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Biosynthetic transport of the asialoglycoprotein receptor H1 to the cell surface occurs via endosomes

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ABSTRACT **Signals for endocytosis and for basolateral and lysosomal sorting are closely related in a number of membrane proteins, suggesting similar sorting mechanisms at the plasma membrane and in the trans-Golgi network (TGN). We tested the hypothesis that basolateral membrane proteins are transported to the cell surface via endosomes for the asialoglycoprotein receptor H1. This protein was tagged with a tyrosine sulfation site (H1^{TS}) to allow specific labeling with [^{35}S]sulfate in the TGN. Madin–Darby canine kidney cells expressing H1^{TS} were pulse-labeled and chased for a period of time insufficient for labeled H1^{TS} to reach the cell surface. Upon homogenization and gradient centrifugation, fractions devoid of TGN were subjected to immunoisolation of compartments containing mannose 6-phosphate receptor, which served as an endosomal marker. H1^{TS} in transit to the cell surface was efficiently coisolated, whereas a labeled secretory protein and free glycosaminoglycan chains were not. This indicates an indirect pathway for the asialoglycoprotein receptor to the plasma membrane via endosomes and has important implications for protein sorting in the TGN and endosomes.**

Identification and characterization of intracellular sorting signals for membrane proteins has revealed that the same or closely related determinants are recognized in both the biosynthetic and the endocytic pathways. For endocytosis via clathrin-coated vesicles, two types of signals have been characterized in the cytoplasmic domains of membrane proteins. One consists of short stretches of 4–6 amino acids including a critical tyrosine residue (1). The other signal involves a dileucine motif (2). In a number of proteins, these internalization motifs, or sequences overlapping with them, were shown to function also as basolateral targeting signals in polarized epithelial cells [e.g., hemagglutinin mutant HAY543, Fc receptor, low density lipoprotein receptor, lysosomal acid phosphatase (3–7)]. In addition, direct transport from the trans-Golgi network (TGN) to endosomes was found to depend on closely related determinants that belong to either dileucine or tyrosine-containing types and that are also functional endocytosis signals [e.g., mannose 6-phosphate receptor, CD3γ and -δ chains, limp II, invariant chain (2, 8–12)]. These findings suggest similar sorting mechanisms at the plasma membrane and at the TGN. This led to the proposal that signal-bearing surface proteins do not exit the TGN together with secretory proteins but rather are transported first to endosomes en route to the basolateral plasma membrane (1, 4).

In this study, we have tested this model and present evidence that H1, the major subunit of the human asialoglycoprotein (ASGP) receptor, takes an indirect route from the TGN to the cell surface in Madin–Darby canine kidney (MDCK) cells. The ASGP receptor (reviewed in ref. 13) is naturally expressed on the basolateral cell surface of hepatocytes as a heterooligomeric complex of two subunits, H1 and H2. H1 is sorted in the cell like the heterooligomeric receptor complex (14, 15). A tyrosine-containing signal in its cytoplasmic domain is responsible for both rapid endocytosis and for specific basolateral expression in polarized MDCK cells (16, 17).

To study TGN-to-cell surface transport, we used a MDCK cell line expressing H1 tagged with a tyrosine sulfation site (H1^{TS}). This permitted labeling of the protein with [^{35}S]sulfate specifically in the TGN. After a subsequent chase incubation too short to allow the protein to reach the cell surface, the presence of labeled H1^{TS} in membranes containing the cation-independent, M_r 300,000 mannose 6-phosphate receptor (MPR300) was analyzed by immunoisolation. H1^{TS} in transit to the cell surface distributed predominantly into MPR300-positive compartments, indicating an indirect biosynthetic route from the TGN to the cell surface via endosomes.

MATERIALS AND METHODS

Cell Culture and Transfection. Culture conditions for MDCK cells (strain II) and derived cell lines, construction of the cDNAs for H1^{TS} and A1Pi^{TS} (A1Pi, α_1-proteinase inhibitor), and preparation of the cell line M1^{TS} have been described (18). To prepare the cell line Mα1^{TS}, the cDNA of A1Pi^{TS} was subcloned into the expression vector pCB6 and transfected according to Kawai and Nishizawa (19). Clonal cell lines resistant to G418 sulfate at 0.5 mg/ml were isolated and screened for expression by immunoblot analysis with an affinity-purified rabbit anti-human A1Pi antibody.

[^{35}S]Sulfate Labeling. Cells expressing H1^{TS} or A1Pi^{TS} were labeled with [^{35}S]sulfate according to the procedure of Régnier-Vigouroux *et al.* (20) using a hypertonic preincubation to enhance sulfate uptake. Sulfate-free minimal essential medium supplemented with [^{35}S]sulfate (1 mCi/ml; 1 Ci = 37 GBq; carrier-free; Amersham) was used. After pulse-labeling, cells were quickly washed with phosphate-buffered saline (PBS) at room temperature and chased at 37°C in minimal essential medium containing twice the concentration of unlabeled sulfate.

The synthesis of free glycosaminoglycan (GAG) chains was induced by addition of 1 mM 4-methylumbelliferyl β-D-xyloside (Sigma; added from a 0.5 M stock solution in dimethyl sulfoxide) to the starvation, labeling, and chase media (21). [^{35}S]Sulfate labeling of GAG chains was performed after isotonic preincubation as described (18).

Transport Kinetics. M1^{TS} cells in 60-mm dishes were pulse-labeled for 7 min at 37°C and chased for different times at 37°C. At the end of the chase, triplicate plates were cooled on ice, washed twice with ice-cold PBS and once with PBS containing 5 mM EDTA, and then incubated in the absence or presence of proteinase K (1 mg/ml) in PBS with 5 mM EDTA

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Abbreviations: A1Pi, α_1-proteinase inhibitor; ASGP, asialoglycoprotein; GAG, glycosaminoglycan; MDCK, Madin–Darby canine kidney; MPR300, M_r 300,000 mannose 6-phosphate receptor; TGN, trans-Golgi network.

for 45 min at 4°C. Proteolysis was stopped by adding 2 mM phenylmethylsulfonyl fluoride. The cells were lysed in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, and $H1^{TS}$ was immunoprecipitated with a polyclonal rabbit antiserum directed against the purified ASGP receptor. Samples were analyzed by SDS/gel electrophoresis and fluorography and quantitated by densitometric scanning.

Subcellular Fractionation. Subcellular fractionation was performed essentially according to Tooze and Huttner (22, 23). Cells were grown to subconfluence in 15-cm dishes (two dishes per experimental condition), pulse-labeled with [^{35}S]sulfate for 5 min at 37°C, and cooled on ice either immediately or after an additional 5-min chase at 37°C. A 20% (vol/vol) suspension of scraped cells in 0.25 M sucrose/1 mM EDTA/1 mM $Mg(OAc)_2$/1.6 mM Na_2SO_4 was passed five or six times through a 22-G needle and homogenized in a cell cracker (24) from EMBL (30 strokes with a 14-μm clearance). The homogenate was centrifuged at 1000 × *g* for 10 min. The resulting postnuclear supernatant was loaded onto an 11-ml linear sucrose density gradient (0.3–1.2 M sucrose in 10 mM Hepes·KOH, pH 7.2) and centrifuged at 70,000 × *g* in a swinging bucket rotor for either 15 or 60 min at final speed. One-milliliter fractions were taken from the top of the gradient and used for immunoprecipitation of $H1^{TS}$ or $A1Pi^{TS}$. For analysis of GAG chains, aliquots of the fractions were subjected to acetone precipitation with 10 μg of chondroitin sulfate as carrier. Samples were analyzed by SDS/gel electrophoresis and fluorography and quantitated by densitometric scanning.

Immunoisolation of MPR300-Positive Compartments. Immunoisolation of MPR300-positive compartments was performed essentially according to Delbrück *et al.* (25) with an affinity-purified antibody raised against the C-terminal 15 amino acids of the cytoplasmic domain of MPR300. As immunosorbent, sheep anti-rabbit Fc antibodies coupled to Eupergit C1Z (Röhm, Weiterstadt, Germany) were used. Sucrose gradient fractions were diluted to a final concentration of 0.25 M sucrose and preincubated with immunosorbent (IgG at 60 μg/ml) for 1 h at 4°C with shaking. The immunosorbent was removed by centrifugation at 2200 × *g* for 10 min, resulting in the loss of ≈25% of the labeled material. The supernatant was divided into two aliquots and incubated with preimmune or anti-MPR300 immunoglobulins (16 μg/ml) for 45 min at 4°C with shaking. After addition of immunosorbent (IgG at 160 μg/ml) and incubation at 4°C for 45 min, the immune complexes were collected by centrifugation and washed four times with 0.25 M sucrose/1 mM EDTA/bovine serum albumin (5 mg/ml)/10 mM Hepes·KOH, pH 7.2. The distribution of MPR300 was analyzed by immunoblotting with a goat anti-human MPR300 antibody. For this, the immunosorbent was extracted with SDS/gel sample buffer for 10 min at 95°C and the supernatant was subjected to trichloroacetic acid precipitation. For analysis of $H1^{TS}$ and $A1Pi^{TS}$, the proteins were immunoprecipitated from the supernatant and from detergent extracts of the immunosorbent. Extraction was performed by incubation for 10 min on ice in PBS with 1% Triton X-100/0.5% deoxycholate/0.25% SDS. GAG chains were analyzed after acetone precipitation of the supernatant and extraction of the immunosorbent with SDS sample buffer for 10 min at 95°C. Samples were analyzed by SDS/gel electrophoresis and fluorography and quantitated by densitometric scanning. For $H1^{TS}$, $A1Pi^{TS}$, and GAG chains, 80–95% of the initial gradient fractions analyzed was recovered (total of material in the immunoisolated fraction, the supernatant fraction, and the immunosorbent used for preadsorption).

RESULTS

Kinetics of $H1^{TS}$ Transport from the TGN to the Cell Surface. To study post-Golgi transport of the ASGP receptor subunit H1, we have previously tagged the protein at its exoplasmic C terminus with a 9-amino acid tyrosine sulfation peptide (18). This modification allows labeling of the tagged protein, $H1^{TS}$, with [^{35}S]sulfate specifically in the TGN and permits us to follow its fate in pulse–chase experiments. $H1^{TS}$ was expressed in a stable transfected MDCK cell line, $M1^{TS}$. Like wild-type H1, $H1^{TS}$ was specifically expressed on the basolateral surface, as judged by surface immunoprecipitation of filter-grown [^{35}S]sulfate-labeled $M1^{TS}$ cells (not shown). Since H1 is constitutively endocytosed from the plasma membrane and recycled, the steady-state distribution of the protein between the cell surface and intracellular compartments reflects its endocytic behavior (17). By immunoblot analysis of cells digested with proteinase K at 4°C and of untreated cells, 47% ± 13% (*n* = 3) of wild-type H1 (17) and 54% ± 3% (*n* = 6) of $H1^{TS}$ were found on the surface of the MDCK cell lines M1 and $M1^{TS}$, respectively. These results confirmed that the tyrosine sulfation tag did not interfere with sorting of $H1^{TS}$ in either the exocytic or the endocytic pathway.

To determine the kinetics of transport of $H1^{TS}$ to the cell surface, $M1^{TS}$ cells were pulse-labeled with [^{35}S]sulfate for 7 min at 37°C and chased for different times at 37°C in the presence of excess unlabeled sulfate. The fraction of intracellular sulfate-labeled $H1^{TS}$ was determined by proteinase K digestion of surface proteins at 4°C and immunoprecipitation of $H1^{TS}$ (Fig. 1). Within the first 5 min of chase, no labeled protein reached the cell surface. The protected fraction of the protein rapidly decreased in the subsequent 5–10 min and leveled off at ≈40%, corresponding to the steady-state distribution of $H1^{TS}$ in the cell. If proteinase K was present during the chase incubation at 37°C, 90% of $H1^{TS}$ was digested within 20 min (Fig. 1, open square), indicating that essentially all the labeled protein reached the plasma membrane in this period. Apparently, in untreated cells, $H1^{TS}$ is rapidly reinternalized.

To analyze the transport route of $H1^{TS}$ in the subsequent experiments, a pulse-labeling time of 5 min followed by a chase incubation of 5 min was used. Under these conditions, any labeled $H1^{TS}$ was either still in the TGN or in a post-Golgi compartment in transit to the cell surface. As markers for secretory vesicles from the TGN to the plasma membrane, we used the secretory protein A1Pi and free GAG chains. A sulfatable version of A1Pi tagged with a tyrosine sulfation site, $A1Pi^{TS}$, had previously been constructed (18), and a stable expressing MDCK cell line, $M\alpha1^{TS}$, was generated. Synthesis of free GAG chains, which are efficiently sulfated in the TGN, was induced in MDCK cells by incubation with xyloside.

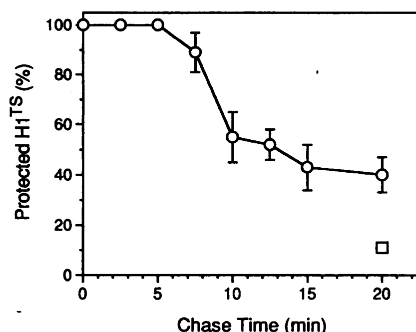

FIG. 1. Transport kinetics of $H1^{TS}$ from the TGN to the cell surface. MDCK cells expressing $H1^{TS}$ were pulse-labeled with [^{35}S]sulfate for 7 min at 37°C, chased at 37°C, and incubated in the absence or presence of proteinase K at 4°C (○). Alternatively, proteinase K was added to the cells during the chase incubation at 37°C (□). Total and protease-resistant $H1^{TS}$ was immunoprecipitated and analyzed by gel electrophoresis. Fluorographs were quantified by densitometric scanning, and the fraction of protease-protected—i.e., intracellular—$H1^{TS}$ was plotted. Means ± SD of three experiments are shown, except for the square, which represents a single experiment.

Separation of Post-Golgi Transport Compartments from TGN. To separate post-Golgi compartments from TGN, we used the procedure developed by Tooze and Huttner (22, 23) to separate secretory vesicles and TGN of PC12 cells. Labeled $M1^{TS}$, $M\alpha1^{TS}$, and xyloside-treated MDCK cells were homogenized by using a ball-bearing cell cracker and subjected to sucrose density-gradient centrifugation at 70,000 × *g* for 15 min. $H1^{TS}$ and $A1Pi^{TS}$ were immunoprecipitated and GAG chains were acetone-precipitated from gradient fractions and analyzed by SDS/gel electrophoresis, fluorography, and densitometric quantitation (Fig. 2 *A, C,* and *D*). After 5 min of labeling with [^{35}S]sulfate, when labeled proteins are still in the TGN (22), [^{35}S]sulfated $H1^{TS}$, $A1Pi^{TS}$, and GAG chains were found mainly in fractions 5–10 (solid circles). After an additional chase of 5 min (open circles), labeled $A1Pi^{TS}$ and GAG chains peaked near the top of the gradient in fractions 2 and 3 (Fig. 2 *C* and *D*), in agreement with the observations by Huttner and colleagues (20, 22, 26). In contrast to these secretory markers, labeled $H1^{TS}$ showed a broad distribution poorly separated from that obtained immediately after pulse-labeling (Fig. 2*A*). An improved separation was achieved by extending the centrifugation time to 60 min (Fig. 2*B*). After a 5-min pulse with [^{35}S]sulfate, $H1^{TS}$ peaked in fractions 9–11 and was virtually absent from fractions 1–7. After a 5-min chase, almost 50% of the protein was recovered in fractions 2–7, which thus contained labeled $H1^{TS}$ in transit to the cell surface. In the following immunoisolation experiments, fractions 3–6 of gradients centrifuged for 60 min were used to analyze $H1^{TS}$, and fractions 1–4 of 15-min gradients were used to analyze $A1Pi^{TS}$ and GAG chains.

Immunoisolation of MPR300-Containing Compartments. The mannose 6-phosphate receptors cycle between endosomes, where they are mainly concentrated, the TGN, and the plasma membrane (27). Since they deliver newly synthesized lysosomal hydrolases from the TGN to endosomes specifically via clathrin-coated vesicles, they can be used as markers for an indirect pathway to the cell surface via endosomes as opposed to the direct pathway via secretory vesicles. Using an immunoisolation procedure based on an antiserum directed against the cytoplasmic portion of MPR300 (25), we analyzed whether $H1^{TS}$ in transit to the cell surface was in the membrane compartments containing MPR300.

We used the immunoisolation procedure developed previously for human HepG2 cells (25) and adopted it to efficiently isolate MPR300 from fractionated MDCK cells. In our 60-min sucrose gradient, the bulk of MPR300 is concentrated in fractions 3–6 (not shown). These fractions were pooled and incubated with either the anti-MPR300 or preimmune immunoglobulins. Immune complexes were then isolated with a secondary antibody coupled to Eupergit C1Z as described. The distribution of MPR300 in the isolated and supernatant fractions analyzed by immunoblotting is shown in Fig. 3. In the control experiment with preimmune antibodies, MPR300 was almost exclusively recovered in the supernatant of the immunoisolation. Using the anti-MPR300 antibody, however, the mature, complex glycosylated form of MPR300 was almost quantitatively associated with the immunosorbent. As previously observed with HepG2 cell homogenates (25), the high-mannose form of MPR300 (and thus the endoplasmic reticulum) was inefficiently isolated and largely remained in the supernatant. Essentially the same result was obtained when the immunoisolation was performed on pooled fractions 1–4 of 15-min gradients (not shown).

To analyze the behavior of $H1^{TS}$, $M1^{TS}$ cells were labeled with [^{35}S]sulfate for 5 min, chased for 5 min, homogenized, and subjected to a 60-min gradient centrifugation. Fractions 3–6 were subjected to immunoisolation as in Fig. 3. $H1^{TS}$ was immunoprecipitated from detergent extracts of the immunosorbent and from the supernatant of the immunoisolation (Fig. 4*A*). In the control experiment with preimmune serum, $H1^{TS}$ was found mainly in the supernatant. Using anti-MPR300 antibody, however, $H1^{TS}$ was efficiently immunoisolated. In several experiments, >90% of $H1^{TS}$ could be coisolated with MPR300-positive compartments (Fig. 5).

$A1Pi^{TS}$ and GAG chains were analyzed by performing the immunoisolation procedure on fractions 1–4 of sucrose gradients identical to those shown in Fig. 2 *C* and *D*. As expected for molecules in secretory vesicles, both $A1Pi^{TS}$ and GAG chains were mainly found in the supernatant fraction when either preimmune or anti-MPR300 antibodies were used (Fig. 4 *B* and *C*). Less than 20% of the labeled material was associated with the immunosorbent (Fig. 5). To exclude the possibility that $A1Pi^{TS}$ had leaked out of its compartments during the immunoisolation procedure, a protease protection

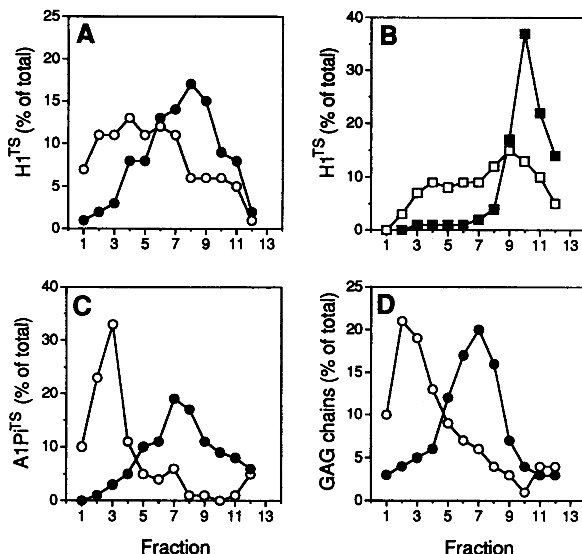

FIG. 2. Separation of proteins in post-Golgi compartments from proteins in the TGN by gradient centrifugation. MDCK cells synthesizing $H1^{TS}$, $A1Pi^{TS}$, or free GAG chains were pulse-labeled with [^{35}S]sulfate for 5 min followed by either no chase (solid symbols) or a chase of 5 min (open symbols). Upon homogenization, the cells were subjected to sucrose density-gradient centrifugation at 70,000 × *g* for 15 min (*A, C,* and *D*) or for 60 min (*B*). From aliquots of the gradient fractions, labeled $H1^{TS}$ and $A1Pi^{TS}$ were immunoprecipitated, and labeled free GAG chains were acetone-precipitated. Fluorographs of SDS gels were quantified by densitometric scanning.

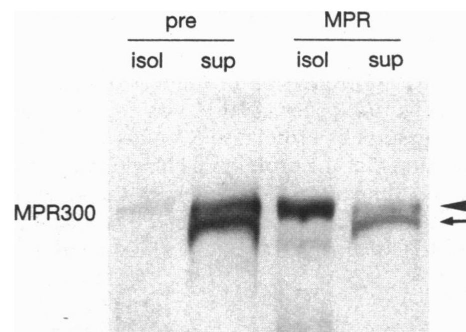

FIG. 3. Immunoisolation of MPR300-positive compartments from gradient fractions. Homogenized MDCK cells were fractionated by sucrose density-gradient centrifugation at 70,000 × *g* for 60 min (as in Fig. 2*B*). Gradient fractions 3–6 were subjected to immunoisolation with an antibody against the cytoplasmic domain of MPR300 (MPR) or, as a control, a preimmune antibody (pre) as described. Aliquots of the isolated material (isol) and the corresponding supernatants (sup) were taken for immunoblot analysis with an anti-MPR300 antiserum. The high-mannose and complex glycosylated forms of MPR300 are indicated by an arrow and an arrowhead, respectively.

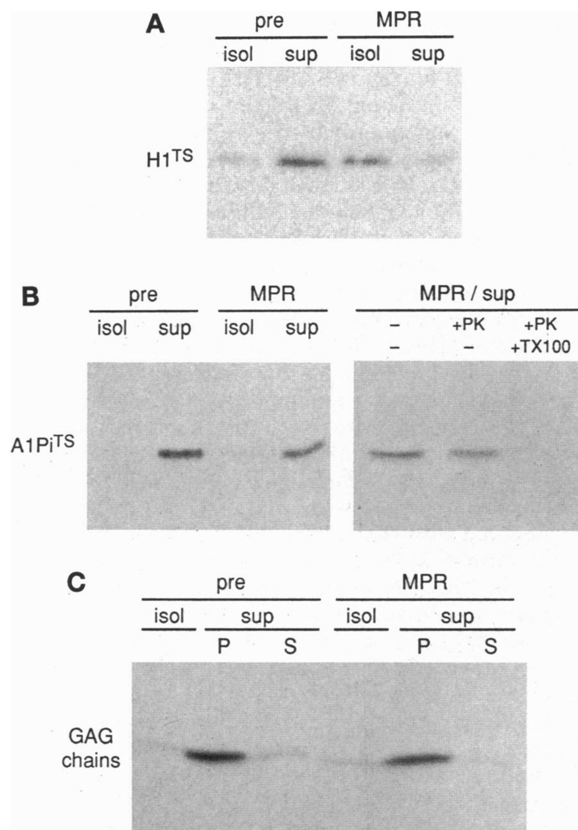

FIG. 4. Distribution of $H1^{TS}$, $A1Pi^{TS}$, and free GAG chains in transit to the cell surface upon immunoisolation of MPR300-positive compartments. MDCK cells expressing $H1^{TS}$ (*A*), $A1Pi^{TS}$ (*B*), or free GAG chains (*C*) were labeled for 5 min with [^{35}S]sulfate, chased for 5 min, homogenized, and fractionated by sucrose gradient centrifugation as in Fig. 2 *B–D*, respectively. isol, Isolated material; sup, supernatant. Fractions containing the labeled molecules after exiting the TGN—i.e., fractions 3–6 for $H1^{TS}$ and fractions 1–4 for $A1Pi^{TS}$ and GAG chains—were pooled and subjected to immunoisolation with an antibody against the cytoplasmic domain of MPR300 (MPR) or a preimmune antibody (pre). The integrity of $A1Pi^{TS}$-containing vesicles in the supernatant of the MPR300 immunoisolation (MPR/sup) was tested by incubation with proteinase K (PK) in the absence or presence of Triton X-100 (TX100). Labeled GAG chains were shown to be associated with membrane compartments by centrifugation at 100,000 $\times$ *g* for 30 min (P, pellet; S, supernatant). $H1^{TS}$ and $A1Pi^{TS}$ were analyzed by immunoprecipitation and free GAG chains were analyzed by acetone precipitation followed by gel electrophoresis and fluorography.

experiment was performed on the supernatant of an immunoisolation (Fig. 4*B Right*). $A1Pi^{TS}$ was found to be mostly resistant to digestion by proteinase K unless Triton X-100 was added, indicating that the protein was inside intact membrane compartments. Similarly, GAG chains in the supernatant of the immunoisolation could be sedimented at 100,000 $\times$ *g* for 30 min (Fig. 4*C*), confirming their association with membrane vesicles.

DISCUSSION

Our results show that the ASGP receptor protein $H1^{TS}$ is transported from the TGN to the cell surface via a MPR300-positive intermediate. MPR300 cycles between endosomes and the TGN as well as the plasma membrane (27). Immunoisolation of MPR300-positive membranes was performed after pulse–chase times insufficient for labeled $H1^{TS}$ to reach the cell surface. This was to exclude isolation of labeled $H1^{TS}$ from the plasma membrane and after reinternalization. To avoid

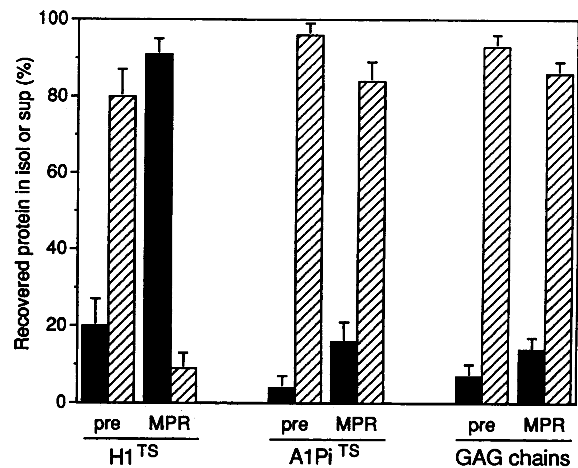

FIG. 5. Distribution of $H1^{TS}$, $A1Pi^{TS}$, and free GAG chains between the immunoisolated subfraction and the corresponding supernatant. Fluorographs of immunoisolation experiments as shown in Fig. 4 were quantified by densitometric scanning. Mean percentage recovered in immunoisolated (isol) (solid bars) and supernatant (sup) (hatched bars) fractions using anti-MPR300 (MPR) or preimmune antibodies (pre) are shown ($\pm$SD; $n = 6$ for $H1^{TS}$ and $A1Pi^{TS}$, $n = 8$ for GAG chains).

isolation of labeled $H1^{TS}$ still present in the TGN, we analyzed gradient fractions that were separate from those containing the compartment of sulfation (i.e., the TGN). We conclude that labeled $H1^{TS}$ was coisolated with MPR300 in endosomal membranes. Therefore, biosynthetic surface transport of $H1^{TS}$ occurs via endosomes rather than via secretory vesicles.

The receptor is thus segregated from secretory proteins in the TGN and packaged into separate vesicles destined for endosomes. Other studies have previously provided evidence that some membrane proteins including the ASGP receptor exit the TGN in vesicles distinct from those carrying secretory proteins: (*i*) In rat hepatocytes, using quantitative immunogold electron microscopy, the ASGP receptor was found to be depleted from budding secretory vesicles, which contained albumin and lipoproteins (28). (*ii*) TGN-to-cell surface transport of basolateral secretory proteins was shown to be strongly suppressed by treatment with microtubule-depolarizing agents, whereas basolateral surface transport of membrane proteins was only slightly affected. This was shown for laminin vs. Na^+,K^+-ATPase and β_1 integrins in MDCK cells using nocodazole (29) and for albumin vs. the polymeric IgA receptor in hepatocytes using colchicine (30). (*iii*) In hepatocytes, albumin and four other secretory proteins were not coimmunoisolated with the polymeric IgA receptor in a membrane preparation enriched in exocytic vesicles, whereas glucose transporter, ASGP receptor, transferrin receptor, and epidermal growth factor receptor were efficiently copurified (31). All the membrane proteins analyzed in these studies are basolateral and several of them are known to contain tyrosine-based sorting signals. Based on our results, we propose that these membrane proteins, like $H1^{TS}$, are segregated at the TGN into an endosomal pathway en route to the cell surface.

Transit of $H1^{TS}$ to the cell surface via MPR300-positive membranes does not necessarily imply that the protein exits the TGN in clathrin-coated vesicles together with MPR300. It is conceivable that additional, clathrin-independent vesicles are involved in TGN-to-endosome transport. In fact, by immunogold electron microscopy of rat hepatocytes, the ASGP receptor was not found to be concentrated in clathrin-coated buds of the TGN (32). Furthermore, an efficient interaction of cytoplasmic sequences with AP-1 adaptor complexes of Golgi clathrin coats has been detected only for the mannose 6-phosphate receptors in *in vitro* experiments but not for the low

density lipoprotein receptor and lysosomal acid phosphatase (33, 34). However, interactions of low affinity cannot be excluded by the available *in vitro* results.

While this manuscript was in preparation, Futter *et al.* (35) reported that newly synthesized transferrin receptor, another basolateral endocytic protein, could be detected in endosomes before reaching the surface in HEp.2 cells. Endosomes were isolated after being loaded with gold-coupled anti-transferrin receptor antibodies. Alkaline phosphatase, a glycosylphosphatidylinositol-anchored protein, and a secretory form of horseradish peroxidase were not found in this endosome preparation. This result and our study are consistent with the model that indirect surface transport via endosomes depends on a cytosolic sequence determinant. It remains to be experimentally tested whether basolateral sorting and biosynthetic surface transport via endosomes indeed depend on the same signals.

The indirect pathway to the plasma membrane gives endosomes an additional role in sorting of membrane proteins en route to the plasma membrane. It remains to be studied whether transport via endosomes is specific for polarized sorting of basolateral membrane proteins or whether it can be observed for other membrane proteins as well. It has recently been shown for the low density lipoprotein receptor and the polymeric IgA receptor that sorting to the plasma membrane in the biosynthetic pathway and return to the plasma membrane following internalization is controlled by the same signals (36, 37). This suggested that the same or similar sorting mechanisms are operative in the TGN and in endosomes. Successive sorting in the TGN and in endosomes could provide a mechanism of increased efficiency. At present, however, it cannot be excluded that all membrane proteins are first transported to endosomes, in which case endosomes would be the main site for polarized sorting of membrane proteins.

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1. Trowbridge, I. S., Collawn, J. F. & Hopkins, C. R. (1993) *Annu. Rev. Cell Biol.* **9,** 129–161.
2. Letourneur, F. & Klausner, R. D. (1992) *Cell* **69,** 1143–1157.
3. Brewer, C. B. & Roth, M. G. (1991) *J. Cell Biol.* **114,** 413–421.
4. Hunziker, W., Harter, C., Matter, K. & Mellman, I. (1991) *Cell* **66,** 907–920.
5. Matter, K., Hunziker, W. & Mellman, I. (1992) *Cell* **71,** 741–753.
6. Prill, V., Lehmann, L., von Figura, K. & Peters, C. (1993) *EMBO J.* **12,** 2181–2193.
7. Hunziker, W. & Fumey, C. (1994) *EMBO J.* **13,** 2963–2969.
8. Johnson, K. F. & Kornfeld, S. (1992) *J. Cell Biol.* **119,** 249–257.
9. Johnson, K. F. & Kornfeld, S. (1992) *J. Biol. Chem.* **267,** 17110–17115.
10. Sandoval, I. V., Arredondo, J. J., Alcalde, J., Noriega, A. G., Vandekerckhove, J., Jimenez, M. A. & Rico, M. (1994) *J. Biol. Chem.* **269,** 6622–6631.
11. Ogata, S. & Fukuda, M. (1994) *J. Biol. Chem.* **269,** 5210–5217.
12. Odorizzi, C. G., Trowbridge, I. S., Xue, L., Hopkins, C. R., Davis, C. D. & Collawn, J. F. (1994) *J. Cell Biol.* **126,** 317–330.
13. Spiess, M. (1990) *Biochemistry* **29,** 1009–1018.
14. Shia, M. A. & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1158–1162.
15. Geffen, I., Wessels, H. P., Roth, J., Shia, M. A. & Spiess, M. (1989) *EMBO J.* **8,** 2855–2861.
16. Fuhrer, C., Geffen, I. & Spiess, M. (1991) *J. Cell Biol.* **114,** 423–431.
17. Geffen, I., Fuhrer, C., Leitinger, B., Weiss, M., Huggel, K., Griffiths, G. & Spiess, M. (1993) *J. Biol. Chem.* **268,** 20772–20777.
18. Leitinger, B., Brown, J. L. & Spiess, M. (1994) *J. Biol. Chem.* **269,** 8115–8121.
19. Kawai, S. & Nishizawa, M. (1984) *Mol. Cell. Biol.* **4,** 1172–1174.
20. Régnier-Vigouroux, A., Tooze, S. A. & Huttner, W. B. (1991) *EMBO J.* **10,** 3589–3601.
21. Miller, S. G. & Moore, H.-P. H. (1992) *Methods Enzymol.* **219,** 234–248.
22. Tooze, S. A. & Huttner, W. B. (1990) *Cell* **60,** 837–847.
23. Tooze, S. A. & Huttner, W. B. (1992) *Methods Enzymol.* **219,** 81–93.
24. Balch, W. E. & Rothman, J. E. (1985) *Arch. Biochem. Biophys.* **240,** 413–425.
25. Delbrück, R., Desel, C., von Figura, K. & Hille-Rehfeld, A. (1994) *Eur. J. Cell Biol.* **64,** 7–14.
26. Rosa, P., Barr, F. A., Stinchcombe, J. C., Binacchi, C. & Huttner, W. B. (1992) *Eur. J. Cell Biol.* **59,** 265–274.
27. Kornfeld, S. & Mellman, I. (1989) *Annu. Rev. Cell Biol.* **5,** 483–525.
28. Geuze, H. J., Slot, J. W. & Schwartz, A. L. (1987) *J. Cell Biol.* **104,** 1715–1723.
29. Boll, W., Partin, J. S., Katz, A. I., Caplan, M. J. & Jamieson, J. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8592–8596.
30. Saucan, L. & Palade, G. E. (1992) *Hepatology* **15,** 714–721.
31. Saucan, L. & Palade, G. E. (1994) *J. Cell Biol.* **125,** 733–741.
32. Geuze, H., Slot, J. W. & Schwartz, A. L. (1992) in *The Trans-Golgi Exits of MPR*, eds. Windler, E. & Greten, H. (Zuckerschwerdt, Munich), pp. 37–43.
33. Glickman, J. N., Conibear, E. & Pearse, B. M. F. (1989) *EMBO J.* **8,** 1041–1047.
34. Sosa, M. A., Schmidt, B., von Figura, K. & Hille-Rehfeld, A. (1993) *J. Biol. Chem.* **268,** 12537–12543.
35. Futter, C. E., Connolly, C. N., Cutler, D. F. & Hopkins, C. R. (1995) *J. Biol. Chem.* **270,** 10999–11003.
36. Matter, K., Whitney, J. A., Yamamoto, E. M. & Mellman, I. (1993) *Cell* **74,** 1053–1064.
37. Areoti, B. & Mostov, K. E. (1994) *EMBO J.* **13,** 2297–2304.