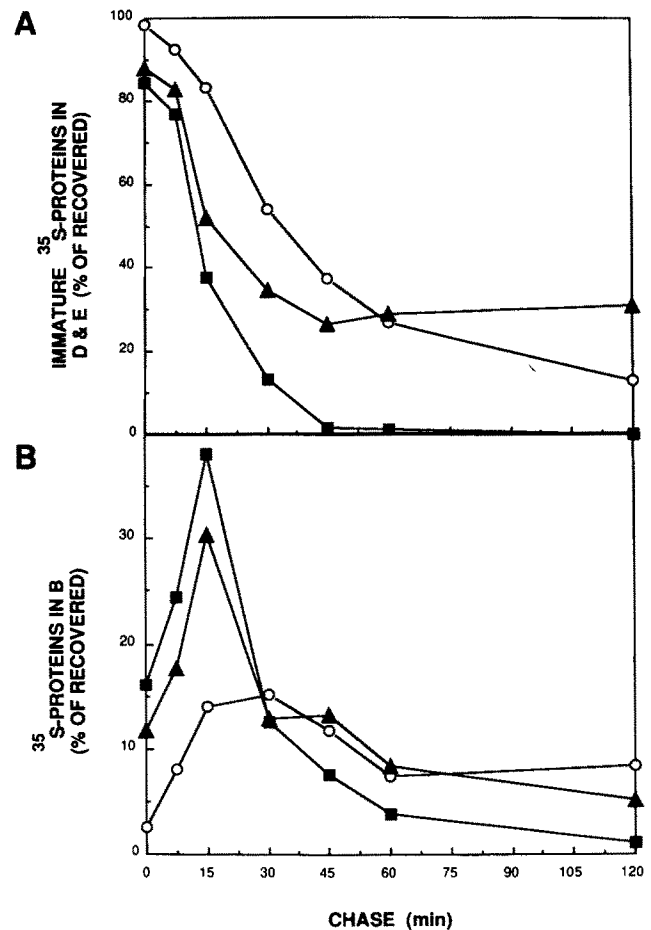

CHASE (min)

Figure 3. Kinetics of intercompartmental movement. (*A*) Exit from the ER. The lower bands in fractions *d* and *e* of the Golgi flotation were quantitated from the fluorograms and expressed as a percent of the total autoradiographic density recovered for each molecule in fractions *b*, *d*, and *e*. Based on the considerable galactosyl transferase activity appearing in these fractions (Table I, 28% for *d* + *e*), a portion of the lower band appearing in these fractions is probably derived not from the ER but from early parts of the Golgi complex. The symbols for the molecules are the same as in Fig. 1 *B*. (*B*) Transit of the labeled cohort through the Golgi apparatus. The upper and lower bands together in fraction *b* were quantitated from the fluorograms and expressed as the percentage of total density for each molecule recovered in fractions *b*, *d*, and *e*. For each point, duplicate immunoprecipitations for each molecule were done from the same pulse-labeled liver; for three of the time points (0, 30, and 45 min), results similar to those shown were obtained when the procedure was repeated on another rat. The average recoveries from homogenates (±SD) of labeled molecules in fractions *b*, *d*, and *e* were 93 ± 16% for 5NT, 100 ± 32% for DPPIV, and 91 ± 33% for pIgAR.

pIgAR has an apparent half-life of 1.1 h (Scott and Hubbard, 1992). The complex glycosylated form of this molecule is continually being shed as secretory component from the apical surface beginning at ~45 min chase. Fig. 1 *B* shows that, by 45 min, the percentage of labeled pIgAR migrating at the mature size (~75%) has already exceeded the steady state level (~60%). Even at 2 h chase, however, the intracellular cohort contains a detectable high mannose subpopulation.

Table I. Percentage Distribution of Recovered Proteins in the One-Step Golgi Flotation

Marker*	a	b	c	d	e
Protein	0.2 ± 0.1	0.9 ± 0.3	5.7 ± 1.5	34.6 ± 1.5	58.1 ± 3.5
Glucose-6-phosphatase	0.1 ± 0.1	0.7 ± 0.3	0.9 ± 0.9	11.0 ± 3.0	87.1 ± 3.8
Galactosyl transferase	6.2 ± 4.0	54.1 ± 8.1	11.5 ± 6.5	14.6 ± 2.6	13.5 ± 0.5
Alkaline phospho-diesterase I	0.2 ± 0.1	1.8 ± 0.6	1.4 ± 0.2	3.9 ± 0.9	92.9 ± 0.3
5NT	0.3 ± 0.2	1.3 ± 0.2	2.0 ± 0.8	9.0 ± 2.0	87.6 ± 3.1
5 NT blot	0.5	6.4	2.3	9.4	81.3
DPPIV blot	ND	2.3	2.3	7.3	88.0
pIgAR blot	ND	6.1	4.4	22.9	66.5
CE9 blot	ND	0.8	1.5	6.0	91.8

Fractions from the one-step fractionation were assayed for protein, enzyme activities, or by quantitative immunoblotting. Assays are expressed as the mean ± SD for the percent distribution of recovered activity for three separate fractionations. Immunoblotting was a single determination, quantitated by densitometry. Fractions were collected from the top of the gradient and designated as follows: a, top of the tube to top of white floated band; b, the white floated band enriched in Golgi membranes; c, between white band and load; d, load fraction; and e, pellet.
* Percentage of the homogenate protein or enzyme activities recovered in the fractions were: protein, 101 ± 7%; glucose-6-phosphatase, 120 ± 15%; galactosyl transferase, 107 ± 17%; alkaline phosphodiesterase I, 103 ± 6%; and 5NT, 106 ± 14%. The percentages of immunoblot density in homogenate recovered in fractions were: 5NT, 66%; DPPIV, 81%; pIgAR, 138%; and CE9, 92%. All enzyme assays and immunoblotting were performed on the same samples as used for the pulse-chase experiments presented in the figures, except that for the galactosyl transferase. For this assay, the metabolic label obscured the radioactive signal for ³H galactose incorporation; assays were performed instead on similarly fractionated unlabeled livers.

One-Step Golgi Flotation

In a single flotation, this procedure isolates Golgi cisternae in good yield and of high purity. Despite the fact that most of the ER and PM fractionate together into the pellet, the high mannose form found at the ER and the complex glycosylated form present at the PM can be discerned easily by their different electrophoretic mobilities. Therefore, in conjunction with pulse-labeling techniques, this procedure is well suited for studying the kinetics with which newly synthesized membrane glycoproteins move through the organelles during synthesis: all three compartments can be discerned from a single fractionation step from the same homogenate, and nearly 100% of the ³⁵S-labeled molecules are followed.

The biochemical characterization of the one-step Golgi flotation is presented in Table I. The results agree generally with those published in the original method (Bergeron et al., 1982). Fraction b contains <1% of the total cellular protein, but ~50% of the total activity of galactosyl transferase, a trans-Golgi marker. Another study (Futerman et al., 1990) has shown that this fraction is also enriched in the cis-Golgi marker mannosidase II, and our most recent experiments have shown this fraction to be enriched in the trans-Golgi marker sialyl transferase and the TGN marker TGN 38 (our own unpublished observations). Contamination of fraction b by markers of ER (glucose-6-phosphatase) is ~1%, and contamination by PM markers (alkaline phosphodiesterase I and 5NT) is <2%. Markers for these organelles are found predominantly in the pellet, fraction e.

Enzyme activities proved to be more accurate than quantitative immunoblotting for characterizing the Golgi flotation, as immunoblotting gave poor recoveries when expressed as a percent of immunoreactive material in homogenate. However, results from one such experiment are included in Table I to show how pIgAR distributes in this gradient. More of this protein remains in the load fraction d compared with PM resident molecules, and considerably more is present in fraction b as well. The distribution of pIgAR in lower density membranes is consistent with its known intracellular distri-

bution at steady state. Most is either in transit through the Golgi complex or resides in transcytotic and endocytic vesicles, all of which are lighter than ER and PM (Perez et al., 1988; Quintart et al., 1989). The time of cellular transit after ligand binding is known to be considerable, since radiolabeled pIgAR injected into the blood does not reach a peak in the bile until 40 min later (Hoppe et al., 1985).

Intercompartmental Transport

After fractionation of the pulse-labeled livers, fractions b, d, and e (together containing >90% of the markers for ER, Golgi complex, and PM) were probed for their content of metabolically labeled 5NT, DPPIV, and pIgAR by quantitative immunoprecipitation. Fig. 2 shows the radiolabeled bands in fractions e and b at various times of chase. To facilitate quantitation, we immunoprecipitated amounts of the fractions that would yield intensities of similar magnitudes on a single fluorogram. Thus, the band intensities of b compared with e (Fig. 2, A and B) do not indicate absolute organellar amounts; the portion of the fraction immunoprecipitated to produce a good signal was greater for b than e (see legend). The time course of disappearance of the lower band in Fig. 2 A indicates the trend of movement out of the ER. At 15 min, bands appear relatively lighter, probably because this is the peak of appearance in fraction b (Fig. 2 B) and d (not shown); variations in metabolic label injection and incorporation may also have contributed. The appearance of a mature-sized band in fraction e indicates molecules that have moved past the TGN, and presumably reside at the PM. Indeed, no mature band is seen in fraction e until the 30-min chase point, a time when the specific radioactivity of newly synthesized molecules in a highly enriched PM fraction has reached about half-maximum and is rising rapidly (Bartles et al., 1987).

Fig. 2 B shows the state of maturation of newly synthesized molecules appearing in fraction b, the Golgi-enriched fraction, at each time point. Each labeled population contains two or more glycosylated forms, with a trend toward a greater proportion of mature-sized forms at later time points.

At some time points, the Golgi fraction contains glycosylated forms in addition to the major high mannose and complex glycosylated bands. These are most likely short-lived intermediates in the glycosylation pathway with partially trimmed mannose residues. Such intermediates have been documented for pIgAR (Sztul et al., 1985b). The reduced resolution of the salicylate-enhanced fluorography prevented a precise determination of these minor forms.

The movement through organelles is more striking when the portion of the molecule in a fraction is expressed as a percent of the total labeled cohort recovered for each time point. Fig. 3 A shows the percent of recovered molecule present as the lower band (the immature form) in fractions d and e. Together these fractions contain 98% of the ER marker glucose-6-phosphatase. A majority of the lower band appearing in these fractions is derived from the ER. The half time for exit from the ER was <15 min for 5NT and pIgAR but ~30 min for DPPIV. Thus, DPPIV appears to be retained longer in the ER. The relative lightness of the immature forms of 5NT at early time points raises the possibility that the mAbs recognize preferentially the mature form of 5NT; if so this would increase slightly the apparent exit of 5NT from the ER shown in Fig. 3 A, but would not affect any gross trends. The second peak observed for pIgAR in fraction b at later time points is probably due to endosomal contamination in the Golgi fraction and is consistent with the possibility that some of this molecule has reached endosomes by 45 min of chase. The apparent incomplete exit from the ER by pIgAR (Fig. 3 A, 2-h time point) may simply over represent the subpopulation of newly synthesized molecules that have not yet left the cell after approximately two half lives (see above).

Fig. 3 B depicts movement through the Golgi complex as a percent of each labeled molecule (upper and lower bands together) appearing in fraction b at each time of chase. The movement through b is especially swift for 5NT. >15% of the newly synthesized 5NT resides in fraction b at the earliest time point examined (0 min chase, 10 min after the pulse); this value rises to almost 40% by 15 min chase, the peak of the time course. By 2 h the percent of the cohort in fraction b has dropped to 1.2%, the same as the steady-state value for 5NT enzymatic activity shown in Table I. Since the recovery of galactosyl transferase activity in fraction b is ~50%, as much as 80% of the newly synthesized 5NT may reside in the Golgi at 15 min of chase. The newly synthesized bolus of pIgAR in fraction b shows a peak value close to that observed for 5NT; by 2 h, 5.2% remains in b.

DPPIV exhibits more sluggish movement. At the earliest time point examined, only 2.5% appears in the Golgi fraction, indicating a delayed exit from the ER, compared with the other two proteins. The broader peak of DPPIV observed in fraction b would be expected for a protein exiting the ER more slowly. Notably, at 2 h chase ~10% of the newly-synthesized molecule still exists in its immature form in fractions d and e (Fig. 3 A), and an additional 10% remains in fraction b (Fig. 3 B). The steady-state value for DPPIV in fraction b, as assayed by immunoblotting, is ~2% (Table I). These data raise the possibility that small pools of newly synthesized immature DPPIV and pIgAR remain intracellular for prolonged periods.

Domain Localization and Transcytosis

We next examined the appearance of newly synthesized molecules in a highly enriched PM fraction, using techniques previously worked out in detail in our laboratory (Bartles et al., 1985b; Bartles and Hubbard, 1990). In this procedure we isolate an enriched fraction of PM sheets, vesiculate by gentle sonication, and then centrifuge to equilibrium on sucrose gradients. Basolaterally and apically derived vesicles migrate to characteristic distributions in these gradients (Fig. 4 C). We have shown previously that the relative specific activities of five newly synthesized molecules in PM sheets reach their maxima between 45 and 60 min chase (Bartles et al., 1987). However, at these early time points, the newly synthesized apical molecules distribute with basolateral markers. Only at time points >2 h do apical resident proteins distribute as they are seen at steady state.

5NT, DPPIV, and pIgAR were immunoprecipitated from the detergent extracts of domain gradient fractions. The results we obtained for the domain localization of DPPIV in this cohort were similar to those we have reported previously (Bartles et al., 1987), i.e., newly synthesized DPPIV first appears basolaterally and approaches steady-state distribution by 150 min of chase. 5NT also exhibited a clear basolateral distribution at the early time points. Fig. 4 A shows the fluorograms for 5NT separated on the domain gradient at the indicated times of chase; this is presented graphically in Fig. 4 B. By 3.5 h of chase, the longest time point examined, a majority of 5NT distributed with apical vesicles. Even at this time, however, the domain distribution is still skewed toward the basolateral, as compared with the steady-state distribution (Fig. 4 C).

The relative rates of transcytosis for 5NT and DPPIV are shown graphically in Fig. 5. The peak densities for apical and basolateral protein markers are fractions 6 and 10, respectively. By dividing the portion of newly synthesized proteins in these fractions by their corresponding steady-state values obtained from immunoblot analysis of the same fractions, we derive an arbitrary polarity ratio. Steady-state polarity has been attained when the upper line (fraction 10) and the bottom line (fraction 6) reach 1.0. The polarity ratio for DPPIV indicates that steady-state levels are reached between 2.5 and 3.5 h of chase (Fig. 5, o). In contrast, the apical polarity ratio for 5NT has reached only 0.66 at 3.5 h of chase (Fig. 5, ■). Thus the faster protein through the secretory pathway is the slower protein during transcytosis. These results show that 5NT, similar to all other apical proteins we have studied in hepatocytes, travels to the apical domain via an indirect route, and that the rate of transcytosis for 5NT is slower than that which is observed for a transmembrane protein.

To test the possibility that we were actually observing two pools of 5NT (one GPI-anchored and one transmembrane), we treated apical and basolateral peak fractions with GPI-specific phospholipase C from bacillus cereus. The percent of 5NT released by the treatment varied from 70–90% in these fractions and did not correlate with domain localization.

The PM domain gradient analysis was not useful for studying the transcytosis of pIgAR. At all time points examined, this molecule had essentially the same distribution on the
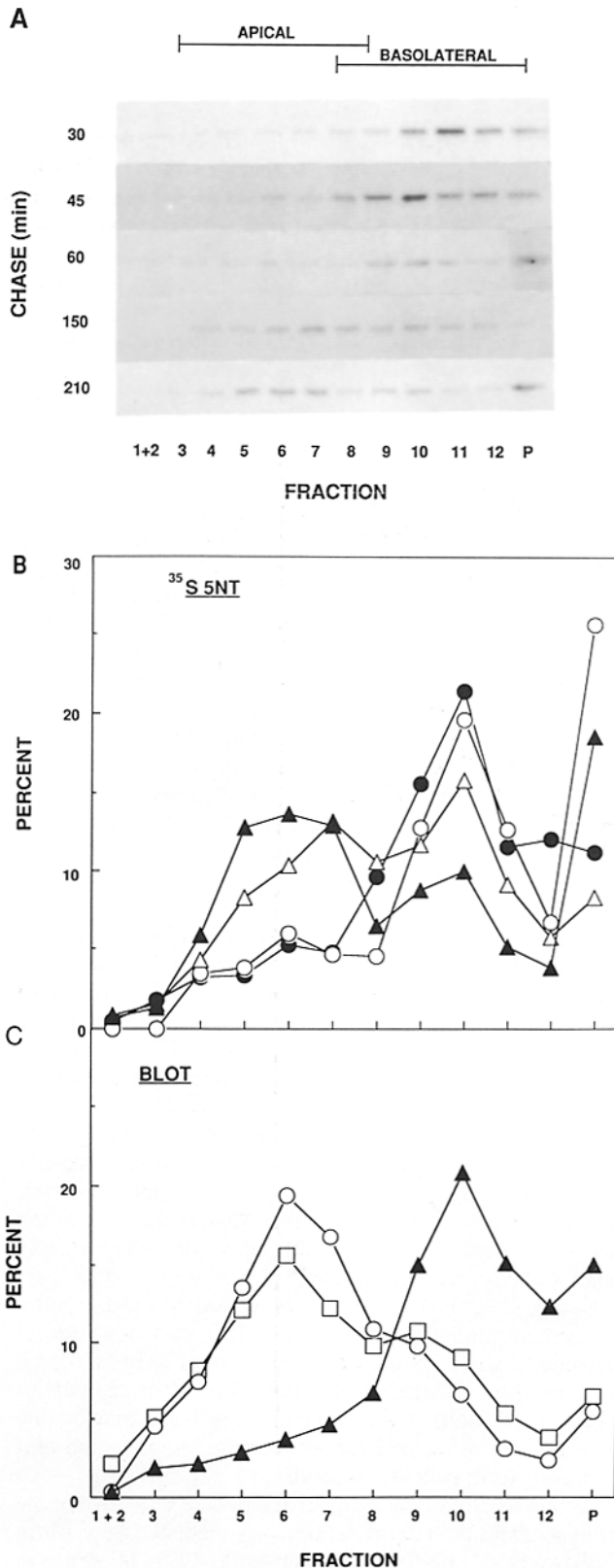
A

B

C

Figure 4. Domain localization of newly synthesized 5NT. Hepatocyte PM sheets were isolated from rats pulse labeled for 10 min with L-[³⁵S]methionine and then chased with cold methionine for the times designated. To resolve domains, PM sheets were vesiculated and the vesicles separated on sucrose density gradients. 5NT
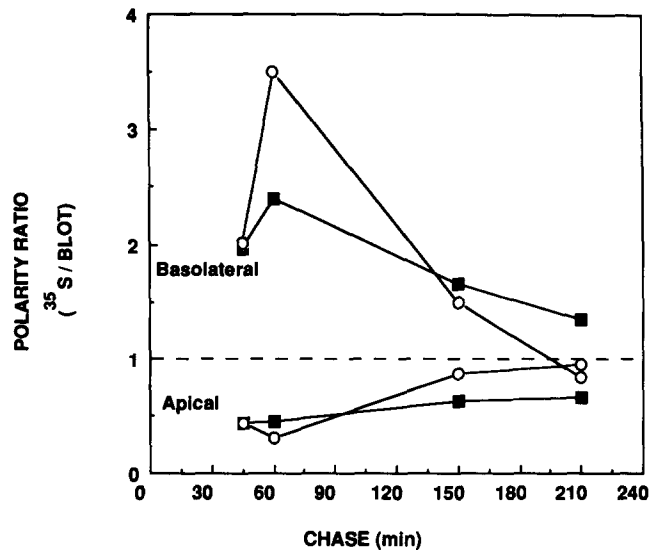

Figure 5. Polarity of newly synthesized molecules compared to steady state. The percent of the ³⁵S-labeled molecule in fraction 6 (the apical peak) or fraction 10 (the basolateral peak) at each time point depicted in Fig. 4 *B* was divided by the steady-state percentages in the same fractions as calculated by immunoblotting. A polarity ratio of 1.0 (dashed line) indicates that the newly synthesized molecule in this fraction has distributed the same as the chemical amounts. A declining polarity ratio over time in fraction 10 (basolateral peak) indicates disappearance of newly synthesized protein from this domain, while the rising polarity ratio in fraction 6 indicates the trend of transcytosis to the apical domain (o) DPPIV; (■) 5NT.

gradient. This distribution was intermediate to that seen for basolateral and apical markers, with the peak radioactivity always appearing in fraction 7 or 8. A similar behavior for pIgAR on this gradient has been previously reported (Solari et al., 1989), and we have previously noted a similar distribution for ¹²⁵I-labeled endosomes (Bartles et al., 1987). Due to the short half-life of this molecule the signal in the

was immunoprecipitated from the gradient fractions and the distribution of the recovered radiolabeled molecule determined by densitometric scanning of the fluorograms. (*A*) Fluorograms 5NT immunoprecipitates at various times of chase. The brackets define the position of the apical and basolateral domain markers (see *C*). The 30-min time point is an 8-wk exposure, and the 45-min time point is a 6-wk exposure; the three later time points were 4-wk exposures. (*B*) Graph of the data obtained from the densitometric analysis of the fluorograms shown in *A*. The proportion of the radiolabeled molecule in each fraction is expressed as a percent of the total molecule recovered from the gradient. The amount of labeled molecule appearing in the pellet fraction reflects variations in the sonication procedure, and does not indicate domain localization (Bartles et al., 1985). (●) 45, (o) 60, (△) 150, and (▲) 210 min. (*C*) Distributions of the apical markers DPPIV and 5NT and the basolateral marker CE9 in the domain gradient. The distributions of these markers were determined by immunoblot analysis of the four gradients shown in *B*; the points shown are the mean of the four determinations. The standard deviation did not exceed 4% for any fraction except the pellet. (□) 5NT; (o) DPPIV; and (▲) CE9.

PM fraction at 150 min chase was greatly decreased; by 210 min it was almost undetectable. The behavior of pIgAR on the domain gradient is consistent with what is known about its localization in liver (Sztul et al., 1985b; Luzio et al., 1986; Perez et al., 1988; Quintart et al., 1989). The appearance of this receptor at the basolateral surface is brief, and internalization into a pool of endosomal/transcytotic vesicles occurs rapidly. Transcytotic transit, and not basolateral residence, is the rate-limiting step in the movement of this molecule to the apical surface. pIgAR never exhibits a characteristic apical localization in the domain analysis because it is rapidly released into the bile upon reaching the apical surface.

Discussion

These experiments compare directly the posttranscriptional trafficking of a GPI-anchored protein with two single-span transmembrane proteins of hepatocytes in vivo. GPI-anchored PM proteins are thought to follow the same general biosynthetic itinerary as their transmembrane counterparts, i.e., ER to Golgi to PM. This pathway has been confirmed directly for GPI-anchored proteins in trypanosomes (Bangs et al., 1986; Duszenko et al., 1988). Previous studies of 5NT in vitro (Wada et al., 1986) and in hepatoma cells (van den Bosch et al., 1986) also suggest this itinerary. We demonstrate here that 5NT in hepatocytes uses this route in vivo. The rapid appearance and disappearance of newly synthesized 5NT in the ER and Golgi (Fig. 3) shows that the intracellular transport of 5NT to the PM is at least as efficient as for transmembrane proteins, and may in fact be more efficient. The newly synthesized populations of both pIgAR and DPPIV contain minor portions of molecules that appear to persist as immature forms long after the population of 5NT in the same cohort has reached 100% maturity (Fig. 2, 120-min time points). One explanation for this result is that GPI-anchored proteins might somehow be excluded from pathways that divert or retain proteins on their way to the cell surface. Alternatively, molecules of 5NT not receiving a GPI anchor immediately after translation might be rapidly degraded. In intestinal cells, a small portion of newly synthesized DPPIV is retained intracellularly due to improper folding, and our data suggest that this retention may also occur for DPPIV in liver (Matter and Hauri, 1991).

The chief finding of this work is that, after exit from the Golgi apparatus, most or all of the apically destined 5NT is inserted into the basolateral domain. We have shown previously (Bartles et al., 1987) that the time required for PM proteins to reach 50% of maximal specific radioactivity in a highly enriched PM fraction is ~30 min chase (40 min postinjection). Maximum specific radioactivity in the PM is attained between 45 min and 1 h. By performing the Golgi flotation experiments in conjunction with PM fractionations, we have provided additional support to our previously published evidence that contamination of the PM sheet preparation by late elements of the Golgi complex is not a concern in this analysis. 12.6% of newly synthesized 5NT resides in the Golgi-enriched fraction at 30 min chase, and this value has fallen about threefold (to 3.8%) by 1 h. Yet the relative specific radioactivity of proteins in the PM fraction approximately doubles over this same period, and, more importantly, remains basolateral. Because the peaks of basolateral

and apical markers overlap (Fig. 4 C), our analysis cannot rigorously eliminate the possibility that a small minority of the newly synthesized population of PM proteins travels directly from the TGN to the apical surface. Although 5NT is enriched at the apical domain (bile canaliculus) of hepatocytes, a smaller population exists at the basolateral domain at steady state (Fig. 4 C, fraction 10) (Matsuura et al., 1984; Luzio et al., 1986). The indirect route that we have shown for 5NT provides an obvious mechanism by which 5NT can reach the basolateral domain.

A soluble form of 5NT with enzymatic and immunological characteristics identical to the GPI-anchored form is found in blood (Klemens et al., 1990; discussed in Zimmerman, 1992). Our data raise the possibility that some of this pool is cleaved by a phospholipase C or D at the sinusoid and never reaches the apical domain.

We observed an incomplete release of 5NT from membranes by phospholipase C, and this is in accordance with many previous reports (discussed in Zimmerman, 1992 and references therein). These observations have led to the notion that a portion of 5NT is anchored by a transmembrane domain (Klemens et al., 1990). However, when this possibility has been addressed using other biochemical or molecular biological tests (Bailyes et al., 1990; Misumi et al., 1990; Ogata et al., 1991), all evidence supports an exclusive GPI anchorage for 5NT. Most notably, detergent-solubilized, immunoaffinity-purified 5NT contains equimolar amounts of inositol and protein (Bailyes et al., 1990), and the mAb used for that purification was also used for the immunoprecipitations in the present study. If two alternately anchored pools of 5NT exist in hepatocytes, they could not account for the results of the present study and are probably not the basis of distinction between the basolateral and apical pools. A complex of 5NT with a transmembrane protein at the basolateral domain (which could facilitate internalization or prevent it) is one possible mechanism by which the hepatocyte could maintain basolateral and apical pools of 5NT.

Our results demonstrate that 5NT is sorted via an indirect pathway to the apical domain. However, newly synthesized 5NT has not yet reached steady state at the 3.5 h time point, and extrapolation of the curves suggests that it would not reach steady state until some time between 5 and 6 h chase. This is at least 4.5 h after the first 5NT is detected at the basolateral domain. Thus it takes approximately twice as long for 5NT to reach steady state as it does for DPPIV. Furthermore, DPPIV is in fact transcytosed somewhat more slowly than aminopeptidase N and B10, two other apical transmembrane proteins which have been subject to the same analysis (Bartles et al., 1987; M. Maurice, unpublished observation). A slow rate of transcytosis may be due to a slow rate of internalization, inefficient segregation into the transcytotic pathway, or both.

Certain motifs on the cytoplasmic tails of transmembrane proteins affect the rate of endocytosis mediated by clathrin (Collawn et al., 1990; Brewer and Roth, 1991; Miettinen et al., 1992). Clathrin-coated pits act as one of a number of molecular filters in the cell (Bretscher et al., 1980; reviewed in Hopkins, 1992). GPI-anchored proteins possess no cytoplasmic tail and are mostly excluded from such sorting mechanisms. Indeed, it is established that GPI-anchored proteins are excluded from coated pits and enter the endocytic pathway via clathrin-independent endocytosis (Roth-

berg et al., 1990; Lemansky et al., 1990; Hansen et al., 1991; Bamezai et al., 1992; Keller et al., 1992). Morphological evidence that predates the discovery of the GPI anchor has shown 5NT to be largely excluded from coated pits (Stanley et al., 1983; Matsuura et al., 1984). The internalization rates for molecules subject to nonclathrin endocytosis are reported to be 2–4 times slower than those mediated by clathrin (Tran et al., 1987; Bamezai et al., 1992; Keller et al., 1992). Thus our results are consistent with 5NT being internalized at the slower rate expected for nonclathrin endocytosis, though the rates of internalization for GPI-anchored proteins in polarized epithelial cells have not been reported.

At short times after endocytosis, molecules internalized via clathrin and nonclathrin mechanisms occupy distinct vesicles (Hansen et al., 1991; Bamezai et al., 1992). By 10 min, however, these pathways have converged, and molecules internalized by both pathways are localized in tubulovesicular endosomes (Tran et al., 1987). The microenvironments of such structures are the likely location for the segregation of molecules into the smaller vesicles of the transcytotic pathway (Hubbard, 1989; Hopkins et al., 1990). Along with the slow internalization discussed above, an inefficient segregation of GPI-anchored molecules at any step in this pathway could contribute to the slow rate of transcytosis we observe for 5NT.

Recycling to the PM could also contribute to the slow transcytosis of 5NT. The intracellular or "latent" pool of 5NT in some cultured cells is as high as 50% (Stanley et al., 1980; Widnell et al., 1982; Baron and Stanley, 1987). Cell fractionation suggests that the intracellular pool of 5NT is small in vivo (Quinart et al., 1989). Morphological studies show that an intracellular pool of 5NT remains 2 h after cycloheximide treatment in cells in culture (van den Bosch et al., 1988) but not in vivo (Geuze et al., 1984). Some and perhaps all GPI-anchored proteins undergo repeated internalizations into spiral-coated vesicles classically called caveolae (Rothberg et al., 1990, 1992; reviewed in Anderson et al., 1992); whether these vesicles pinch off to form nonclathrin vesicles is unclear. The different rates of internalization into these structures might explain the broad range in the size of the intracellular pool reported for 5NT under various conditions.

It has been suggested that newly synthesized GPI-anchored proteins use the direct route in hepatocytes, based on the appearance of two out of six GPI-anchored proteins in an enriched fraction of late endosomes (Ali and Evans, 1990). 5NT was one of the four GPI-anchored proteins that did not appear in this fraction. Such data do not directly address the question of GPI sorting and in fact argue equally well for the indirect route. Since we have studied only one molecule, the possibility remains that some other GPI-anchored proteins might use the direct route.

Our results do not exclude the possibility that the sorting of GPI-anchored molecules into lipid microdomains occurs at the PM or during the endocytic pathway. In fact, a model whereby some or all newly synthesized sphingolipids arrive first at the basolateral domain and are then transcytosed would allow GPI-anchored proteins to remain associated with sphingolipids throughout the entire pathway. Alternatively, internalized 5NT might meet up in endosomes with newly synthesized glycosphingolipids taking a direct route to the apical domain; in this model, segregation into microdomains could act as a means of sorting GPI-anchored molecules for transcytosis. Such issues would be greatly clarified by a characterization of the pathways of lipid traffic in hepatocytes.

References

Ali, N., and W. H. Evans. 1990. Priority targeting of glycosyl-phosphatidyl-inositol-anchored proteins to the bile-canalicular (apical) plasma membrane of hepatocytes. *Biochem. J.* 271:193–199.

Anderson, R. G. W., B. A. Kamen, K. G. Rothberg, and S. W. Lacey. 1992. Potocytosis: sequestration and transport of small molecules by caveolae. *Science (Wash. DC).* 255:410–411.

Aronson, N. A., and O. Touster. 1974. Isolation of rat liver plasma membrane fragments in isotonic sucrose. *Methods Enzymol.* 31:90–102.

Bailyes, E. M., M. Soos, P. Jackson, A. C. Newby, K. Siddle, and J. P. Luzio. 1984. The existence and properties of two dimers of rat liver ecto-5'-nucleotidase. *Biochem. J.* 221:369–377.

Bailyes, E. M., M. A. J. Ferguson, C. A. L. S. Colaco, and J. P. Luzio. 1990. Inositol is a constituent of detergent-solubilized immunoaffinity-purified rat liver 5'-nucleotidase. *Biochem. J.* 265:907–909.

Bamezai, A., V. S. Goldmacher, and K. L. Rock. 1992. Internalization of glycosyl-phosphatidylinositol (GPI)-anchored lymphocyte proteins II. GPI-anchored and transmembrane molecules internalize through distinct pathways. *Eur. J. Immunol.* 22:15–21.

Bangs, J. D., N. W. Andrews, G. W. Hart, and P. T. Englund. 1986. Posttranslational modification and intracellular transport of a Trypanosome variant surface glycoprotein. *J. Cell Biol.* 103:255–263.

Baron, M. D., and J. P. Luzio. 1987. The synthesis and turnover of 5'-nucleotidase in primary cultured hepatocytes. *Biochim. Biophys. Acta.* 927:81–85.

Bartles, J. R., and A. L. Hubbard. 1988. Plasma membrane protein sorting in epithelial cells: do secretory pathways hold the key? *Trends Biochem. Sci.* 13:181–184.

Bartles, J. R., and A. L. Hubbard. 1990. Biogenesis of the rat hepatocyte plasma membrane. *Methods Enzymol.* 191:825–841.

Bartles, J. R., L. T. Braiterman, and A. L. Hubbard. 1985a. Biochemical characterization of domain-specific glycoproteins of the rat hepatocyte plasma membrane. *J. Biol. Chem.* 260:12792–12802.

Bartles, J. R., L. T. Braiterman, and A. L. Hubbard. 1985b. Endogenous and exogenous domain markers of the rat hepatocyte plasma membrane. *J. Cell Biol.* 100:1126–1138.

Bartles, J. R., H. M. Feracci, B. Stieger, and A. L. Hubbard. 1987. Biogenesis of the rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* 105:1241–1251.

Bergeron, J. J. M., R. A. Rachubinski, R. A. Sikstrom, B. I. Posner, and J. Paiement. 1982. Galactose transfer to endogenous acceptors within Golgi fractions of rat liver. *J. Cell Biol.* 92:139–146.

Brandli, A. W., R. G. Parton, and K. Simons. 1990. Transcytosis in MDCK cells: identification of glycoproteins transported bidirectionally between both plasma membrane domains. *J. Cell Biol.* 111:2909–2921.

Bretscher, M. S., J. N. Thomson, and B. M. F. Pearse. 1980. Coated pits act as molecular filters. *Proc. Natl. Acad. Sci. USA.* 77:4156–4159.

Brewer, C. B., and M. G. Roth. 1991. A single amino acid change in the cytoplasmic domain alters the polarized delivery of influenza virus hemagglutinin. *J. Cell Biol.* 114:413–421.

Brown, D. A., and J. K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* 68:533–544.

Casanova, J. E., Y. Mishumi, Y. Ikehara, A. L. Hubbard, and K. E. Mostov. 1991. Direct apical sorting of rat liver dipeptidylpeptidase IV expressed in Madin-Darby Canine Kidney cells. *J. Biol. Chem.* 266:24428–24432.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.

Collawn, J. F., M. Stangel, L. A. Kuhn, V. Esekogwu, S. Jing, I. S. Trowbridge, and J. A. Tainer. 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell.* 63:1061–1072.

Cross, G. A. M. 1990. Glycolipid anchoring of plasma membrane proteins. *Annu. Rev. Cell Biol.* 6:1–39.

Duszenko, M., I. E. Ivanov, M. A. J. Ferguson, H. Plesken, and G. A. M. Cross. 1988. Intracellular transport of a variant surface glycoprotein in *Trypanosoma brucei. J. Cell Biol.* 106:77–86.

Futerman, A. H., B. Stieger, A. L. Hubbard, and R. E. Pagano. 1990. Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus. *J. Biol. Chem.* 265:8650–8657.

Geuze, H. J., J. W. Slot, G. J. Strous, J. P. Luzio, and A. L. Schwartz. 1984. A cycloheximide-resistant pool of receptors for asialoglycoproteins and mannose 6-phosphate residues in the Golgi complex of hepatocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2677–2685.

Hansen, S. H., K. Sandvig, and B. van Deurs. 1991. The preendosomal compartment comprises distinct coated and noncoated endocytic vesicle populations. *J. Cell Biol.* 113:731–741.

Harb, J., K. Meflah, Y. Duflos, and S. Bernard. 1983. Purification and properties of bovine liver plasma membrane 5′ nucleotidase. *Eur. J. Biochem.* 137:131–138.

Hopkins, C. R. 1992. Selective membrane protein trafficking: vectorial flow and filter. *Trends Biochem. Sci.* 17:27–32.

Hopkins, C. R., A Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. *Nature (Lond.).* 346:335–339.

Hoppe, C. A., T. P. Connolly, and A. L. Hubbard. 1985. Transcellular transport of polymeric IgA in the rat hepatocyte: biochemical and morphological characterization of the transport pathway. *J. Cell Biol.* 101:2113–2123.

Hubbard, A. L. 1989. Endocytosis. *Curr. Opin. Cell Biol.* 1:675–683.

Hubbard, A. L., D. A. Wall, and A. Ma. 1983. Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J. Cell Biol.* 96:217–229.

Keller, G.-A., M. W. Siegel, and I. W. Caras. 1992. Endocytosis of glycophospholipid-anchored and transmembrane forms of CD4 by different endocytic pathways. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:863–874.

Klemens, M. R., W. R. Sherman, N. J. Holmberg, J. M. Ruedi, M. G. Low, and L. F. Thompson. 1990. Characterization of soluble vs. membrane-bound human placental 5′-nucleotidase. *Biochem. Biophys. Res. Commun.* 172:1371–1377.

Lemansky, P., S. H. Fatemi, B. Gorican, S. Meyale, R. Rossero, and A. M. Tartakoff. 1990. Dynamics and longevity of the glycolipid-anchored membrane protein, Thy-1. *J. Cell Biol.* 110:1525–1531.

Lisanti, M. P., and E. Rodriguez-Boulan. 1990. Glycophospholipid membrane anchoring provides clues to the mechanism of protein sorting in polarized epithelial cells. *Trends Biochem. Sci.* 15:113–118.

Lisanti, M. P., A. Le Bivic, A. R. Saltiel, and E. Rodriguez-Boulan. 1990. Preferred apical distribution of glycosyl-phosphatidylinositol (GPI) anchored proteins: a highly conserved feature of the polarized epithelial cell phenotype. *J. Membr. Biol.* 113:155–167.

Lisanti, M. P., M. Sargiacomo, L. Graeve, A. R. Saltiel, and E. Rodriguez-Boulan. 1988. Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. *Proc. Natl. Acad. Sci. USA.* 85:9557–9561.

Luzio, J. P., E. M. Bailyes, M. Baron, K. Siddle, B. M. Mullock, H. J. Geuze, and K. K. Stanley. 1986. The properties, structure, function, intracellular localization, and movement of hepatic 5′ nucleotidase. *In* Cellular Biology of Ectoenzymes, G. W. Kreutzberg, editor. Springer-Verlag, New York. 89–116.

Matsuura, S., S. Eto, K. Kato, and Y. Tashiro. 1984. Ferritin immunoelectron microscopic localization of 5′-nucleotidase on rat liver cell surface. *J. Cell Biol.* 99:166–173.

Matter, K., and H.-P. Hauri. 1991. Intracellular transport and conformational maturation of intestinal brush border hydrolases. *Biochemistry.* 30:1916–1923.

Matter, K., M. Brauchbar, K. Bucher, and H.-P. Hauri. 1990. Sorting of endogenous plasma membrane proteins occurs from two sites in cultured human intestinal epithelial cells (Caco-2). *Cell.* 60:429–437.

Miettinen, H. M., K. Matter, W. Hunziker, J. K. Rose, and I. Mellman. 1992. Fc receptor endocytosis is controlled by a cytoplasmic domain determinant that actively prevents coated pit localization. *J. Cell Biol.* 116:875–888.

Misumi, Y., S. Ogata, S. Hirose, and Y. Ikehara. 1990. Primary structure of rat liver 5′-nucleotidase deduced from the cDNA. *J. Biol. Chem.* 265:2178–2183.

Misumi, Y., S. Ogata, K. Ohkubo, S. Hirose, and Y. Ikehara. 1990. Primary structure of human placental 5′-nucleotidase and identification of the glycolipid anchor in the mature form. *Eur. J. Biochem.* 191:563–569.

Mostov, K. E., and D. L. Deichter. 1986. Polymeric immunoglobulin receptor expressed in MDCK transcytoses IgA. *Cell.* 46:613–621.

Mostov, K. E., G. Apodaca, B. Aroeti, and C. Okamoto. 1992. Plasma membrane protein sorting in polarized epithelial cells. *J. Cell Biol.* 116:577–583.

Ogata, S., Y. Hayashi, Y. Misumi, and Y. Ikehara. 1990. Membrane-anchoring domain of rat liver 5′-nucleotidase: identification of the COOH-terminal serine-523 covalently attached with a glycolipid. *Biochemistry.* 29:7923–7927.

Perez, J. H., W. J. Branch, L. Smith, B. M. Mullock, and J. P. Luzio. 1988. Investigation of endosomal compartments involved in endocytosis and transcytosis of polymeric immunoglobulin A by subcellular fractionation of perfused isolated rat liver. *Biochem. J.* 251:763–770.

Quintart, J., P. Baudhuin, and P. J. Courtoy. 1989. Marker enzymes in rat liver vesicles involved in transcellular transport. *Eur. J. Biochem.* 184:567–574.

Rothberg, K. G., Y. Ying, J. F. Kolhouse, B. A. Kamen, and R. G. W. Anderson. 1990. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J. Cell Biol.* 110:637–649.

Rothberg, K. G., J. E. Heuser, W. C. Donzell, Y.-S. Ying, J. R. Glenney, and R. G. W. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell.* 68:673–682.

Scott, L. J., and A. L. Hubbard. 1992. Dynamics of four rat liver plasma membrane proteins and polymeric IgA receptor. Rates of synthesis and selective loss into the bile. *J. Biol. Chem.* 267:6099–6106.

Siddle, K., E. M. Bailyes, and J. P. Luzio. 1981. A monoclonal antibody inhibiting rat liver 5′-nucleotidase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 128:103–107.

Simons, K., and G. van Meer. 1988. Lipid sorting in epithelial cells. *Biochemistry.* 27:6197–6202.

Solari, R., E. Schaerer, C. Tallichet, L. T. Braiterman, A. L. Hubbard, and J.-P. Kraehenbuhl. 1989. Cellular location of the cleavage event of the polymeric immunoglobulin receptor and fate of its anchoring domain in the rat hepatocyte. *Biochem. J.* 257:759–768.

Stanley, K. K., M. R. Edwards, and J. P. Luzio. 1980. Subcellular distribution and movement of 5′-nucleotidase in rat cells. *Biochem. J.* 186:59–69.

Stanley, K. K., B. Burke, T. Pitt, K. Siddle, and J. P. Luzio. 1983. Localization of 5′-nucleotidase in a rat liver cell line using a monoclonal antibody and indirect immunofluorescent labeling. *Exp. Cell Res.* 144:39–46.

Sztul, E. S., K. E. Howell, and G. E. Palade. 1983. Intracellular and transcellular transport of secretory component and albumin in rat hepatocytes. *J. Cell Biol.* 97:1582–1591.

Sztul, E. S., K. E. Howell, and G. E. Palade. 1985a. Biogenesis of the polymeric IgA receptor in rat hepatocytes. I. Kinetic studies of its intracellular forms. *J. Cell Biol.* 100:1248–1254.

Sztul, E. S., K. E. Howell, and G. E. Palade. 1985b. Biogenesis of the polymeric IgA receptor in rat hepatocytes. II. Localization of its intracellular forms by cell fractionation studies. *J. Cell Biol.* 100:1255–1261.

Tran, D., J.-L. Carpentier, F. Sawano, P. Gorden, and L. Orci. 1987. Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. *Proc. Natl. Acad. Sci. USA.* 84:7957–7961.

van den Bosch, R., H. J. Geuze, A. P. M. du Maine, and G. J. Strous. 1986. Transport and metabolism of 5′-nucleotidase in a rat hepatoma cell line. *Eur. J. Biochem.* 160:49–54.

van den Bosch, R. A., A. P. M. du Maine, H. J. Geuze, A. van der Ende, and G. J. Strous. 1988. Recycling of 5′-nucleotidase in a rat hepatoma cell line. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3345–3351.

van Meer, G., E. H. K. Stelzer, R. W. Wijnaendts-van-Resandt, and K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby Canine Kidney) cells. *J. Cell Biol.* 105:1623–1635.

van't Hof, W., and G. van Meer. 1990. Generation of lipid polarity in intestinal epithelial (Caco-2) cells: sphingolipid synthesis in the Golgi complex and sorting before vesicular traffic to the plasma membrane. *J. Cell Biol.* 111:977–986.

Verdon, B., and E. G. Berger. 1983. Galactosyltransferase. *In* Methods of Enzymatic Analysis: Enzymes I: Oxoreductases and Transferases. Vol. III. J. Bergemeyer and M. Grassl, editors. Verlag Chemie, Basel, Switzerland. 374–381.

Wada, I., M. Himeno, K. Furuno, and K. Kato. 1986. Biosynthesis and intracellular transport of rat liver 5′-nucleotidase. *J. Biol. Chem.* 261:2222–2227.

Widnell, C. C., Y.-J. Schneider, B. Pierre, P. Baudhuin, and A. Trout. 1982. Evidence for a continual exchange of 5′-nucleotidase between the cell surface and cytoplasmic membranes in cultured rat fibroblasts. *Cell.* 28:61–70.

Wollner, D. A., K. A. Krzeminski, and W. J. Nelson. 1992. Remodeling the cell surface distribution of membrane proteins during the development of epithelial cell polarity. *J. Cell Biol.* 116:889–899.

Zimmerman, H. 1992. 5′-nucleotidase: molecular structure and functional aspects. *Biochem. J.* 285:345–365.