A Vesicular Intermediate in the Transport of Hepatoma Secretory Proteins from the Rough Endoplasmic Reticulum to the Golgi Complex

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Abstract. We have identified a vesicle fraction that contains α1-antitrypsin and other human HepG2 hepatoma secretory proteins en route from the rough endoplasmic reticulum (RER) to the cis face of the Golgi complex. [35S]Methionine pulse-labeled cells were chased for various periods of time, and then a post-nuclear supernatant fraction was resolved on a shallow sucrose-D2O gradient. This intermediate fraction has a density lighter than RER or Golgi vesicles. Most α1-antitrypsin in this fraction (P1) bears N-linked oligosaccharides of composition similar to that of α1-antitrypsin within the RER; mainly Man3GlcNAc2 with lesser amounts of Man2GlcNAc2 and Man4GlcNAc2; this suggests that the protein has not yet reacted with α-mannosidase-I on the cis face of the Golgi complex. This light vesicle species is the first post-ER fraction to be filled by labeled α1-antitrypsin after a short chase, and newly made secretory proteins enter this compartment in proportion to their rate of exit from the RER and their rate of secretion from the cells: α1-antitrypsin and albumin faster than preC3 and α1-antichymotrypsin, faster, in turn, than transferrin. Deoxynojirimycin, a drug that blocks removal of glucose residues from α1-antitrypsin in the RER and blocks its intracellular maturation, also blocks its appearance in this intermediate compartment. Upon further chase of the cells, we detect sequential maturation of α1-antitrypsin to two other intracellular forms: first, P2, a form that has the same gel mobility as P1 but that bears an endoglycosidase H-resistant oligosaccharide and is found in a compartment—probably the medial Golgi complex—of density higher than that of the intermediate that contains P1; and second, the mature sialylated form of α1-antitrypsin.

Newly made secretory proteins are localized to the lumen of the rough endoplasmic reticulum (RER). Their subsequent transport to cis Golgi vesicles is thought to be mediated by small transport vesicles, but identification and characterization of these species has proven elusive. The RER of cells specialized for secretion contains smooth segments, termed transitional zones, that are thought to be the sites of budding of vesicles (Palade, 1975) but there is no direct evidence to support this conclusion.

Exit of proteins from the RER appears to be the step at which maturation of secretory and plasma membrane proteins is regulated. In human and mouse hepatoma cells, and in the exocrine pancreas, newly made secretory proteins are exocytosed at very different rates; the rate-limiting and distinct step in intracellular maturation for each protein is its transport from the RER (Strous and Lodish, 1980; Lodish et al., 1983; Scheele and Tartakoff, 1985; Ledford and Davis, 1983; Fries et al., 1984). Many integral plasma membrane and viral surface glycoproteins mature to the cell surface at different rates and, again, the distinctive and limiting step is exit from the RER (Fitting and Kabat, 1982; Williams et al., 1985). Transport through the Golgi stack and to the cell surface occurs at essentially the same rate for all secretory and plasma membrane proteins in a given cell (Lodish et al., 1983; but see Yeo et al., 1985).

Many human genetic diseases are due to mutations in secretory or plasma membrane proteins that cause their intracellular maturation to be blocked at the level of the RER (Perlmuter et al., 1985 and references therein; Yamamoto et al., 1986). Similarly, defined alterations—either single amino acid changes or small deletions—in proteins such as vesicular stomatitis virus (VSV) glycoprotein or immunoglobulin light chains block their intracellular maturation at the level of the RER (Rose and Bergmarm, 1983; Wu et al., 1983; Machamer et al., 1985; Gallione and Rose, 1985). In yeast, amino acid substitutions in either the signal sequence or mature segment of a secretory protein can slow or block its

1. Abbreviations used in this paper: endo H, endoglycosidase H; RER, rough endoplasmic reticulum.
maturation at the level of the RER (Emr et al., 1984; Schauer et al., 1985; Hagenauer-Tsapis et al., 1986).

These and other studies have led to the notion of a receptor(s) that mediates the selective incorporation of secretory proteins into the transitional region of the RER and thus into transport vesicles destined for the Golgi complex. Alternatively, a receptor or "gatekeeper" could selectively retain certain proteins—possibly because they are abnormally folded or glycosylated—in the ER lumen. Thus, it is of some interest to identify the vesicles that transport secretory proteins from the RER. We report here the initial characterization of vesicles that have all of the expected properties of these transport intermediates.

Materials and Methods

Materials

1-Deoxynojirimycin, N-glycanase, and endoglycosidase H (endo H) were obtained from Genzyme Corp. (Boston, MA). Rabbit antiserum directed toward human albumin, transferrin, α1-antitrypsin, α1-antichymotrypsin, and C3 complement were purchased from DAKOPATTS, Copenhagen. Fixed *Staphylococcus aureus* cells were purchased from the New England Enzyme Center, Inc. (Boston, MA), and [35S]methionine was from the Radiochemical Center, Amersham Corp. (Arlington Heights, IL).

MicroPak AX-5 and AX-10 HPLC columns were purchased from Varian Associates, Inc. (Palo Alto, CA); Amberlite MB-3, α-mannosidase (Jack bean), and sodium borohydride were from Sigma Chemical Co. (St. Louis, MO); Dowex AG50-W8 was from Bio-Rad Laboratories (Richmond, CA).

Growth and Labeling of HepG2 Cells

Culture dishes of 100 mm diameter were seeded with 4 × 10⁵ HepG2 cells, and incubated at 37°C in MEM (GIBCO, Grand Island, NY) supplemented with 10% FCS (Schwartz et al., 1981). Cultures were generally fed on the second day after seeding and used on the fifth, at which time the cells had approximately doubled. Similar to our previous study (Lodish and Kong, 1984), the cells were washed once in methionine-free growth medium, placed in 2.0 ml methionine-free growth medium (containing 10% dialyzed FCS), and incubated at 32°C. After 20 min, 150 μCi [35S]methionine was added, and the culture incubated for a further 5 min (pulse). The plates were washed once with chase medium and 5 ml chase medium (growth medium plus 10% FCS and 1 mM methionine) was added to each. After incubation at 32°C for the appropriate time (chase), the cells were placed at 0–4°C, washed three times in complete PBS, and scraped from the dish in ice-cold PBS.

Homogenization and Gradient Analysis

All procedures were conducted at 0–4°C. The pooled cell pellets were resuspended in 5 vol homogenization buffer (0.01 M Hepes, pH 7.4, 0.25 M sucrose), and allowed to swell for 10 min. They were homogenized with 15 strokes of a tight-fitting Dounce homogenizer, resulting in over 85% cell breakage. Nuclei were removed by centrifugation at 1,000 rpm for 10 min. Of the postnuclear supernatant, 2.0 ml was layered on a gradient for the SW41 Beckman rotor, consisting of 1 ml of each of the following sucrose solutions (wt/vol) all in D2O containing 10 mM HEPES, pH 7.4: 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, and 50%. After centrifugation at 4°C for 3 h at 36,000 rpm, the gradient was fractionated into 32 0.15 ml fractions using an ISCO gradient collector.

Immunoprecipitations

To 50 μl of each gradient fraction, pooled pairwise, was added 5 μl normal rabbit serum and 300 μl Tris-buffered saline (Owen et al., 1980) containing 1% sodium deoxycholate and 1% NP-40 (buffer 1). After incubation at 4°C for 1 h, the mixture was pre-cleared by addition of 50 μl of a 10% suspension of *S. aureus* cells (Owen et al., 1980). After centrifugation to remove the bacteria, 5 μl of specific antiserum was added. After a 2-h overnight incubation at 4°C, 50 μl of a 10% suspension of *S. aureus* was again added. After a 1-h incubation at 4°C, the immunoprecipitate was recovered by centrifugation in a microfuge, washed twice in buffer 2 (0.142 M NaCl, 0.24 M KCl, 0.008 M Na2HPO4; 0.0004 M KH2PO4, 0.5% sodium deoxycholate, 1% Triton X-100), resuspended in 50 μl gel sample buffer containing SDS, and boiled. Analysis was by electrophoresis through 10% polyacrylamide gels.

Controls established that these conditions were sufficient to recover 85% of the labeled protein in question. In particular, <10% additional labeled protein was recovered if the first supernatant from the immunoprecipitation was reacted with additional antiserum. In some cases, the immunoprecipitate was split into two portions; one was digested with endo H and the other mock digested, as detailed previously (Zilberstein et al., 1980).

Labeling with [3H]Mannose

One 90% confluent 100-mm plate of HepG2 cells was washed twice with glucose-free MEM, and placed in 2.0 ml glucose-free MEM containing 10% dialyzed FCS and 0.02 mg/ml glucose. After incubation at 32°C for 15 min, 1.0 ml: [3H]Mannose (a gift from Dr. P. Robbins, Massachusetts Institute of Technology) was added, and incubation continued for an additional 40 min. Homogenization and gradient analysis were as detailed above. For oligosaccharide analysis, 200 μl each of fractions 16–18 of the gradient were pooled as the "light vesicles," as were 200 μl each of fractions 25–29 (RER). To each pool, 2.0 ml buffer 1 and 30 μl normal rabbit serum was added. After a 1-h incubation, 300 μl of a 10% *S. aureus* suspension was added, and the mixture incubated overnight. After centrifugation, 20 μl anti-α1-antitrypsin was added to the supernatant. After a 2-h incubation at 4°C, 300 μl of a 10% *S. aureus* suspension was added. The bacterial pellet was recovered by centrifugation, washed twice in buffer 2, and resuspended in 200 μl of a solution containing 0.2 M NaPO4, pH 8.6, 0.5% SDS, and 1% 2-mercaptoethanol. The mixture was boiled for 3 min and centrifuged. The supernatant was used for analysis of oligosaccharide size and charge. Upon electrophoresis in a 10% SDS gel only one band, of mobility P (Fig. 1), was seen, and over 90% of the [3H]radioactivity in this immunoprecipitate was released by endo H digestion (not shown).

To the supernatant, sufficient 0.2 M sodium phosphate, pH 8.6, was added to decrease the SDS concentration to 0.2%. After addition of NP-40 to a final concentration of 1.4%, the sample was treated for 18 h at 37°C with N-glycanase (12.5 U/ml) which had previously been dialyzed against the above buffer.

At the end of the incubation period, 3 vol of ice-cold ethanol were added to the reaction. The sample was centrifuged (5,000 g) for 10 min and the pellet was washed with 2–3 ml of 75% ethanol. The combined supernatants were evaporated by a stream of N2. To the residue 0.3 ml of 0.2 M sodium borate, pH 9.8, and 20 μl of 0.1 M NaBH4 were added and incubated at 30°C for 4 h. The reaction was terminated by acidifying the mixture with 1 M acetic acid. The reaction mixture was passed through a column (4 ml bed volume) of Dowex AG50 and the column was washed with four bed volumes of distilled water. The eluate and washings were combined and evaporated. An aliquot of the sample was analyzed on a MicroPak AX-10 column using published procedures (Baenziger and Natowicz, 1981). Since no charged oligosaccharide species were detected, the remainder of the sample was desalted with Amberlite MB-3 (1 ml column) and analyzed on MicroPak AX-5 (Mells and Baenziger, 1981).

Standard high mannose-type oligosaccharides bearing 5–9 mannose residues were prepared from ribonuclease B (Hirani, S., and J. Rasmussen, manuscript in preparation).

α-Mannosidase Digestion

Digestion with Jack bean α-mannosidase was performed in 0.1 M sodium acetate buffer, pH 5.0, containing 0.4 mM Zn2+. The reduced oligosaccharides were incubated with 1 U of the enzyme for 24 h at 37°C. The digestions were terminated by boiling for 2 min and desalted by passing over a column of Amberlite MB-3 (1 ml) in water. The digests were analyzed on MicroPak AX-5 before.

Results

Intracellular Maturation of Secretory Proteins in HepG2 Cells

In this study we used the human hepatoma cell HepG2, which synthesizes and secretes a number of well-character-

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Intracellular forms of $\alpha$-antitrypsin. Four 100-mm plates of HepG2 cells were labeled with $[^{35}\text{S}]$methionine for 5 min at 32°C, then chased for 25 min at 32°C. As detailed in Materials and Methods, 2.0 ml of the 50-ml postnuclear supernatant was resolved on a D$_2$O–sucrose gradient. From pairwise-pooled gradient fractions, $\alpha$-antitrypsin was recovered by immunoprecipitation. Half of each fraction was digested with endo H (+) while half was mock digested (−). The fraction number is that of the first of the two fractions pooled, and the numbers run from least to most dense (see Fig. 2). M and P denote the mature and precursor forms, respectively, in the nondigested samples; P1 and P2 denote the endo H–sensitive and –resistant forms generated from P by digestion with endo H. As noted in the text, we also use P1 and P2 to denote that portion of P (before endo H digestion) that is sensitive and resistant, respectively, to endo H. Samples 25 and 26 were lost from this analysis. We do not know the identity of material that is in fractions 29 and 31 with gel mobility of M but that is sensitive to endo H.

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Figure 1. Intracellular forms of $\alpha$-antitrypsin. Four 100-mm plates of HepG2 cells were labeled with $[^{35}\text{S}]$methionine for 5 min at 32°C, then chased for 25 min at 32°C. As detailed in Materials and Methods, 2.0 ml of the 50-ml postnuclear supernatant was resolved on a D$_2$O–sucrose gradient. From pairwise-pooled gradient fractions, $\alpha$-antitrypsin was recovered by immunoprecipitation. Half of each fraction was digested with endo H (+) while half was mock digested (−). The fraction number is that of the first of the two fractions pooled, and the numbers run from least to most dense (see Fig. 2). M and P denote the mature and precursor forms, respectively, in the nondigested samples; P1 and P2 denote the endo H–sensitive and –resistant forms generated from P by digestion with endo H. As noted in the text, we also use P1 and P2 to denote that portion of P (before endo H digestion) that is sensitive and resistant, respectively, to endo H. Samples 25 and 26 were lost from this analysis. We do not know the identity of material that is in fractions 29 and 31 with gel mobility of M but that is sensitive to endo H.

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Figure 2. Subcellular distribution of HepG2 secretory proteins after a 5-min pulse or after a 25-min chase. Four plates of HepG2 cells were labeled with [35S]methionine for 5 min (pulse) or for 5 min followed by a 25-min chase with unlabeled methionine. (These are the same samples used in Fig. 1). After D2O-sucrose gradient analysis, specific labeled proteins were recovered by immunoprecipitation, and analyzed by SDS gel electrophoresis and autoradiography. As in Fig. 1, α1-antitrypsin immunoprecipitates were analyzed further by endo H digestion. The autoradiograms were scanned with an integrating LKB laser microdensitometer, and the areas under each peak recorded. Plotted is the fraction of labeled protein in each paired fraction, relative to the total recovered from the gradient. In most cases, several exposures of the film were scanned and/or several amounts of each sample analyzed, to be sure that the recorded area was linearly proportional to the amount of labeled protein in the gel band. (a) Distribution of labeled α1-antitrypsin after a 5-min pulse. (b) Distribution of the three forms of α1-antitrypsin after a 25-min chase. These forms are depicted in Fig. 1. The total amount of the labeled M form is normalized to 100%; the total of the P1 and P2 forms of P is also normalized to 100%. The actual ratio of radioactivity in M to P (i.e., to P1 plus P2) was as 1:6. (Solid circles) P1; (open squares) P2; (solid triangles) M. (c) Distribution of labeled transferrin after the pulse or pulse-chase. (d) Distribution of labeled albumin after a pulse or pulse-chase.

from vesicles, and is ignored in the following analysis. The most dense fractions (27-32), at the 30–50% sucrose interface, contain α1-antitrypsin, labeled P in Fig. 1, that co-migrates with α1-antitrypsin labeled after a 5-min pulse (not shown). All of this material is completely sensitive to endo H, generating a form labeled P1 (lanes 27+, 29+, and 31+). We will also use the term P1 to denote that portion of α1-antitrypsin of gel mobility P (before endo H digestion) that generates the P1 form upon endo H digestion. As expected from our previous work (Lodish et al., 1983), in cells labeled for 5 min but not chased, all labeled α1-antitrypsin is in the P1 form and is in these RER fractions (Fig. 2 A); i.e., it migrates as P before and as P1 after endo H digestion.

In cells labeled for 5 min and chased for 25 min, the P1 form of α1-antitrypsin is also found in low density membrane (12.5 to 17.5% sucrose, fractions 11-16 of Fig. 1 and Fig. 2 B). As detailed below, this material defines the RER-Golgi transport intermediate. More dense membranes (22% sucrose, fractions 17-22) contain a form of α1-antitrypsin that co-migrates with the ER precursor, P, but that is essentially resistant to endo H generating the form labeled P2 (Fig. 1, lanes 17+, 19+, and 21+). We also use P2 to refer to that portion of P that generates P2 after endo H digestion. Intermediate P2 represents a later stage of maturation than P1, in either RER or light vesicles and presumably is material that has passed through the medial Golgi complex (wherein is localized α-mannosidase II, the enzyme whose action renders the oligosaccharide resistant to endo H; reviewed in...
Figure 3. Kinetics of formation of intermediates in maturation of α1-antitrypsin. Two plates of HepG2 cells were pulse-labeled with [35S]methionine for 5 min; two were pulsed and chased for 12.5 min; two for 25 min, and two for 40 min, all at 32°C. As in Fig. 1, a postnuclear supernatant was analyzed on a D2O-sucrose gradient. α1-Antitrypsin was recovered from paired fractions by immunoprecipitation, and digested with endo H. The amounts of the three forms of α1-antitrypsin (P1, P2, and M) were determined by scanning the autoradiograms, as in Fig. 2. The total amount of labeled α1-antitrypsin (i.e., P1 plus P2 plus M) recovered from each of the four gradients was set at 100%. The "light vesicles" are fractions 9-16, and the RER fractions 27-32. (Fig. 4 shows a further analysis of these profiles.) The % sucrose (wt/wt) in gradient fractions is shown in the top panel, as determined from the refractive index.

Kornfeld and Kornfeld, 1985). Finally, the slowest migrating intracellular α1-antitrypsin, M, co-migrates with the secreted form, is resistant to endo H, and contains sialic acid (as evidenced by sensitivity to neuraminidase; not shown). Clearly this material is in or has passed through the trans face of the Golgi complex, the locale of sialyltransferase (reviewed in Kornfeld and Kornfeld, 1985). The mean density of vesicles containing M is greater than that of the light vesicles containing P1, but lighter than those with intermediate P2 (Fig. 2 B).

When similar gradients were centrifuged for 24 h to equilibrium, there was no change in the profile of α1-antitrypsin (or albumin or transferrin) within the gradient itself (fractions 7 to the bottom). The only difference from Figs. 1 and 2 was that the released proteins sedimented farther into the gradient with a peak at about fraction 9 (data not shown). Thus, the relative densities noted above for light and RER-containing vesicles P1 and vesicles containing P2 and M are equilibrium values.

Kinetics of Maturation of α1-Antitrypsin

The study in Fig. 3 establishes that the intracellular intermediates of α1-antitrypsin maturation are formed sequentially, in the order P1 (RER) → P1 (light vesicle) → P2 → M → exocytosis. After a 5-min pulse, all labeled α1-antitrypsin is the P1 form, and (neglecting released material at
Figure 4. Kinetics of movement of secretory proteins from the RER to the "light vesicles." The gradients of Fig. 3 were analyzed for labeled transferrin (a), α1-antichymotrypsin (c), albumin (d), and pre C3 (e) as well as for α1-antitrypsin P1 (b, as depicted in Fig. 3). In calculating the subcellular distribution of the labeled proteins, only fractions 7-32 (i.e., the gradient proper minus released protein) were used. Plotted is the fraction of each labeled protein in the "light vesicles" (Nos. 9-16) and RER (Nos. 27-32) relative to the total labeled protein in fractions 7-32.

The top of the gradient is exclusively in the RER fractions at the 30-50% sucrose interface. After a 12-min chase, no labeled P2 or M forms have been formed. Importantly, ~18% of the intracellular labeled α1-antitrypsin has moved to light vesicles (Fig. 3, see the lower panel; see also Fig. 4) and is still in the P1 (endo H-sensitive form). After a 25-min chase, both P2 and M forms appear. Note that P2 is always in a more dense fraction than is the "light vesicle" P1. The amount of M increases from 25 min of chase to 40 min, while the amount of P2 and "light vesicle" P1 decrease, thus documenting the above order.

The Asparagine-linked Oligosaccharides of "Light Vesicle" P1 α1-Antitrypsin

In the study depicted in Fig. 5, cells were labeled for 40 min with [3H]mannose, and α1-antitrypsin was isolated from RER and "light vesicle" fractions using sucrose gradients similar to those of Fig. 1. The kinetics of labeling α1-antitrypsin with [3H]mannose are nonlinear with time; about eight times more radioactivity is found in this protein after a 40-min labeling period than after 20 min of labeling (not shown). Probably this is due to a large pool of mannose or

Figure 5. Analysis of RER and "light vesicle" α1-antitrypsin asn-oligosaccharides by HPLC on a MicroPak AX-5 column. Immunoprecipitated α1-antitrypsin from gradient fractions 27-31 (a) and 11-16 (b) were treated with N-glycanase (12.5 U/ml) for 18 h at 37°C. After addition of ethanol to precipitate protein, the supernatants were concentrated, dissolved in sodium borate, pH 9.8, and reduced with NaBH4. After acidification with acetic acid, the samples were concentrated, desalted with Amberlite MB3, and analyzed by HPLC on a MicroPak AX-5 column (see Materials and Methods). The standard markers are as follows: M0, Man9GlcNAc2Man9GlcNAc2; M1, Man9GlcNAcMan9GlcNAc2; M2, Man9GlcNAcMan9GlcNAc2; M3, Man9GlcNAcMan9GlcNAc2; M4, Man9GlcNAcMan9GlcNAc2; M5, Man9GlcNAcMan9GlcNAc2.
mannose derivatives. This means that most of the α1-antitrypsin was labeled during the last 20 min. Indeed, all of the [3H]-labeled protein in both fractions had the P mobility on SDS gels, and over 90% of the [3H]radioactivity was released by endo H (not shown).

As judged by HPLC analysis on an AX-10 column, all of the [3H]-oligosaccharide released by N-glycanase from the RER form of α1-antitrypsin fractionated with uncharged (i.e. sialic acid-deficient) oligosaccharides (not shown). On an AX-5 sizing column, most of the [3H]radioactivity co-migrated with Man9GlcNAc2 (Fig. 5 a). Smaller amounts of Man5GlcNAc2 and Man6GlcNAc2 were present, but there were no smaller oligosaccharides. This distribution is characteristic of the RER forms of many proteins – both those that are transiently in the RER before maturation in the Golgi complex and those that are resident RER proteins (see Kornfeld and Kornfeld, 1985).

Of the [3H]radioactivity released from the “light vesicle” form of α1-antitrypsin by N-glycanase, 60% was Man9GlcNAc2, Man10GlcNAc2, or Man11GlcNAc2 (Fig. 5 b). Jack bean α-mannosidase treatment degraded these high mannose oligosaccharides to mannose and Man-GlcNAc2 (not shown). Thus, at least 60% of the α1-antitrypsin in this fraction has oligosaccharides characteristic of the RER. Similar results (not shown) were obtained from cells labeled for 30 min with [3H]mannose. Clearly these oligosaccharides have not yet reacted with the α-mannosidase I at the cis face of the Golgi complex, the enzyme that converts the oligosaccharides to a Man9GlcNAc2 intermediate. About 40% of the released radioactivity migrated with Man6GlcNAc2 or with smaller oligosaccharides and was resistant to α-mannosidase digestion (not shown). The exact structure of these has not been determined, but their properties suggest that they have been subject to additional α-mannosidase reaction, either in the RER (Bischoff et al., 1986) or at the cis side of the Golgi complex. Clearly, these “light vesicle” fractions are not pure, and probably contain α1-antitrypsin in other organelles such as the Golgi complex.

**Rate of Maturation of Secretory Proteins from the RER to the “Light Vesicle” Intermediate**

As noted, different secretory proteins exit from the RER at very different rates. The experiment in Fig. 4 shows that secretory proteins enter the “light vesicle” fraction in proportion to their rate of movement from the RER. Though the resolution of this experiment is compromised by the relatively large region of the sucrose gradient that is denoted “light vesicles,” and by the fact that this fraction is in no way pure, it does suggest that this “light vesicle” fraction is the first post-RER intermediate.

Even after a 40-min chase, there is no change in the amount of labeled transferrin in the RER, and <4% is found in the “light vesicle” fraction (Fig. 5 a; see also Fig. 2 c). This is consistent with previous findings that transferrin exits the RER with a half-time in excess of 120 min (Lodish et al., 1983). By contrast, the amount of labeled pre-C3 and α1-antichymotrypsin in the “light vesicle” fraction increases during the 40-min chase from a background value of ∼4% to a maximum of 8–12% (c and e). This rate of movement to light vesicles is faster than that of transferrin, but slower than that of albumin or α1-antitrypsin (b and d; see also Fig. 2 d) consistent with their intermediate rate of exit from the RER (see Lodish et al., 1983).

All of the labeled α1-antichymotrypsin in the “light vesicle” fraction co-migrates with the material recovered from the RER, and yields the identical faster-migrating species after endo H digestion (not shown). Because of the low level of labeling with [3H]mannose, we have not attempted to determine the oligosaccharide structure on the various forms of α1-antichymotrypsin. However, this result is consistent with the notion that α1-antichymotrypsin in this “light vesicle” fraction represents a post-RER, pre-cis Golgi species, analogous to that of α1-antitrypsin. As endo H digestion of pre-C3 does not affect its gel mobility, and as albumin is not glycosylated, intracellular forms of these proteins have not been analyzed. All intracellular transferrin has oligosaccharides Man-GlcNAc2, Man5GlcNAc2, Man6GlcNAc2, and GlcMan6GlcNAc2, as shown previously (Lodish et al., 1983).

Fig. 4 also proves that the “light vesicles” are not pieces of RER vesicles that have lost their ribosomes. If that were the case, all labeled secretory proteins would be found in these vesicles in the same proportion they are found in the RER, and that certainly is not the case.

**Entry of α1-Antitrypsin into “Light Density” Transport Vesicles Is Regulated**

Previously we showed that rapid movement of α1-antitrypsin and α1-antichymotrypsin from the RER to the Golgi complex requires removal of three glucose residues from each high mannose asn-linked oligosaccharide (Lodish and Kong, 1984; see also Gross et al., 1983). The drug 1-deoxynojirimycin, an inhibitor of glucosidase II (Saunier et al., 1982), causes accumulation of the Glc1,3Man5GlcNAc2 processing intermediate of asn-linked oligosaccharides. It has no effect on secretion of albumin, transferrin, or C3 but it dramatically slows secretion of α1-antitrypsin and α1-antichymotrypsin, and causes glucosylated α1-antitrypsin and α1-antichymotrypsin to accumulate in the RER (Lodish and Kong, 1984). Fig. 6 shows that pretreatment of cells with 1-deoxynojirimycin totally blocks maturation of pulse-labeled α1-antitrypsin into “light density” transport intermediates during a 12-min chase (Fig. 6 a). As expected, it has no effect on movement of albumin (Fig. 6 b). Also as expected, transferrin does not move to these vesicles whether or not the drug is added (Fig. 6 c). This study confirms our conclusion that entry of secretory proteins from the RER into this vesicle fraction is regulated, and appears to be the distinctive and rate-limiting step in eventual secretion of the protein from the cell.

**Discussion**

We have identified a novel vesicle fraction, of light density (∼15% sucrose in D2O) that appears to be an intermediate in transport of α1-antitrypsin and other hepatoma secretory proteins from the RER to the Golgi complex. The key to the discovery of this fraction appears to be shallow sucrose gradients in D2O, which were used to resolve the postnuclear fractions of human hepatoma HepG2 cells pulse-labeled with [35S]methionine or chased with unlabeled methionine for various periods of time.

Several pieces of evidence demonstrate that these vesicles are not fragments of the RER, and are the first post-ER inter-
mediates detectable: after a 5-min pulse, no labeled secretory proteins are localized in this fraction; all co-purify with RER (Figs. 2–4). During the chase, radiolabeled secretory proteins enter this fraction in proportion to their previously determined rate of exit from the RER (Fig. 4) and eventual secretion from the cell (Lodish et al., 1983). The density of this fraction is lighter than the RER, and lighter also than vesicles that contain later Golgi-processing intermediates termed P2 and M (Fig. 2). When banded to equilibrium in H2O-sucrose gradients (data not shown), these vesicles have a density of 1.115 g/cm3, much lighter than the vesicles that contain P2 (1.14 g/cm3) or those that contain M (1.12 to 1.14 g/cm3). Finally, most of the α1-antitrypsin in this fraction has an oligosaccharide composition similar to that of the protein in the RER (Fig. 5).

Considerable evidence also indicates that secretory proteins in this vesicle fraction have not yet passed through the cis face of the Golgi complex. First, the majority of α1-antitrypsin in these vesicles is sensitive to digestion with endo H, and bears asn-linked oligosaccharides of structure identical to that on RER-localized protein (Fig. 5). In particular, at least 60% of the labeled α1-antitrypsin has not been acted upon by Golgi α-mannosidase-I, the enzyme that trims asn-linked oligosaccharides to Man9GlcNAc2. Since deoxynojirimycin-sensitive RER α-mannosidase can trim high mannose oligosaccharides to Man6GlcNAc2 (Bischoff et al., 1986), the profile of Fig. 5 b is consistent with the possibility that more than 60% of the α1-antitrypsin in this fraction has not yet seen Golgi α-mannosidase I. The localization of Golgi α-mannosidase I to the cis subcompartment is not definitive, but most recent work is consistent with this assignment (see Kornfeld and Kornfeld, 1985). Importantly, in fibroblasts definitive marker enzymes from the cis face of the Golgi complex (e.g., N-acetylgalcosaminylphosphotransferase) are in more dense vesicles than are those that contain definitive markers of the medial Golgi complex (N-acetylgalcosaminyltransferase I) (Goldberg and Kornfeld, 1983; Dunny and Rothman, 1985). However, HepG2 cells have not yet been subjected to this type of analysis, nor have these enzymes been localized to vesicles (from fibroblasts or hepatoma cells) resolved by our D2O-sucrose gradients. Our putative RER-Golgi transfer vesicles are lighter in density than the medial vesicles that contain the more mature P2 form of α1-antitrypsin (Figs. 1 and 2 b). This latter form co-migrates on SDS gels with the form of α1-antitrypsin found in the RER and "light-vesicle" fractions, but is resistant to digestion with endo H. Though we have not determined the oligosaccharide on P2, it is evident that it has already passed through the medial Golgi complex. Since equilibrium density centrifugation of Golgi vesicles forms a variety of cells that invariably yields the rank order trans lighter than medial lighter than cis (e.g., Goldberg and Kornfeld, 1983), it is fair to conclude that the light hepatoma vesicles that contain the P1 form of α1-antitrypsin are pre-cis Golgi vesicles. This conclusion must be considered tentative until Golgi α-mannosidase I is definitively localized to the cis face of the Golgi complex. In any case, these vesicles are the most proximal to the RER of any compartment in the secretory pathway yet detected by subcellular fractionation.

The ER is a continuous network of cisternae that is continuous with the outer nuclear envelope, and upon homogenization is sheared into smaller, sealed, microsomal vesicles. It is possible that our intermediate vesicles are generated during homogenization from the smooth transitional zone of the ER (Palade, 1975) or from other smooth regions of ER, and do not exist, as such, in the cell.

In the introduction, we summarized much of the evidence that indicates that exit from the RER of secretory and plasma
membrane glycoproteins is the rate-limiting and distinctive step in their intracellular maturation. Though it is not known what factors regulate the maturation of different wild-type or mutant proteins, much work suggests that, at a minimum, the protein needs to acquire its mature tertiary or quaternary membrane glycoproteins is the rate-limiting and distinctive step in intracellular maturation of hepatoma secretory proteins (Fig. 4). Further, an inhibitor of RER α-glucosidase blocks the secretion of α-antitrypsin and α-antichymotrypsin at the level of the RER (Lodish and Kong, 1984); it also blocks entry of these proteins into the light density fraction (Fig. 6 and data not shown).

It is obviously necessary to purify and characterize these novel light density transport vesicles in more detail. Much recent work suggests that they should not bear a clathrin coat (Payne and Scheckman, 1985), but otherwise it is unknown what protein(s) might be on their surface that could direct or target them to their cis Golgi destination. Also, it may be possible to detect the formation of these vesicles from RER vesicles in vitro, and their fusion with the cis face of the Golgi complex much in the way Rothman and his colleagues showed that in vitro, vesicles can bud from one Golgi compartment and fuse with a successive one (reviewed by Dunphy and Rothman, 1985).

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