The Moran Foundation
DEPARTMENT OF PATHOLOGY
BAYLOR COLLEGE OF MEDICINE
TEXAS MEDICAL CENTER
HOUSTON, TEXAS 77030

June 23, 1992

Richard N. Sifers, Ph.D.
Department of Pathology
Baylor College of Medicine

Dear Dr. Sifers:

Please update me on the status of your Moran Foundation project (1-91-0052) entitled "Characterization of a Chaperone Protein Associated with Normal and Malfolded Variants of Human Alpha-1 Antitrypsin".

Since approval and funding is generally for a one-year period, all projects approved in or prior to June 1991 should now be "complete", or nearly so.

I need a progress and/or final report regarding your project, including dates and times of any presentations, and information regarding any publications.

Please submit this to me within the next 30 days.

Sincerely yours,

Philip J. Migliore, M.D.
Research Director

PJM/ms

c: Dr. Michael Lieberman
Mr. John Moran

FINAL REPORT for Moran Foundation Project (1-91-0052)

Date: 6/25/92

Project Title: "Characterization of a Chaperone Protein Associated with Normal and Malfolded Variants of Human Alpha1-antityrpsin"

Investigator: Richard N. Sifers, Ph.D.
Assistant Professor
Department of Pathology

PROGRESS REPORT:

Co-immunoisolation and identification of the molecular chaperone bound to the Z variant of human alpha1-antitrypsin (AAT). Our results indicate that the interaction between the molecular chaperone and the Z variant polypeptide is very weak and easilly disrupted. Detergents, temperature, pH, and salt can interfere with the association between these molecules. After many attempts, we were able to identify very low stringency conditions to immunoisolate the chaperone protein along with the Z variant. Using several criteria, we have now identified the chaperone as immunoglobulin heavy chain binding protein (BiP). This macromolecule is a major constituent of the endoplasmic reticulum in all cells and associates with many newly synthesized malfolded secretory proteins in the endoplasmic reticulum. It is becoming more evident that BiP is somehow utilized in catalyzing the folding of newly synthesized proteins in the endoplasmic reticulum. Also, BiP is able to target malfolded polypeptides for degradation in the cell.

We are the first to identify an association between BiP and the Z variant. This finding begins to explain the mechanism used for retaining the protein in the endoplasmic reticulum which ultimately leads to the development of cirrhosis.

Several molecules of BiP are bound to a single Z variant polypeptide. Using an improved method for sedimentation velocity centrifugation studies, we have observed multiple BiP-Z variant complexes appear following the formation of the complex. It is likely that these various forms reflect the binding of multiple molecules of the chaperone (estimated to be three molecules) to a single Z variant polypeptide. This is the first account of actually quantitating the stoichiometry of BiP binding to a polypeptide.

The retained BiP-Z variant complex dissociates in vivo prior to intracellular degradation of the Z variant polypeptide. Molecules of BiP begin to dissociate from the Z variant complex about 30 minutes following synthesis of AAT in the endoplasmic reticulum. Release of BiP from the Z variant is absolutely required before the polypeptide is degraded intracellularly (i.e., in the endoplasmic reticulum). Thus, the dissociation of the Z variant from BiP regulates the rate of its entrance into the intracellular degradative pathway.

Cation-dependent hydrolysis of ATP causes the release of the Z variant from BiP. It is known that binding and also dissociation of BiP from various polypeptides requires a magnesium-dependent ATPase activity that is inherent to the BiP macromolecule. Furthermore, this

activity is inhibited by calcium ions. Indeed, we have determined that the BiP-Z variant complex dissociates when cells are lysed in the presence ATP plus $MgCl_2$, and that the chelation of calcium ions accelerates this process.

Depletion of intracellular calcium has no effect on the release of the Z variant from BiP. To determine whether calcium in the ER might somehow regulate the dissociation of the Z variant from BiP, we manipulated intracellular calcium levels using calcium ionophores and thapsigargin. These manipulations had no effect on the dissociation of the Z variant from BiP and suggest that calcium is most likely not utilized in regulating the dissociation process *in vivo*.

BiP is used to retain a truncated variant of human AAT and catalyze the folding of the normal M1 polypeptide. The truncated $Null_{Hong\ Kong}$ variant of human AAT also binds BiP in a manner identical as that used for the Z variant. Thus, BiP-binding is a general mechanism for retaining mutant forms of AAT in the endoplasmic reticulum.

Using short-term metabolic radiolabeling of cells plus immunoisolation techniques, we have verified that three molecules of BiP associate with the normal M1 variant of human AAT for approximately 2 minutes. During that period, BiP catalyzes the folding of the AAT polypeptide and then releases it for its secretion from the cell.

The results of these studies have allowed us to identify the mechanism used for (1) catalyzing the folding of human AAT and (2) targetting malfolded mutant variants for degradation. Both of these findings are major accomplishments in the fields of intracellular protein transport and alpha1-antitrypsin deficiency.

PRESENTATIONS AND PUBLICATIONS:

Presentation at the Gordon Conference 6/30/92:

Le, A., and Sifers, R.N. "Degradation of the Z Alpha1-antitrypsin Variant in the Endoplasmic Reticulum". This abstract will not be published.

Publications:

We are in the process of writing a manuscript that deals with the identification and characterization of the BiP-Z complex. The manuscript will be submitted to the *Journal of Biological Chemistry*. As always, I will acknowledge the support of the Moran Foundation and will send a reprint of the article to the Scientific Advisory Committee, following its publication.

I also want the members of the Scientific Advisory Committee to be aware that I was recently chosen to write a "News and Views" article for *Nature* because of recent findings in the field of alpha1-antitrypsin deficiency. This is truly an honor for me. Although no mention of funding sources were permitted in this article, I am fully aware that support from the Moran Foundation is responsible for my selection as the author. Therefore, I am sending a copy of that article along with this final report.

PROTEIN TRANSPORT

Z and the insoluble answer

Richard N. Sifers

ACCUMULATION in the liver of the Z variant of human α_1-antitrypsin can lead to cirrhosis, but the structural identity of the accumulated protein has been unknown. On page 605 of this issue[1], Lomas *et al.* unveil a totally unexpected finding — the Z variant forms insoluble homopolymers in the endoplasmic reticulum of the liver, and does so by a well-defined mechanism.

Human α_1-antitrypsin (AAT) is a single, folded polypeptide that inhibits the action of serine proteases. Although the macromolecule is one of the main components of serum, its predominant physiological role is to prevent degradation of elastin fibres in the lung. It accomplishes this task by forming a tight complex with elastase, a protease secreted from neutrophils in the lung, which ultimately inhibits its hydrolytic activity[2]. Heritable forms of AAT deficiency are most often associated with the synthesis of a variant macromolecule, which bears a mutation in the polypeptide[3] that somehow hinders its ultimate secretion from hepatocytes[4] (the principal site of synthesis). Decreased secretion lowers the level of serum AAT, and also the total elastase inhibitory activity in the lung. The resulting degradation of elastin fibres is implicated in emphysema[2].

A subset of AAT variants accumulates within the endoplasmic reticulum of the liver, and is associated with cirrhosis[5]. One such variant, designated Z, contains a glutamate-to-lysine substitution at residue 342 (ref. 2). Because of its frequency in the population, and the severity of its impaired secretion, the Z variant has served as a prototype for analysing intrahepatic protein retention and accumulation.

Discovery of the Z-variant accumulation mechanism resulted from analysis of a structural feature shared by this and other members of a class of serine protease inhibitors known as serpins[6]. Briefly, the reactive inhibitory site of serpins centres on two amino acids in a 14-amino-acid loop at the surface of the folded polypeptide[6,7]. For AAT and other inhibitory members of this family, amino-terminal peptides of the reactive centre loop are inserted into a gap in the major structural feature of the folded macromolecule, β-pleated sheet A (see Fig. 2 of the paper of Lomas *et al.*, page 606). This 'stressed' conformation is required for the reactive centre to function as a substrate to react with and inhibit targeted proteases. Indeed, the absence of inhibitory activity for some members of the serpin family may result from the substitution of amino acids within the reactive centre loop, thereby preventing its partial insertion into sheet A and the formation of the stressed conformation[7].

A clue to the mechanism of Z accumulation in liver was reported by Carrell *et al.*[8] last year, following their observation that under mild denaturing conditions the entire reactive centre-loop peptide of an intact inhibitor, such as AAT, could 'lock' into sheet A. This event is akin to the major structural rearrangement of AAT when the loop has been relaxed by proteolytic cleavage at its reactive inhibitory site and actually becomes strand 4 of the sheet. As described in the new report[1], incubation of the purified, secreted form of the normal M variant under these conditions led to its unexpected polymerization *in vitro*. Polymerization between macromolecules pre-bound with a synthetic peptide homologous to the reactive centre loop, which had inserted into sheet A, did not occur. This and subsequent analyses implied that polymerization under these conditions results from the stable insertion of the mobile reactive centre loop of one molecule into sheet A of another. Increased polymerization of the Z variant (that is, spontaneous polymerization under nondenaturing conditions) was then observed *in vitro*, as the authors had predicted. This prediction was based on the ability of the glutamate-to-lysine substitution at the hinge region of the reactive centre loop to prevent its insertion into sheet A, thereby leaving it in an 'opened' conformation ready to accept the appropriate loop from another Z molecule.

Confirmation that loop-sheep polymerization of the Z variant does occur in liver endoplasmic reticulum comes from the striking electron micrographs showing tangled polymers of the macromolecule purified from human liver biopsies (see Fig. 3, page 606). Furthermore, the morphology and physical characteristics of the liver-derived Z polymers are identical to those of the serum-derived protein polymerized *in vitro*. The authors did not test the effect of nonspecific peptides on *in vitro* polymerization of either the M or Z variant, but their hypothesis of loop-sheet polymerization is borne out by physical and biochemical analyses.

The tendency of the secreted Z variant to undergo spontaneous aggregation[9] led most, if not all, researchers in the field to conclude that a similar mechanism was responsible for its retention and accumulation in the liver endoplasmic reticulum. But this conclusion was never quite secure because it could not explain why the macromolecule isolated from liver inclusion bodies could exhibit full protease inhibition[10]. Conceivably, a randomly aggregated molecule would be grossly misfolded and therefore display little, if any, inhibitory activity after isolation. The evidence for the accumulation of polymerized Z protein now satisfies this discrepancy.

In terms of protein retention in the endoplasmic reticulum, it is reasonable to conclude that polymerization of the Z variant could dramatically hinder its export from that compartment, as the

Out of the frying pan

THE Saharan silver ant *Cataglyphis bombycina* treads a narrow line between heat exhaustion and being eaten by lizards. As R. Wehner *et al.* demonstrate on page 586 of this issue, the ants prefer to run the risk of the former than face the near-certainty of the latter. Most desert creatures, lizards included, seek shade when surface temperature exceed about 45 °C. In response, the ants emerge to forage only when temperatures rise above 46.5 °C, and can tolerate temperatures of up to about 53.6 °C. Confined to this narrow thermal 'window', they operate at the limits of their tolerance and adopt additional strategies to minimize the heat burden. The ant pictured here, for example, is resting on a stalk of grass where the ambient temperature is slightly less intense than that at ground level. H. G.

authors suggest. But one must now consider a new question — quite simply, is loop-sheet polymerization used for retaining all secretion-impaired AAT variants or is there an alternative mechanism in some cases? Because most secretion-impaired variants do not show any evidence of accumulating in hepatocytes[3,4], the second hypothesis has to be valid. For example, how else can one explain the retention mechanism employed for the truncated $Null_{Hong\ Kong}$ variant, in which the entire reactive centre loop is missing[11]?

Overall, given that molecular chaperones are increasingly implicated in catalysing the folding and causing the retention of a variety of secretory and membrane-associated polypeptides in the liver endoplasmic reticulum[12], it might be premature to imply that loop-sheet polymerization is the sole mechanism for retaining the Z variant, or any other AAT variant. The identification of a soluble, high-molecular-weight form of the newly synthesized Z variant in transfected mouse hepatoma cells[13] might reflect its association with a chaperone, rather than support the hypothesis suggested by Lomas *et al.*[1].

What, if anything, does the discovery of polymerized Z protein tell us about AAT-related liver disease? Apparently, accumulation of the insoluble human Z variant in hepatocytes of transgenic mice is enough to induce liver damage in those animals[14]. Unexpectedly, the normal M variant of the human protein can also accumulate in the hepatic endoplasmic reticulum in transgenic mice, but only as a result of its increased synthesis[15]. Unlike the Z variant, the accumulated M protein is completely soluble following cell lysis[15] and these animals exhibit no apparent liver damage[14]. This observation raises the possibility that it is the insolubility of the accumulated Z variant that might actually be the cause of liver damage, at least in mice. However, understanding the mechanism of Z accumulation should at least aid in the design of methods to suppress that event, and allow all the retained protein to be degraded[13].

As a possible way forward from Lomas and colleagues' findings, it has been observed that accumulation of the normal M variant in the liver endoplasmic reticulum causes a concomitant accumulation of the endogenous murine AAT polypeptide[15]. Although this might represent the formation of human–mouse AAT heteropolymers, the exact identity of this material remains to be determined. But these findings do show that transgenic mice might be a helpful model for further analysing the loop-sheet polymerization mechanism as it pertains to the association of nonidentical serpin molecules in the endoplasmic reticulum, and also to test methods for suppressing the accumulation of the polymerized Z variant. □

Richard N. Sifers is in the Departments of Pathology and Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA.

1. Lomas, D. A., Evans, D. L., Finch, J. T. & Carrell, R. W. *Nature* **357**, 605–607 (1992).
2. Gadek, J. E. & Crystal, R. G. *Metabolic Basis of Inherited Disease*, 5th edn (McGraw-Hill, Minneapolis, 1982).
3. Brantly, M., Nukiwa, Y. & Crystal, R. G. *Am. J. Med.* **84**, 13–31 (1988).
4. Sifers, R. N., Finegold, M. J. & Woo, S. L. C. *Am. J. Respir. Cell molec. Biol.* **1**, 341–345 (1989).
5. Sharp, H. L., Bridges, R. A., Krivit, W. & Freier, E. F. *J. Lab. clin. Med.* **73**, 934–939 (1969).
6. Huber, R. & Carrell, R. W. *Biochemistry* **28**, 8951–8966 (1989).
7. Wright, H. T., Quian, H. X. & Huber, R. *J. molec. Biol.* **213**, 518–528 (1990).
8. Carrell, R. W., Evans, D. L. & Stein, P. E. *Nature* **353**, 576–578 (1991).
9. Cox, D. W., Billingsley, G. D. & Callahan, J. W. *FEBS Lett.* **205**, 255–260 (1986).
10. Bathurst, I. C., George, P. M., Travis, J. & Carrell, R. W. *FEBS Lett.* **117**, 179–183 (1984).
11. Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muensch, H. & Woo, S. L. C. *J. biol. Chem.* **263**, 7330–7335 (1988).
12. Gething, M. J. & Sambrook, J. *Nature* **355**, 33–44 (1992).
13. Le, A., Ferrell, G. A., Dishon, D. S., Le, Q.-Q. & Sifers, R. N. *J. biol. Chem.* **267**, 1072–1080 (1992).
14. Carlson, J. A. *et al. J. clin. Invest.* **83**, 1183–1190 (1989).
15. Sifers, R. N., Rogers, B. B., Hawkins, H. K., Finegold, M. J. & Woo, S. L. C. *J. biol. Chem.* **264**, 15696–15700 (1989).

SOLAR SYSTEM

Wandering on a leash

Carl D. Murray

MENTION of chaos in the motion of objects in the Solar System conjures up images of colliding planets, impacting asteroids and a variety of catastrophic events. However, a number of recent discoveries have shown that although gravitational perturbations can lead to chaotic orbits, the nature of the chaos can be quite subtle. A numerical investigation of the orbit of the asteroid 522 Helga, reported by Milani and Nobili on page 569 of this issue[1], shows that it is probably an example of 'stable chaos', where the orbit is chaotic yet confined to a particular region of the asteroid belt. Although the concept may seem counter-intuitive, stable chaotic motion has now been identified in a number of orbits in the Solar System.

The explanation of this apparent paradox has more to do with the imprecision of language than any fundamental problem with the dynamics. Using everyday definitions it is difficult to envisage a political or economic situation that is simultaneously stable and chaotic; they appear to be mutually exclusive states. However, using more precise definitions, such a combination is commonplace in a number of dynamical systems. Chaos can be thought of as a sensitive dependence on initial conditions, or the exponential divergence of nearby trajectories and the rate at which this occurs can be calculated. A definition of 'stable' is more difficult because it means different things to different people in different circumstances. For example, an incomplete list by Szebehely[2] gives 47 different concepts of stability. An intuitive definition is that a given system is stable if it does not deviate from its original configuration by more than some specified amount. In this sense a stable orbit is one which is confined or bounded such that deviations take place within strict limits. Stable chaos occurs when such deviations are chaotic. A simple analogy is the motion of a ball on a roulette wheel; although dissipation and the skill of the croupier are important, the ball's trajectory is chaotic yet usually confined to the vicinity of the wheel.

Examples of stable chaos can be seen in a variety of dynamical systems ranging from simple systems with one degree of freedom to the highly complex gravitational interactions of the planets. Numerical investigations of the orbit of the outer planets[3,4] by Wisdom and colleagues showed good evidence that the orbit of Pluto is chaotic with a timescale for exponential divergence of 20 million years (Myr). Laskar[5,6] used an integration of the averaged equations of motion to show that the divergence timescale for the inner planets is as low as 5 Myr. In neither case was there any indication of gross instability; the planets remain in their orbits and the chaos just acts to limit the predictive power of celestial mechanics. However, conclusions from such numerical integrations are only as good as the results for the last time step — as yet there is no numerical or analytical proof that the orbits of the planets are stable for timescales approaching the age of the Solar System, 5 billion years.

522 Helga is not the first object in the Solar System found to be exhibiting stable chaos, but the result of Milani and Nobili is unusual in two respects: the rate of orbit divergence and the location of the stable chaos. Although the integration time was short (7 Myr), it corresponds to 1,000 times the divergence timescale, compared with factors of 50 and 40 obtained for Pluto and the inner planets respectively. This gives the authors the confidence to state that the orbit of the asteroid is an example of stable chaos. But it has to be remem-

Abstract for Gordon Conference
6/30/92
Andover, New Hampshire

Funding sources are not presented in these abstracts

Degradation of the Z Alpha1-antitrypsin Variant in the ER. Ann Le and Richard N. Sifers, Depts. of Pathology and Cell Biology, Baylor College of Medicine, Houston, Texas 77030.

Approximately 80% of the human Z alpha$_1$-antitrypsin variant is retained and degraded in the ER of transfected mouse hepatoma cells. Results from sedimentation velocity centrifugation analyses and low stringency immunoisolation techniques suggest that three molecules of the molecular chaperone BiP are bound to a single retained Z polypeptide. Binding of BiP to the Z variant delays the complete trimming of its N-linked oligosaccharides in the ER. Likewise, the complete BiP-Z complex fails to bind Con A-Sepharose *in vitro* and suggests that delayed oligosaccharide trimming results from steric hinderance of these moieties caused by the binding of the molecular chaperone to the Z polypeptide. Significantly, a discrete size reduction of the Z variant, observed by SDS-PAGE, occurs during its degradation and this phenomenon is inhibited by the mannose analogue 1-deoxymannojirimycin (dMM). Finally, both the dMM-inhibitable oligosaccharide modification event and degradation of the Z variant also occur in cells where the plasma membrane has been selectively permeabilized with streptolysin O which prevents ER-to-Golgi vesicular transport. Overall, these findings indicate that release of the Z variant from BiP controls the onset of its degradation in the ER and suggests that a dMM-inhibitable alpha-mannosidase is involved in the hydrolysis of its oligosaccharides.

Galley Proof

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 267, No. ■, Issue of ■, pp. ■, 1992
Printed in U.S.A.

Soluble Aggregates of the Human PiZ α_1-Antitrypsin Variant Are Degraded within the Endoplasmic Reticulum by a Mechanism Sensitive to Inhibitors of Protein Synthesis*

(Received for publication, July 30, 1991)

Anhquyen Le‡, Gail A. Ferrell‡§, Dana S. Dishon‡¶, Quyen-Quyen A. Le‡, and Richard N. Sifers‡‖**

From the ‡Department of Pathology, Section of Molecular Pathobiology and the ‖Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Greater than 85% of the transport-impaired PiZ variant of human α_1-antitrypsin is retained within transfected mouse hepatoma cells and is subjected to intracellular degradation (Le, A., Graham, K., and Sifers, R. N. (1990) *J. Biol. Chem.* 265, 14001–14007). The retained protein undergoes a discrete size reduction that results from the modification of its endoglycosidase H-sensitive oligosaccharides and is inhibited by 1-deoxymannojirimycin. Metabolic poisons and inhibitors of protein synthesis perturb the intracellular degradation of the retained protein but do not affect its size reduction. The ability of metabolic poisons to influence the degradation of the PiZ variant in cells treated with brefeldin A indicates that export of the macromolecule from the endoplasmic reticulum (ER) is not the energy-dependent component of its degradation. Subcellular fractionation experiments have verified that both the size reduction and degradation of the retained PiZ variant occur within the rough ER. Finally, sedimentation velocity centrifugation analysis of radiolabeled cell extracts has indicated that approximately 80% of the PiZ variant consists as soluble aggregates immediately after its synthesis. An inability to detect more extensive aggregation during the retention period supports our previous conclusion that only a small fraction of the macromolecules actually form large insoluble aggregates (Graham, K. S., Le, A., and Sifers, R. N. (1990) *J. Biol. Chem.* 265, 20463–20468). Overall, these findings indicate that soluble aggregates of the PiZ variant are degraded within the ER by a mechanism sensitive to inhibitors of protein synthesis.

In mammalian cells, proteins that transit the compartments of the exocytic pathway are first inserted into the lumen of the rough endoplasmic reticulum (ER)[1] during their biosynthesis (1). Evidence exists that proteins exit the ER via their "bulk" flow (2) into nonselective carrier vesicles that deliver their contents to the *cis* region of the Golgi complex (3). Thus, it is believed that the residence of a protein within any particular compartment of the exocytic pathway results from a "retention" signal associated with that macromolecule (4). An apparent prerequisite for the normal export of many newly synthesized proteins from the ER is that they must first assemble into their native conformation during their transit through that organelle (4). In many cases, proteins that fail to achieve this requirement are retained intracellularly and eventually undergo degradation (5–8). Whether a common pathway is utilized for the disposal of most transport-impaired proteins is, as yet, only speculative.

α_1-Antitrypsin (AAT) is a monomeric secretory protein synthesized most abundantly by hepatocytes (9, 10). Following its secretion from cells, AAT functions as the predominant serine protease inhibitor in human sera (11). Human AAT is a very polymorphic protein. At present, over 75 allelic electrophoretic variants of this macromolecule have been identified (12). The most abundant electromorph of human AAT is designated PiM (11). According to the results of x-ray crystallographic measurements (13), it is predicted that the *Glu* to *Lys* substitution at position 342 in the PiZ variant (9, 14) causes the newly synthesized macromolecule to misfold within the ER. Evidence, albeit circumstantial, that at least some of the PiZ variant might misfold is the presence of large insoluble aggregates of the PiZ variant present within distended rough ER cisternae of hepatocytes in the livers of PiZZ individuals (15) and PiZ-bearing transgenic mice (16).

Gene expression analysis in stably transfected mouse hepatoma cells has revealed that approximately 85% of the newly synthesized PiZ variant is retained, rather than secreted (7). Identical to the fate of many other transport-incompetent proteins, the retained fraction of the newly synthesized PiZ variant undergoes degradation in a pre-Golgi, nonlysosomal compartment (7). Because a major physiologic role of human AAT is to prevent the degradation of elastin fibers in the lung, a severe deficiency of AAT in the sera of PiZ homozygous individuals allows for the uninterrupted degradation of elastin fibers in this organ and predisposes affected individuals toward the development of pulmonary emphysema (11). Furthermore, accumulation of insoluble aggregates of the PiZ

* This research was supported in part by Grant 1-91-0052 (to R. N. S.) from the Moran Foundation and Grants BRSG SO7 RR05425 and DK42806 (to R. N. S.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Participant in the Summer Medical and Research Training (SMART) program at Baylor College of Medicine and recipient of an undergraduate Pew Scholarship.

¶ Participant in the Summer Medical and Research Training (SMART) program at Baylor College of Medicine.

** To whom correspondence should be addressed: Dept. of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

[1] The abbreviations used are: ER, endoplasmic reticulum; AAT, α_1-antitrypsin; Pi, protease inhibitor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis, endo H, endo-β-*N*-acetylglucosaminidase; dNJ, 1-deoxynorjirimycin; dMM, 1-deoxymannojirimycin; nocodazole, *methyl*-[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate; BFA, brefeldin A; PBS, phosphate-buffered saline; HEPES, 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid.

variant within the hepatic ER that can act, in part, as an etiologic agent toward the development of liver damage in humans (15) and in transgenic mice (17, 18).

An important problem in cell biology is to identify and characterize the molecular mechanisms utilized to recognize and target transport-impaired proteins for their degradation within the exocytic pathway. We have utilized the PiZ variant as a molecular reagent to begin to more fully characterize the various components associated with the intracellular degradation of this particular transport-impaired secretory protein. Here, we report that approximately 80% of the newly synthesized PiZ variant forms stable soluble aggregates, possibly in the form of homotrimers, within the ER. Whereas the aggregated protein underoes a discrete size reduction that is inhibited by 1-deoxymannojirimycin, biochemical and physical evidence substantiates that the size reduction and ultimate degradation of the retained protein occurs within the ER. Finally, degradation of the retained protein exhibits sensitivity toward perturbers of intracellular ATP levels and inhibitors of protein synthesis.

MATERIALS AND METHODS

Chemicals and Reagents—TRAN^{35}S Label (specific activity, >1000 Ci/mmol) was purchased from ICN Biomedicals. Aquasol scintillation fluid was obtained from Du Pont-New England Nuclear. Protein G-Sepharose 4FF was purchased from Pharmacia LKB Biotechnology Inc. Recombinant endoglycosidase H (cloned from *Streptomyces plicatus*), 1-deoxynojirimycin, 1-deoxymannojirimycin, and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim. Brefeldin A was purchased from Epicentre Technologies. Deuterium oxide, 3-methyladenine, *methyl*-[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate (nocodazole), 2,4-dinitrophenol, 2-deoxy-D-glucose, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-*N*-acetylglucosaminide, bovine hemoglobin, and bovine liver catalase were purchased from Sigma. Bovine γ-globulin was purchased from Bio-Rad. Media and fetal bovine sera used in mammalian tissue culture were purchased from GIBCO-BRL.

Stably Transfected Mouse Hepatoma Cell Lines—Stably transfected mouse hepatoma cells (line Hepa 1a) expressing a recombinant PiZ variant (line H1A/RSVATZ-8) (7) or normal PiM1 human AAT (line H1A/M-15) (19) have been described previously.

Antibody—An IgG fraction of a polyclonal goat anti-human AAT antibody preparation was purchased from Organon Teknika-Cappel.

Metabolic Radiolabeling of Human AAT—Nearly confluent monolayers of cells in 100-mm diameter dishes were split 1:2 and refed with fresh growth medium. The following day, monolayers of cells were washed with Dulbecco's phosphate-buffered saline (PBS) and then subjected to a 15-min methionine starvation period by incubation at 37 °C with 3 ml of methionine-free Dulbecco's modified essential medium containing 1 × glutamine and 0.1% fetal calf serum (pulse medium). Two ml of medium were then removed and 300–500 μCi of TRAN^{35}S Label were added to the 1 ml of medium remaining in each dish. Pulse-radiolabeling of cells was performed by the continuous manual rocking of the dish in a 37 °C warm room. Cells were then washed with 37 °C PBS and incubated in 3 ml of 37 °C pulse medium containing unlabeled methionine at a concentration of 120 mg/liter (chase medium). At the appropriate time, the media was collected, cells were washed with ice-cold PBS, and then lysed as described previously (16).

Immunoisolation of Radiolabeled Human AAT—Quantitative immunoisolation of radiolabeled proteins from cell lysates and media was performed by using an excess of antibody and Protein G-Sepharose 4FF as described previously (19). Immunocomplexes were heated in disruption buffer, subjected to SDS-PAGE (8% polyacrylamide), and radiolabeled proteins were detected by fluorographic enhancement of gels as described previously (7). Quantitation of radiolabeled proteins was performed by scintillation counting of excised gel pieces (7). In some cases, immunoisolated protein was subjected to digestion with endoglycosidase H as described previously (19).

Redistribution of Golgi-associated Enzymes into the Endoplasmic Reticulum with Brefeldin A—Brefeldin A was used to cause the retrograde movement of Golgi-associated enzymes into the endoplasmic reticulum as has been described by Lippincott-Schwartz *et al.* (20). For this, brefeldin A (2.5 μg/ml) was included in the media during the methionine starvation, radiolabeling, and chase periods in the appropriate pulse-chase experiments.

Subcellular Fractionation on Sucrose/D_2O Gradients—Cells were subjected to subcellular fractionation using shallow discontinuous D_2O/sucrose gradients as described by Lodish *et al.* (3). For this, pulse-radiolabeled cells from four to six 100-mm diameter dishes were scraped and collected with a pliable rubber spatula into ice-cold PBS. Cells were combined, centifuged at 1000 × *g* for 5 min, and the cell pellet was suspended in ice-cold 10 mM HEPES-HCl, pH 7.4, containing 0.25 M sucrose and 5 mM EDTA to prevent aggregation of organelles. Following an 8-min incubation on ice, cells were subjected to Dounce homogenization at 4 °C. The percent cellular breakage was monitored by Trypan blue exclusion analysis, and homogenization was continued until approximately 50–70% of cells were broken. Intact cells and nuclei were removed from the homogenate by centrifugation at 1000 × *g* for 4 min at 4 °C. To enrich for intact subcellular vesicles, postnuclear supernatants were layered onto 5 ml of 0.33 M sucrose prepared in 10 M HEPES-HCl, pH 7.4, which was layered onto a 2-ml cushion of 2 M sucrose, all in a tube used with the Beckman SW41 rotor. Following a 30-min centrifugation at 40,000 rpm and 4 °C, the visible band of intact vesicles was isolated from the top of the 2 M sucrose cushion. The intact vesicles were gently resuspended and diluted to a final concentration of 0.25 M sucrose with ice-cold 10 mM HEPES-HCl, pH 7.4. The intact vesicles in approximately 2.5 ml were layered onto a shallow discontinuous sucrose gradient prepared in a tube for the Beckman SW41 rotor. The gradient consisted of 1 ml each of 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5, 30, and 50% sucrose in D_20 containing 10 mM HEPES-HCl, pH 7.4. Following a 3-h centrifugation at 36,000 rpm and 4 °C, the gradient was fractionated (0.45 ml/fraction) from the top of the tube using a Densi-Flow II apparatus (Buchler Instruments). An equal volume of 2 × cell lysis buffer was added to gradient fractions and, as described by Lodish *et al.* (3), fractions were pooled pairwise. In some experiments, ribosomes were stripped from rough ER microsomes as described by Wikstrom and Lodish (21) by incubating intact subcellular vesicles with 200 μg/ml RNAse A and 15 mM EDTA at 4 °C for 10 min. The vesicles were then applied to a gradient consisting of 1 ml each of 10, 15, 20, 25, 30, 34, 42, 46, and 50% sucrose in D_20 containing 10 mM HEPES-HCl, pH 7.4, as previously described (21). Following centrifugation as described above, the gradient was fractionated into approximately 15 fractions. Human AAT was immunoisolated from all gradient fractions, subjected to SDS-PAGE (8% polyacrylamide), and the radiolabeled protein was detected by fluorographic enhancement of the gel, as described above. Neutral α-glucosidase activity was assayed in a 100-μl solution containing 50 mM sodium phosphate, pH 6.9, 0.1% bovine serum albumin, cell extract, and 2.5 mM *p*-nitrophenyl-α-D-glucopyranoside. The specific inhibition of both ER α-glucosidase I and II activity with this drug (22) was performed by including 10 mM 1-deoxynorjirimycin in the reaction. Acidic β-hexosaminidase activity was detected in a 100-μl solution containing 0.1 M sodium citrate buffer, pH 5.2, 0.1% bovine serum albumin, cell extract, and 2.5 mM *p*-nitrophenyl-β-D-*N*-acetylglucosaminide. Both reactions were stopped after 4–5 h of incubation at 37 °C by the addition of 1 ml of 0.2 M glycine-NaOH, pH 11.0. Activity was measured by the production of *p*-nitrophenol which was measured by absorbance at 420 nm.

Sedimentation Velocity Centrifugation—Radiolabeled cells from two to four 100-mm diameter dishes were lysed in a total of 0.7 ml of cell lysis buffer (0.05 M Tris-HCl, pH 8.0, 0.5% Nonident P-40, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.01 mg/ml leupeptin, and 0.01 mg/ml pepstatin) and centrifuged at 10,000 × *g* for 5 min to remove cell debris. Approximately 0.5 ml of the supernatant was layered onto a chilled 12-ml linear 5%-20% sucrose gradient containing 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.01% NaN_3 that had been prepared in a tube designed for the Beckman SW41 rotor. Following centrifugation at 40,000 rpm for 24 h, the gradient was fractionated from the top of the tube using a Densi-Flow II apparatus (Buchler Instruments). An equal volume of 2 × cell lysis buffer was added to the odd-numbered fractions, and human AAT was immunoisolated. Immunocomplexes were fractionated by SDS-PAGE (8% polyacrylamide) and human AAT was identified and quantitated as described above. For standardization of the sucrose gradient, specific protein markers were dissolved in cell lysis buffer and subjected to sedimentation velocity centrifugation as described above. Following fractionation of the gradient, calf alkaline phosphatase and bovine liver catalase were assayed as previously described (23). Bovine hemoglobin was identified by its absorption at 575 nm. Bovine γ-globulin was identified after an aliquot from each fraction was sub-

jected to SDS-PAGE, and the protein was detected after the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

RESULTS

Size Reduction of the Retained PiZ Variant Results from the Modification of Its Asparagine-linked Oligosaccharides—Three asparagine-linked oligosaccharides are covalently attached to the human AAT polypeptide during its cotranslational insertion into the lumen of the ER (24). Pulse-chase analysis of the normal PiM1 human AAT variant stably expressed in transfected mouse hepatoma cells (cell line H1A/M-15) demonstrated that the newly synthesized macromolecule exhibits a mass of 50 kDa on SDS-PAGE (Fig. 1*A*). The entire cohort of the radiolabeled macromolecules appeared in the media of these cells within 1 h following their synthesis and exhibited a mass of 56 kDa (Fig. 1*A*) which results from the addition of charged sialic acid residues to the oligosaccharide moieties within the *trans* Golgi network prior to secretion (25). In contrast, only 15% of the pulse-radiolabeled PiZ variant was secreted from transfected mouse hepatoma cells (cell line H1A/RSVATZ-8) during a 3-h chase period (Fig. 1*A*). Thus, as much as 85% of these newly synthesized macromolecules were retained intracellularly, rather than secreted. The immunoisolation of only 27% of the pulse-radiolabeled protein from the cell extract after the 3-h chase period indicated that 58% of the retained macromolecules had been degraded.

As previously reported (7), the retained fraction of the PiZ variant undergoes a discrete size reduction that can be identified by SDS-PAGE (Fig. 1*A*, *asterisk*). To determine the cause of this anomaly, the PiZ variant immunoisolated from both the 0- and 3-h chase periods was deglycosylated by digestion with endoglycosidase H. Although the retained protein is subjected to intracellular degradation during the 3-h chase period, the mobility of the deglycosylated polypeptide was identical to that of the pulse-radiolabeled protein in SDS-PAGE (Fig. 1*B*). These findings indicated that the size reduction of the retained protein did not result from the modification of the radiolabeled polypeptide, but rather, must have involved a modification of the oligosaccharide moieties.

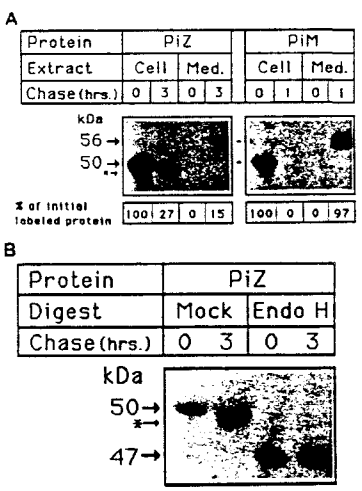

FIG. 1. **The PiZ variant undergoes an oligosaccharide-dependent size reduction during its intracellular retention and degradation.** Stably transfected mouse hepatoma cells synthesizing either the normal PiM1 human AAT variant (*PiM*) (cell line H1A/M15) or the transport-impaired PiZ variant (*PiZ*) (cell line H1A/RSVATZ-8) were pulse-radiolabeled with 300 μCi of TRAN^{35}S Label for 5 min and then incubated in chase medium for either 0 or 3 h as described under "Materials and Methods." *A*, Human AAT immunoisolated from cell extracts and media was fractionated by SDS-PAGE. Radiolabeled proteins were detected by fluorography and quantitated by scintillation counting of the appropriate excised gel piece. *B*, immunocomplexes from the cell extracts containing similar quantities of radioactive PiZ variant were either mock digested (*Mock*) or digested with endoglycosidase H (*Endo H*). Samples were subjected to SDS-PAGE and radiolabeled proteins were detected by fluorography. The apparent mass of each protein is shown. An asterisk (*) denotes the mobility of the intracellular form of the radiolabeled PiZ variant after it has undergone a discrete size reduction.

1-Deoxymannojirimycin Inhibits the Size Reduction of the Retained PiZ Variant, But Not the PiM Protein—The initial processing of asparagine-linked oligosaccharides occurs as a cotranslational event when all 3 terminal glucose residues are hydrolyzed from the $Glc_3Man_9(GlcNAc)_2$ moiety by the action of α-glucosidases I and II within the ER (22). To determine whether the oligosaccharide-dependent size reduction of the retained PiZ variant is caused by a delayed hydrolysis of glucose residues, H1A/RSVATZ-8 cells were preincubated and then radiolabeled in media containing 1-deoxynorjirimycin (dNJ) to specifically inhibit the action of ER α-glucosidases I and II. This manipulation retarded the mobility of the immunoisolated PiZ variant in SDS-PAGE (Fig. 2*A*) and digestion of the protein with endo H produced a radiolabeled polypeptide that exhibited a mobility identical to that of the control protein (Fig. 2*A*). These results verifed that the ER α-glucosidase activities had been inhibited by dNJ and demonstrated that the cotranslational hydrolysis of glucose residues from the oligosaccharides of the PiZ variant occurs normally and is not responsible for its size reduction.

The next step in the trimming of asparagine-linked oligosaccharides involves the hydrolysis of 4 mannose residues by α-mannosidase activities located within the ER and the *cis* Golgi compartment (26). Because both of these subcellular compartments contain α-mannosidases that are inhibited by the mannose analogue 1-deoxymannojirimycin (dMM) (27, 28), we tested whether this compound would hinder the oligosaccharide-dependent size reduction of the retained PiZ variant. For this, the protein was immunoisolated from pulse-radiolabeled H1A/RSVATZ-8 cells that had been chased for 2.5 h in media containing dMM. Evidence that this manipulation had indeed inhibited the appropriate intracellular α-mannosidase activity was demonstrated by the secretion of AAT that exhibited a mass of only 50 kDa (Fig. 2*B*). These findings were expected because the inhibition of α-mannosidases should abolish the hydrolysis of mannose residues and prevent the subsequent production of an oligosaccharide species that would serve as an appropriate substrate for the addition of charged sialic acid residues in the *trans* Golgi network. Furthermore, results from neuraminidase digestion of the secreted protein verified that its oligosaccharides were deficient in sialic acid (data not shown). Treatment of H1A/RSVATZ-8 cells with media containing 0.5–1 mM dMM completely abolished the size reduction of the retained PiZ variant without significantly affecting the rate of its intracellular degradation (Fig. 2*B*). These findings suggest that the size reduction of the retained PiZ variant results from the post-translational hydrolysis of mannose residues by a dMM-inhibitable α-mannosidase. These results were quite different from that observed for the normal PiM protein when H1A/M15 cells were pulse-radiolabeled for 5 min and then chased at 15 °C. These conditions prevent proteins from entering the Golgi complex and allow their oligosaccharides to be trimmed by the ER α-mannosidase (29). Although this manipulation resulted in a size reduction of the retained PiM protein (Fig. 2*C*, *asterisk*) that resulted from the modification of its oligosaccharides within the ER (endo H digestion not shown), this phenomenon was unaffected by 1 mM dMM (Fig. 2*C*).

Metabolic Poisons Inhibit the Intracellular Degradation, But

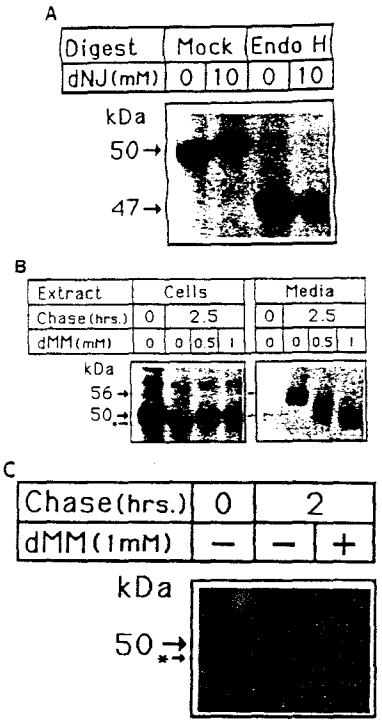

FIG. 2. **1-Deoxymannojirimycin inhibits the size reduction of the retained PiZ variant.** *A*, identical dishes of H1A/RSVATZ-8 cells were incubated for 1 h at 37 °C in regular growth media containing 10 mM 1-deoxynorjirimycin (*dNJ*). Cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label ("Materials and Methods") containing an identical concentration of the drug. Human AAT was immunoisolated from cell extracts and either mock digested (*Mock*) or digested with endoglycosidase H (*Endo H*). Samples were fractionated by SDS-PAGE and radiolabeled proteins were detected by fluorography. *B*, dishes of H1A/RSVATZ-8 cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label and chased for 2.5 h in chase medium containing either 0.5 or 1 mM of 1-deoxymannojirimycin (*dMM*). Human AAT was immunoisolated from cell extracts (*Cells*) and media (*Media*). Immunocomplexes were fractionated by SDS-PAGE and radiolabeled human AAT was detected by fluorography. *C*, dishes of H1A/M15 cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label and chased for 2.5 h in chase media at 15 °C with (±) or without (−) 1 mM of 1-deoxymannojirimycin (*dMM*). Human AAT was immunoisolated from cell extracts and immunocomplexes were fractionated by SDS-PAGE. Radiolabeled human AAT was detected by fluorography. The apparent mass of each protein is shown. An asterisk (*) denotes the mobility of the intracellular form of the radiolabeled protein after it has undergone a discrete size reduction.

Not the Size Reduction of the Retained PiZ Variant—As described above, dMM-inhibitable α-mannosidase activities have now been identified within the ER (27) and *cis* Golgi compartment (28). Thus, our findings did not help to localize the subcellular site where the size reduction of the PiZ variant had occurred. Nascent secretory proteins are delivered to the *cis* Golgi compartment after they enter nonselective vesicles that "bud" from the surface of the smooth ER (3). Because the formation of these vesicles is an energy-dependent event (29), we tested whether the perturbation of intracellular ATP levels would influence either the size reduction or intracellular degradation of the retained PiZ variant. In fact, intracellular degradation (Table I), but not the size reduction of the retained PiZ variant (Fig. 3) was inhibited when pulse-radiolabeled H1A/RSVATZ-8 cells were chased in media containing increasing concentrations of sodium azide and sodium fluoride. A similar result was observed when the radiolabeled cells were chased in various concentrations of 2,4-dinitrophenol and 2-deoxyglucose as another combination of metabolic poisons (Table I). These findings indicated that the intracellular degradation of the PiZ variant, but not its oligosaccharide-dependent size reduction, exhibits an energy-dependent component.

TABLE I

Effect of various compounds on the intracellular degradation of the retained PiZ variant

H1A/RSVATZ-8 cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label and then incubated for the appropriate period in chase medium ("Materials and Methods") containing the compound shown below. The radiolabeled PiZ variant immunoisolated from cell extracts and media was fractionated by SDS-PAGE, detected by fluorography, and quantitated by scintillation counting of the appropriate excised gel pieces. 2DG, .

Treatment[a]	% retained[b]	% degraded[c]	% Inhibition of degradation
Control	77	45	
NaN_3 (20 mM), NaF (5 mM)	82	21	53
NaN_3 (40 mM), NaF (10 mM)	100	20	56
Control	84	61	
DNP (1 mM), 2DG (5 mM)	96	48	35
DNP (5 mM), 2DG (10 mM)	100	21	65
Control	89	74	
BFA (2 μg/ml)[d]	100	78	0
BFA (2 μg/ml), NaN_3 (40 mM), NaF (10 mM)[d]	100	25	68
Control	80	60	
Cycloheximide (50 μg/ml)	75	3	95
Puromycin (50 μg/ml)	82	2	97
Control	86	65	
Nocodazole (20 μg/ml)[e]	85	67	0

[a] The chase period for all experiments was 2.5 h, except in the experiments using brefeldin A where cells were chased for 3 h.

[b] Protein retention was calculated by subtracting the amount of radiolabeled PiZ variant secreted into the media during the chase period from the total amount of the radiolabeled protein synthesized. Percent of PiZ variant retained is shown.

[c] Protein degradation was calculated by subtracting the amount of radiolabeled PiZ variant present in cells following the chase period from the total PiZ variant retained intracellularly. Percent of the PiZ variant degraded is shown.

[d] Duration of the methionine starvation was 25 min, and brefeldin A was included in all media during the experiment.

[e] Cells were first pre-incubated for 2 h in regular growth medium containing nocodazole prior to radiolabeling and chasing in medium containing the drug.

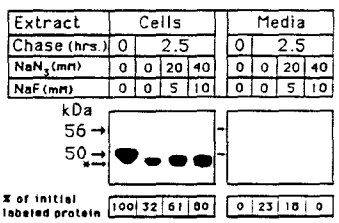

FIG. 3. **Metabolic poisons inhibit the intracellular degradation of the PiZ variant.** Identical dishes of H1A/RSVATZ-8 cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label ("Materials and Methods") and then incubated in chase medium containing various concentrations of sodium azide and sodium fluoride. Human AAT immunoisolated from cell extracts and media were fractionated by SDS-PAGE. Radiolabeled proteins were detected by fluorography and quantitated by scintillation counting of the appropriate excised gel pieces. The apparent mass of each protein is shown. An asterisk (*) denotes the mobility of the intracellular form of the radiolabeled PiZ variant after it has undergone a discrete size reduction.

Energy-dependent Degradation of the PiZ Variant Occurs in Cells Treated With Brefeldin A—Using either combination of metabolic poisons as described above, the greatest inhibition of degradation occurred when protein secretion was totally blocked (Table I) which might have reflected a requirement for the retained protein to exit the ER for proteolysis in a separate compartment. Whereas the ER and Golgi complex are separate and distinct organelles within cells, recent studies (20, 30) have demonstrated that the fungal metabolite brefeldin A (BFA) causes the rapid retrograde distribution of *cis* and *medial* Golgi enzymes into the ER. As predicted, preincubation and pulse-radiolabeling of H1A/RSVATZ-8 cells in media containing 2 μg/ml BFA completely blocked the secretion of the PiZ variant (Fig. 4*A*, Table I) and caused the asparagine-linked oligosaccharides of the retained protein to become resistant to digestion by endoglycosidase H (Fig. 4*B*) which does not occur in control cells (Fig. 1*B*). These findings indicated that the PiZ variant became accessible to enzymes of the Golgi complex and verified that BFA had caused the retrograde distribution of Golgi-associated enzymes into the ER. BFA-treatment of cells did not hinder the intracellular degradation of the pulse-radiolabeled PiZ variant (Fig. 4*A*, Table I). This suggested that the proteases involved in the degradation of the retained protein are either resident to the ER or were diverted to that organelle as the result of the BFA treatment. Conceivably, if degradation of the retained PiZ variant occurs within the Golgi complex then the energy-dependent component associated with its degradation might actually represent its entry into transitions vesicles *en route* to the *cis* Golgi compartment. If this is correct, then one would not expect that metabolic poisons would inhibit degradation of the retained PiZ variant in BFA-treated cells because enzymes of the Golgi complex and the ER reside together in the latter compartment. However, degradation was totally inhibited in BFA-treated cells that were radiolabeled and then chased in media containing 40 mM sodium azide and 10 mM sodium fluoride (Table I). These findings indicated that the exit of the PiZ variant from the ER is not the energy-dependent component associated with its intracellular degradation and suggested that degradation of the protein does not occur within the Golgi complex.

Inhibitors of Protein Synthesis Perturb the Degradation of the Retained PiZ Variant—Because protein synthesis is affected by intracellular ATP levels, we determined whether the energy-dependent intracellular degradation of the PiZ variant might actually be caused by the inhibition of protein synthesis. For this, pulse-radiolabeled H1A/RSVATZ-8 cells were chased for 2.5 h in media containing either cycloheximide or puromycin at 50 μg/ml which totally inhibited protein synthesis in these cells. At this concentration, either compound was able to inhibit the degradation of the retained PiZ (Fig. 5) by greater than 90% (Table I) without significantly influencing protein secretion (Fig. 5). This finding indicated that active protein synthesis is required for the degradation of the retained PiZ variant, and suggested that the ATP-dependence of degradation might actually reflect an inhibition of protein synthesis.

Size Reduction and Degradation of the Retained PiZ Variant Occur within the ER—Subcellular fractionation was utilized as a physical method to identify the intracellular site where the size reduction and degradation of the retained PiZ variant occurs. Lodish *et al.* (3) have reported the separation of ER microsomes from post-ER transition vesicles and Golgi vesicles derived from the human hepatoma cell line Hep G2 by the use of shallow discontinuous sucrose gradients prepared in D_20. This methodology was adapted for analysis of the mouse hepatoma cells used in our experiments. Following a 5-min pulse-radiolabeling of H1A/M15 cells, subcellular vesicles were separated on a D_20/sucrose gradient and the radiolabeled PiM1 protein was immunoisolated from fractions of the gradient. The newly synthesized protein was concentrated in "dense" vesicles (fractions 23–27) located just above the 50% sucrose cushion (Fig. 6*A*) and cofractionated with neutral α-glucosidase activity (Fig. 6*A*), a specific marker for the ER (22, 26). It should be noted that the actual gradient consisted of fractions 7–30, and fractions 1–5 corresponded to the material applied to the top of the gradient. Thus human AAT present within fractions 1–5 is not associated with the gradient and most likely originated from the leakage of damaged vesicles during their preparation. During an extended radio-

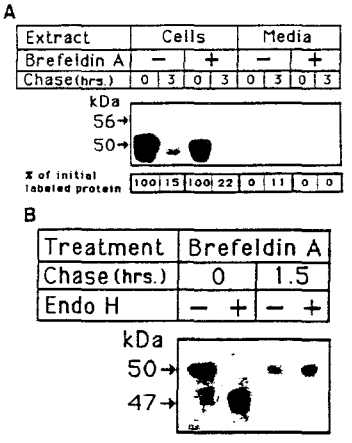

FIG. 4. **The PiZ variant is degraded in cells treated with brefeldin A.** *A*, identical dishes of H1A/RSVATZ-8 cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label, and then incubated in chase media. Brefeldin A (2 μg/ml) was included in the methionine starvation, radiolabeling, and chase media (±) or no drug was added (−). Human AAT was immunoisolated from cell extracts and media and fractionated by SDS-PAGE. Radiolabeled proteins were detected by fluorography and quantitated by scintillation counting of the excised gel pieces. *B*, identical dishes of H1A/RSVATZ-8 cells were subjected to methionine starvation, pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label, and incubated in chase media, all containing brefeldin A (2 μg/ml). Human AAT was immunoisolated from cell extracts and then either mock digested (−) or digested with (±) endoglycosidase H (*Endo H*). The immunocomplexes were fractionated by SDS-PAGE and detected by fluorography. The apparent mass of each protein is shown.

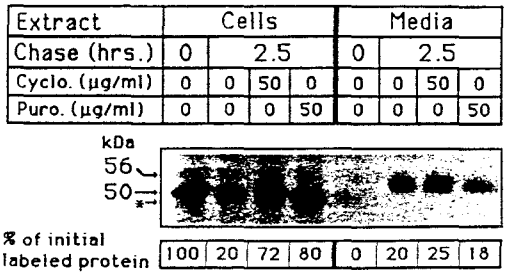

FIG. 5. **Specific inhibitors of protein synthesis prevent the degradation of the retained PiZ variant.** Identical dishes of H1A/RSVATZ-8 cells were pulse-radiolabeled for 5 min with 300 μCi of TRAN^{35}S Label ("Materials and Methods"). Cells were then incubated in chase medium containing either cycloheximide (*Cyclo.*) or puromycin (*Puro.*) for 2.5 h. Human AAT was immunoisolated from cell extracts and media and fractionated by SDS-PAGE. Radiolabeled proteins were detected by fluorography and quantitated by scintillation counting of the excised gel pieces. The apparent mass of each protein is shown. An asterisk (*) denotes the mobility of the intracellular form of the radiolabeled PiZ variant after it has undergone a discrete size reduction.

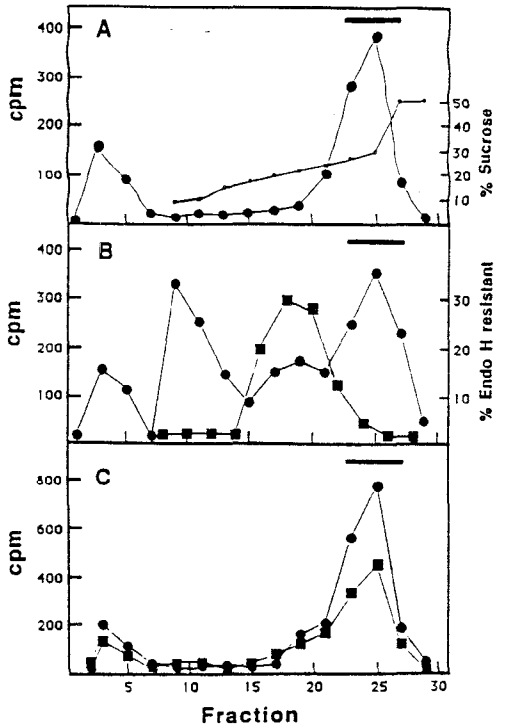

FIG. 6. **Subcellular fractionation on shallow discontinuous D_20/sucrose gradients.** 100-mm dishes of cells were subjected to pulse-chase experiments using 300–500 μCi of TRAN^{35}S Label. Cell homogenates were fractionated on shallow discontinuous sucrose gradients prepared in D_20 as described under "Materials and Methods." Gradients were fractionated and human AAT was immunoisolated from pairwise-pooled fractions. Following fractionation by SDS-PAGE, radiolabeled human AAT was detected by fluorography and quantitated by scintillation counting of excised gel pieces. Quantitation of AAT from the gradient is shown. *A*, H1A/M-15 cells pulse-radiolabeled for 5 min. PiM1 protein (*large solid circles*). Percent sucrose/fraction (*small solid squares*). *B*, H1A/M-15 cells pulse-radiolabeled for 15 min. PiM1 protein (*solid circles*). PiM1 protein bearing endo H-resistant oligosaccharides (*solid squares*). *C*, H1A/RSVATZ-8 cells pulse-radiolabeled for 15 min (*solid circles*) or pulse-radiolabeled for 15 min and chased for 2.5 h (*solid squares*). The *solid bar* at the top of each panel shows the fractions containing the major peak of neutral α-glucosidase activity inhibited by 1-deoxynorjirimycin.

labeling period of 15 min, the PiM1 protein was no longer confined to just the dense vesicles, but 36% was also located within vesicles of an "intermediate" density (fractions 15–21) and approximately 23% was present in vesicles exhibiting a "light" density (fractions 9–13) (Fig. 6*B*). The immunocomplexes from even numbered fractions from the gradient were then subjected to digestion with endo H. As shown in Fig. 6*B*, macromolecules bearing endo H-resistant oligosaccharides were limited to fractions 16–22. This indicated that the vesicles of intermediate density were derived from the Golgi complex where modification of the oligosaccharides of human AAT to an endo H-resistant form occurs (26, 31). Thus, the aforementioned findings were in agreement with those reported by Lodish *et al.* (3) for the intracellular fate of normal human AAT in the human hepatoma cell line HepG2. In that study, the newly synthesized protein initially resides within dense vesicles of the ER, enters a light density fraction of post-ER transition vesicles, and then is delivered to vesicles of the Golgi complex that exhibit an intermediate density and contain the macromolecule bearing endo H-resistant oligosaccharides. Overall, these findings proved the potential usefulness of the D_20/sucrose gradients for identifying the subcellular location of the retained PiZ variant in the transfected mouse hepatoma cells.

In the following experiment, H1A/RSVATZ-8 cells were pulse-radiolabeled for 15 min, and the cell homogenate was subjected to subcellular fractionation as described above. Unlike the PiM protein, the entire cohort of the newly synthesized PiZ variant remained concentrated in the dense vesicles (fractions 23–27) (Fig. 7*A*) that cofractionated with neutral α-glucosidase activity (Fig. 6*C*). Subsequently, subcellular fractionation was performed on a homogenate of H1A/RSVATZ-8 cells that had been pulse-radiolabeled for 15 min and then chased for 2.5 h. This time point was chosen because, by this period, intracellular degradation of the PiZ had been initiated and all of the retained macromolecules exhibited the characteristic size reduction (Fig. 1*A*). As shown in Fig. 7*B*, following the 2.5-h chase period all of the retained PiZ variant resided within the dense vesicles that cofractionated with neutral α-glucosidase activity (Fig. 6*C*). To determine whether the PiZ variant resided within ER microsomes, half of the intact subcellular vesicles derived from a pulse-chase experiment as described above were incubated for 10 min at 4 °C with 200 μg/ml RNAse and 15 mM EDTA to strip ribosomes from the surface of ER microsomes ("Materials and Methods"). Treated and untreated homogenates were then subjected to subcellular fractionation on identical D_20/sucrose gradients ("Materials and Methods"), and the PiZ variant was immunoisolated following fractionation of the gradient. As shown in Fig. 8*A*, this treatment caused vesicles exhibiting neutral α-glucosidase activity to shift to a less dense region of the gradient as compared to that observed for untreated vesicles. It should be noted that this treatment had no effect on the buoyant density of vesicles containing acidic hexosaminidase activity (Fig. 8*A*) which was presumably a marker for lysosomes. Finally, as shown in Fig. 8*B*, vesicles containing the retained PiZ variant cofractionated with those containing neutral α-glucosidase activity before and after treatment with RNAse A and EDTA. This finding provided physical evidence that the retained fraction of the PiZ variant is located within vesicles derived from the rough ER.

To test the possibility that the retained protein might be recycled from a post-ER compartment back to the ER for its degradation, H1A/RSVATZ-8 cells were treated with the microtubule perturber nocodazole (32), which has been shown to totally inhibit the microtubule-dependent recycling of proteins from the Golgi and salvage compartments back to the ER (33). The inability of this compound to hinder the degradation of the PiZ variant (Table I) suggests that recycling of the retained protein between a post-ER compartment and the ER most likely does not occur.

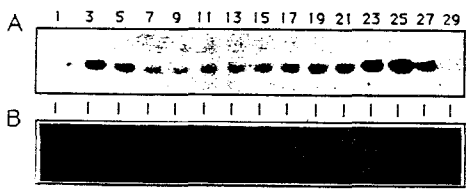

FIG. 7. **The PiZ variant remains in dense vesicles.** H1A/RSVATZ-8 cells were either pulse-radiolabeled for 15 min (*A*), or pulse-radiolabeled for 15 min and chased for 2.5 h (*A*). Cell homogenates were prepared and subjected to subcellular fractionation on shallow discontinuous sucrose gradients prepared in D_20 as described under "Materials and Methods." Gradients were fractionated and human AAT was immunoisolated from pairwise-pooled fractions. The immunoisolated PiZ variant is shown following fractionation by SDS-PAGE and detection by flurography. The fraction number from the gradient is shown at the top of *panel A*. Quantitation of the radiolabeled protein is shown in Fig. 6*C*. Neutral α-glucosidase, a marker for the ER, was identified in fractions 23–27 (Fig. 6) and cofractionated with the PiZ variant.

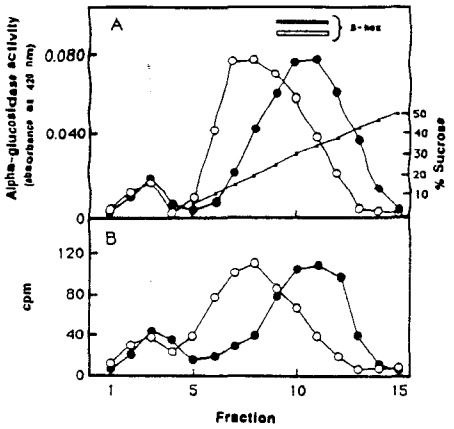

FIG. 8. **The PiZ variant resides in vesicles associated with ribosomes.** H1A/RSVATZ-8 cells were pulse-radiolabeled for 15 min and chased for 2.5 h as described in the legend to Fig. 7*B*. Intact vesicles were prepared from cell homogenates. Vesicles were then either mock digested (*closed symbols*) or incubated for 10 min at 4 °C with 200 μg/ml RNAse and 15 mM EDTA (*open symbols*) to strip ribosomes from the surface of ER microsomes ("Materials and Methods"). Vesicles were then subjected to subcellular fractionation on D_20/sucrose gradients ("Materials and Methods"). *A*, analysis of marker enzymes. Fractions containing neutral α-glucosidase activity (*circles*) and the peak fractions of acidic beta-hexosaminidase (*β-hex*) activity (*bar*) are shown. *B*, quantitation of the radiolabeled PiZ variant in each fraction (*circles*). The percent sucrose in each fraction (*small squares*) is shown in *panel A*.

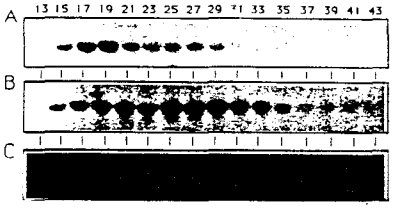

FIG. 9. **Sedimentation profiles of the PiM1 and PiZ variant in 5–20% sucrose gradients.** Dishes of cells were subjected to pulse-chase experiments using 300–500 μCi of TRAN^{35}S Label. Cell lysates were subjected to sedimentation velocity centrifugation in linear 5–20% sucrose gradients as described under "Materials and Methods." Gradients were fractionated and human AAT was immunoisolated from odd-numbered fractions. The immunoisolated PiZ variant is shown following fractionation by SDS-PAGE and detection by flurography. Only fractions containing human AAT are shown. The fraction number from the gradient is shown at the top of *panel A*. Gradients consisted of 43 fractions. *A*, H1A/M-15 cells pulse-radiolabeled for 8 min. *B*, H1A/RSVATZ-8 cells pulse-radiolabeled for 8 min. *C*, H1A/RSVATZ-8 cells pulse-radiolabeled for 8 min and chased for 1.5 h.

The PiZ Variant Forms Soluble Aggregates within the ER—We (16) have previously demonstated that a small fraction (estimated at <1%) of the newly synthesized PiZ variant escapes degradation and is deposited as insoluble aggregates within the ER of hepatocytes from livers of PiZ-bearing transgenic mice (16, 17). To analyze the time course and extent of aggregation of the newly synthesized protein, extracts from pulse-radiolabeled H1A/M-15 cells and H1A/RSVATZ-8 cells were subjected to sedimentation velocity centrifugation in 5–20% linear sucrose gradients. As expected, the newly synthesized normal PiM1 protein sedimented in the gradient (Fig. 9*A*) in a fashion similar to what would be expected for a 50-kDa monomer (Fig. 10*A*). In contrast, the newly synthesized PiZ variant sedimented as a much broader peak within the gradient (Fig. 9*B*). Quantitation of the radiolabeled bands revealed that whereas a small fraction (approximately 20%) of the newly synthesized PiZ variant exhibited a migration similar to that of the PiM1 protein, the majority of the macromolecules sedimented in a fashion that would be expected for a homotrimer of approximately 150 kDa (Fig. 10*B*). This result demonstrates that the newly synthesized PiZ variant does not exist as a monomer within the ER, but rather, forms soluble aggregates immediately following its synthesis.

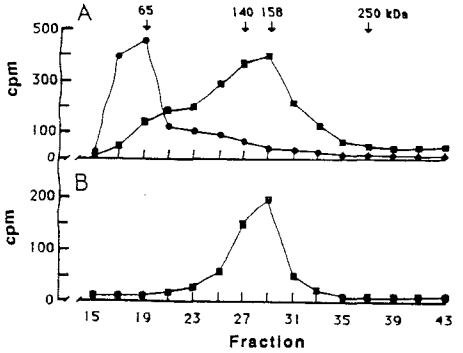

FIG. 10. **The PiZ variant sediments as an apparent oliogomer in 5–20% sucrose gradients.** Sedimentation profile of the PiM1 protein and PiZ variant immunoisolated following sedimentation velocity centrifugation of radiolabeled cell lysates as described in Fig. 8. *A*, H1A/M-15 cells (*solid circles*) and H1A/RSVATZ-8 cells (*solid squares*) pulse-radiolabeled for 8 min. *C*, H1A/RSVATZ-8 cells pulse-radiolabeled for 8 min and chased for 1.5 h (*solid squares*). *Numbers* at the top of *panel A* represent the sedimentation of various marker proteins as described under "Materials and Methods." The fraction containing the most abundant concentration of each marker protein is designated by an *arrow*. Bovine hemoglobulin, 65 kDa; calf alkaline phosphatase, 140 kDa; bovine γ-globulin, 158 kDa; bovine liver catalase, 250 kDa.

Next, we tested whether the newly synthesized PiZ underwent a greater degree of aggregation during the time course of its intracellular retention. For this, H1A/RSVATZ-8 cells were pulse-radiolabeled and then chased for 1.5 h, a time point when approximately 50% of the protein has been degraded (7). Analysis of the PiZ variant demonstrated that its relative rate of sedimentation through the gradient was similar to that of the newly synthesized protein (Fig. 9*C*). Thus, utilizing this methodology, we were unable to detect the formation of larger aggregates of the PiZ variant during the 1.5-h retention period.

DISCUSSION

Degradation of the PiZ Variant within the ER—Recently, we (7) reported that the addition of the tetrapeptide KDEL, but not KDAS, to the carboxyl terminus of a recombinant truncated PiZ variant protected the protein from intracellular degradation. Because there is evidence that the KDEL sequence is utilized as a specific signal for the retention of normal luminal proteins within the ER via their recycling from a post-ER salvage compartment back to the ER (34), we had interpreted these results as evidence that the PiZ variant exits the ER prior to its intracellular degradation. Thus, a major purpose of the present study was to identify the subcellular site where transport-impaired human AAT variants are retained and degraded. In the present study, results from subcellular fractionation experiments utilizing shallow discontinuous sucrose gradients in D_20 provided physical evidence that the retained protein was not delivered to the Golgi complex nor did it even enter light density post-ER transition vesicles during its intracellular retention. Pre-Golgi retention of the PiZ variant was also supported by the observation that the oligosaccharides of the protein do not become endo H-resistant in control cells and that nocodazole did not affect

its intracellular degradation. Because pulse-chase studies have indicated that degradation of the PiZ variant is initiated either prior to or concomitant with its size reduciton (16), these findings provide evidence that the retained protein is degraded in the ER. In light of this conclusion, one must question how the KDEL tetrapeptide can prevent the intracellular degradation of the truncated PiZ variant (7). One possible explanation for this phenomenon is that the KDEL tetrapeptide might cause the recombinant macromolecule to sequester in a region of the ER that is deficient in the appropriate proteolytic activity. Future studies will be directed toward elucidating the protective effect of the KDEL tetrapeptide.

It is now apparent from the work of several laboratories (5–8, 35) that many partially assembled membrane-associated proteins are retained and subsequently degraded within the ER. Similar to the degradation of the retained PiZ variant, Wikstrom and Lodish (21) have reported that the unassembled H2a subunit of the asialoglycoprotein receptor is degraded within the ER by a mechanism that is inhibited by perturbers of intracellular ATP levels and inhibitors of protein synthesis. Thus, it is possible that degradation of transport-impaired membrane-associated proteins and secretory proteins within the ER might follow a similar, but perhaps not identical, pathway. Furthermore, whether a specific segment of the aggregated PiZ variant functions as a "degradation signal" as has been demonstrated for the transmembrane domain of the α subunit of the T cell antigen receptor (36, 37), remains to be investigated.

Aggregation as a Putative ER Retention Signal—As described earlier, present experimental evidence suggests that the residence of a protein within the ER results from a "retention signal" associated with that macromolecule (4). Whereas immunoglobulin heavy chain binding protein has been implicated in retaining many misfolded and unassembled proteins within the ER (for a review, see Ref. 4), no association between binding protein and the PiZ variant has yet been identified (16, 38). As shown in the present study, inhibition of protein synthesis prevented degradation of the retained PiZ variant but did not allow any of the retained macromolecules to be secreted. Therefore, retention of the PiZ variant is permanent, and thus, so must be any retention signal associated with the macromolecule. Approximately 80% of the newly synthesized PiZ variant associates into soluble aggregates which exhibit a mobility in sucrose gradients that would be expected of a homotrimer. It is noteworthy that aggregation of the protein is permanent during the retention period and that the percent of the newly synthesized macromolecules that undergo this structural alteration correlates well with the amount that is retained. Thus at present, aggregation of the PiZ variant appears to be the most likely candidate to cause its retention within the ER. Although aggregation of the PiZ variant, a phenomenon that has also been identified in the secreted form of the protein (39), might result from the misfolding of the variant polypeptide, further experiments must be performed to substantiate this possibility.

As described earlier (16), a small fraction of the newly synthesized PiZ variant is deposited within the ER as degradation-resistant insoluble aggregates. A common ultrastructural feature for a class of diseases designated as the lysosomal storage disorders (40) is that undegraded material accumulates within swollen lysosomes because of the deficiency of a particular acid hydrolase that is involved in the normal degradation of the accumulating macromolecule. Thus, accumulation of the undegraded insoluble PiZ variant within distended cisternae of the ER could be considered as a type of "ER storage disorder" and, in the case of the PiZ variant, is associated with the development of liver disease (15, 17, 18). Obviously, in contrast to the lysosomal storage disorders, accumulation of the insoluble PiZ variant results from its inability to be degraded rather than because of a deficiency of a particular hydrolytic activity. Whether the insoluble PiZ accumulates because it cannot be hydrolyzed *versus* the possibility that this fraction of the protein never reaches a degradative region of the ER is, as yet, only speculative.

dMM-inhibitable Oligosaccharide Modification of the PiZ Variant in the ER—An unexpected characteristic concerning the size reduction of the retained PiZ variant within the ER was that this phenomenon could be inhibited by the mannose analogue dMM. Whereas α-mannosidase I associated with the *cis* Golgi compartment is inhibited by dMM (42), the activity of the well-studied ER α-mannosidase capable of trimming asparagine-linked oligosaccharides to the $Man_6GlcNAc_2$ structure is unaffected by this compound (26). However, Rizzolo and Kornfeld (27) have recently identified a dMM-inhibitable α-mannosidase activity associated within the ER of COS cells. Thus, it is possible that the oligosaccharides of the retained PiZ variant might serve as a substrate for this particular dMM-inhibitable ER α-mannosidase. The inability of dMM to affect the oligosaccharide trimming of the normal PiM1 protein when its ER-to-Golgi transport is blocked suggests that the oligosaccharides of the normal monomeric protein do not serve as a substrate for the dMM-inhibitable ER α-mannosidase. Whether this finding reflects a differential partitioning of the PiM1 and PiZ proteins within the ER will be the focus of further investigations.

REFERENCES

1. Walter, P., Gilmore, R., and Blobel, G. (1984) *Cell* **38**, 5–8
2. Wieland, F. T., Gleason, M. L., Serafini, T. A., and Rothman, J. E. (1987) *Cell* **50**, 289–300
3. Lodish, H. F., Kong, N., Hirani, S. and Rasmussen, J. (1987) *J. Cell Biol.* **104**, 221–230
4. Rothman, J. E. (1987) *Cell* **50**, 521–522
5. Lippincott-Schwartz, J., Bonafacino, J. S., Yuan, L. C., and Klausner, R. D. (1988) *Cell* **54**, 209–220
6. Amara, J. F., Lederkremer, G., and Lodish, H. F. (1989) *J. Cell Biol.* **109**, 3315–3324
7. Le, A., Graham, K. S., and Sifers, R. N. (1990) *J. Biol. Chem.* **265**, 14001–14007
8. Ivessa, N. E., de Lemos-Chiarandini, C. Tsao, Y.-S., Sabatini, D. D., and Kreibich, G. (1989) *J. Cell. Biol.* **109**, 207a
9. Sifers, R. N., Carlson, J. A., Clift, S. M., DeMayo, F. J., Bullock, D. W., and Woo, S. L. C. (1987) *Nucleic Acids Res.* **15**, 1459–1475
10. Carlson, J. A., Rogers, B. B., Sifers, R. N., Hawkins, H. K., Finegold, M. J., and Woo, S. L. C. (1988) *J. Clin. Invest.* **82**, 26–36
11. Perlmutter, D. H. and Pierce, J. A. (1989) *Am. J. Physiol.* **257**, L147-L162
12. Brantly, M., Nukiwa, Y., and Crystal, R. G. (1989) *Am. J. Med.* **84**, 13–31
13. Loebermann, H., Tukuoka, R., Deisenhofer, J., and Huber, R. J. (1984) *J. Mol. Biol.* **177**, 531–556
14. Nukiwa, T., Satoh, K., Brantly, M. L., Ogushi, F., Fells, G. A., Courtney, M., and Crystal, R. G. (1986) *J. Biol. Chem.* **261**, 15989–15994
15. Sharp, H. L., Bridges, R. A., Krivit, W., and Frier, E. F. (1969) *J. Lab. Clin. Med.* **73**, 934–939
16. Graham, K. S., Le, A., and Sifers, R. N. (1990) *J. Biol. Chem.* **265**, 20463–20468
17. Carlson, J. A., Rogers, B. B., Sifers, R. N., Finegold, M. J., Clift, S. M., DeMayo, F. J., Bullock, D. W., and Woo, S. L. C. (1989) *J. Clin. Invest.* **83**, 1183–1190
18. Dyaico, M. J., Grant, S. G. N., Felts, K., Nichols, W. S., Geller, S. A., Hager, J. H., Pollard, A. J., Kohler, S., W., Short, H. P., Jirik, F. R., Hanahan, D., Sorge, J. A. (1988) *Science* **242**, 1409–1412

©AP OCTMV2 BCA3$$552G BC-Jan-A3 94552 11/4-566ax cm-2674 10-30-91 13-45-57

19. Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muensch, H., and Woo, S. L. C. (1988) *J. Biol. Chem.* **263,** 7330–7335
20. Lippincott-Schwartz, J., Yuan, L. C., Bonafacino, J., S., and Klausner, R. D. (1989) *Cell* **56,** 801–813
21. Wikstrom, L., and Lodish, H. F. (1990) *J. Cell Biol.* **113,** 997–1007
22. Saunier, B., Kilker, R. K. Jr., Tkacz, J. S., Quaroni, A., and Herscovics, A. (1982) *J. Biol. Chem.* **257,** 14155–14161
23. Martin, R. G., and Ames, B. N. (1961) *J. Biol. Chem.* **236,** 1372–1379
24. Carrell, R. W., Jeppsson, J.-O., Laurell, C.-B., Brennan, S. O., Owen, M. C., Vaughn, L., and Boswell, D. R. (1982) *Nature* **298,** 329–334
25. Lodish, H. F., Kong, N., Snider, M., and Strous, G. J. A. M. (1983) *Nature* **304,** 80–83
26. Kornfeld, R., and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **54,** 631–664
27. Rizzolo, L. J., and Kornfeld, R. (1988) *J. Biol. Chem.* **263,** 9520–9525
28. Beckers, C. J. M., and Balch, W. E. (1989) *J. Cell Biol.* **108,** 1245–1256
29. Balch, W. E., Elliott, M. M., and Keller, D. S. (1986) *J. Biol. Chem.* **261,** 14681- 4689
30. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) *J. Cell Biol.* **111,** 2295–2306
31. Dunphy, W. G., and Rothman, J. E. (1985) *Cell* **42,** 13–21
32. De Brabander, M. J., Von de Veire, R. M. L., Aerts, F. E. M., Borgers, M., and Janssen, P. N. J. (1976) *Cancer Res.* **36,** 905–916
33. Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H-P., Yuan, L. C., and Klausner, R. D. (1990) *Cell* **60,** 821–836
34. Pelham, H. R. B. (1988) *EMBO J.* **7,** 913–918
35. Wileman, T., Carson, G. R., and Concino, M. (1990) *J. Cell. Biol* . **110,** 973–986
36. Bonifacino, J. S., Suzuki, C. K., and Klausner, R. D. (1990) *Science* **247,** 79–82
37. Bonafacino, J. S., Cosson, P., and Klausner, R. D. (1990) *Cell* **63,** 503–513
37. Bischoff, J., Liscum, L., and Kornfeld, R. (1986) *J. Biol. Chem.* **261,** 4766–4774
38. McCracken, A. A., Kruse, K. B., and Brown, J. L. (1989) *Mol. Cell. Biol.* **9,** 1406–1414
39. Cox, D. W., Billingsley, G. D., and Callahan, J. W. (1986) *FEBS Lett.* **205,** 255–260
40. Miller, A. L., Freeze, H. H., and Kress, B. C. (1981) in *Lysosomes and Lysosomal Storage Diseases* (Callahan, J. W., and Lowden, J. A., eds) pp. 271–288, Raven Press, New York