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ANNUAL REPORT ON THE PROGRESS OF GRANT #1-89-0040 (Moran Foundation)

Title of project: Intracellular Transport of Alpha$_1$-antitrypsin

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

I. PROGRESS REPORT

A. Intracellular degradation of transport-impaired variants of human alpha$_1$-antitrypsin variants - In our analysis to analyze the normal mechanism of secretion of normal human alpha$_1$-antitrypsin (AAT) from cells, we began to characterize variants of that protein that are hindered in their secretion. We've utilized mouse hepatoma cells that are stably transfected with the transport-impaired human AAT variants designated PiZ and Pi Null$_{Hong\ Kong}$, to study the fate of these macromolecules. Pulse/chase analysis of the metabolically radiolabeled normal and transport-impaired human AAT variants have been performed. Approximately 85% of the newly synthesized PiZ variant and all of the Pi Null$_{Hong\ Kong}$ variant is retained within the cell, rather than secreted. Interestingly, the retained fraction of either variant protein undergoes degradation that is initiated after a 30 minute lag period following protein synthesis. Once degradation has been initiated, the intracellular half-life for either protein is approximately 45-60 minutes.

B. Evidence that lysosomal acid hydrolases are not involved in the intracellular degradation of the transport-impaired variant proteins - Previous studies have shown that growth of cells in the weak bases chloroquine or NH_4Cl will raise the pH of all acidic intracellular compartments, including lysosomes. However, neither of these drugs has a significant effect on the degradation of the retained human AAT variants. Likewise, treatment of cells with leupeptin and leucine methyl ester, potent inhibitors of lysosomal proteolytic activity, do not effect the degradation of the retained proteins. These findings suggest that lysosomes are not involved in this degradative pathway. Finally, the inability of colchicine to inhibit degradation has indicated that lysosomal autophagy is not involved in this degradative pathway.

C. Degradation of the retained human AAT variants occurs in a pre-Golgi compartment - It has been established that N-linked oligosacharides of glycoproteins in the endoplasmic reticulum (ER) exhibit sensitivity to digestion by endoglycosidase H. However, sensitivity of the oligosaccharides to this enzyme is lost when the protein has been transported to the medial stacks of the Golgi complex. The action of glycolytic enzymes present in this subcellular compartment alter the structure of these oligosaccharides such that they no longer serve as substrates for endo H. Thus, we examined the endo H-sensitivity of the oligosaccharides of the retained PiZ and Pi Null$_{Hong\ Kong}$ variants at one hour following their synthesis which was approximately equivalent to their intracellular half-lives. Our studies have shown that the oligosaccharides of either protein continue to be sensitive to digestion by endo H.

Of course, it is entirely possible that the oligosaccharide moieties of these transport-

impaired variant proteins (possibly malfolded) might be sterically hindered for recognition by glycolytic enzymes present in the Golgi. In such a case the endo H sensitivity of *these structures* would not serve as an accurate method to eliminate the possibility of ER-to-Golgi transport of *the retained proteins prior to degradation. Thus, as a more accurate method, degradation of the* PiZ variant was examined following the treatment of cells with media containing a variety of inhibitors of ER-to-Golgi vesicular transport. These drugs included the carboxylic ionophore monensin, the K^+/H^+ exchanger nigericin, and the proton ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), all of which inhibit ER-to-Golgi vesicular transport by a variety of mechanisms. However, none of these drugs significantly alter the kinetics of the degradation of the PiZ variant at concentrations that prevented the secretion the PiM protein.

D. Subcellular degradation of the transport-impaired human AAT variants exhibits pH sensitivity - Although our studies suggested that lysosomes are not involved in the degradation of the transported-impaired human AAT variants (Section B), we tested whether an intracellular pH change would affect this degradative process. For this study, the pH of all subcellular compartments was "clamped" by incubating pulse radiolabeled PiZ-bearing cells in an isotonic solution containing 0.14 M KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 50 uM nigericin (a K^+/H^+ exchanger), and 100 uM valinomycin (a K^+ ionophore), buffered with 50 mM HEPES-HCl at either pH 6, 7, or 8. Pulse labeled cells chased in the isotonic solution at pH 7 demonstate the normal kinetic degradation profile of the PiZ variant. Interestingly however, degradation of the PiZ variant is greatly inhibited when cells are chased at pH 8. In contrast, degradation is markedly accelerated when all subcellular compartments are chased at pH 6. This accelerated degradation at acidic pH is specific for the mutant human AAT variants because this manipulation does not cause the degradation of the normal PiM human AAT protein.

E. Biochemical mapping of the degradative event among compartments of the secretory pathway - Existence of the tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL) at the carboxyl terminus of a soluble protein within the ER functions as a signal for the re-cycling of the protein from a post-ER compartment back to the ER. Therefore, we utilized this sequence to determine whether transport-impaired variants of human AAT exit the ER prior to their intracellular degradation. DNA constructs were prepared that code for the synthesis of a truncated 49 kDa PiZ variant containing either AKDEL or AKDAS as the carboxyl terminal amino acids. The sequence AKDAS (Ala-Lys-Asp-Ala-Ser) was chosen as a negative control sequence because it will not function in the ER-recycling phenomenon. Pulse-chase analysis of mouse hepatoma cells transfected with either of the DNA constructs has revealed that whereas the protein bearing the terminal AKDAS sequence undergoes degradation, the addition of AKDEL totally abolishes the degradation of the misfolded protein. Conceivably, for the AKDEL signal to exhibit an influence on degradation, the mutant protein must exit the ER prior to degradation.

F. Subcellular deposition and degradation of an insoluble fraction of the PiZ variant - Although our studies have demonstrated that the retained fraction of the PiZ variant undergoes degradation, the protein accumulates as large insoluble aggregates within distended cisternae of the hepatic endoplasmic reticulum. The intrahepatic accumulation of the insoluble protein can act as an etiologic agent toward the development of liver disease in humans and transgenic mice. The accumulation of this insoluble protein suggests that a fraction of the newly synthesized PiZ variant must be deposited within the cell at some step along the secretory pathway. The timecourse of the deposition of the insoluble PiZ variant was analyzed by metabolically radiolabeling the PiZ variant in primary hepatocytes isolated from PiZ-bearing transgenic mice. Then, the insoluble protein was extracted from the particulate fraction of the cell lysate

and subsequently identified by immunoprecipitation. The results of this study have shown that <1% of the newly synthesized PiZ variant is actually deposited in cells. Therefore, we conclude that the accumulation of the insoluble protein occurs gradually over a long period of time. The fact that the insoluble protein is intact (i.e., has not been subjected to degradation) and accumulates in swollen cisternae of the hepatic ER provides further evidence that degradation of this protein occurs in a post-ER compartment.

II. PRESENT STATUS OF THE RESEARCH PROJECT

The total amount of support ($7000) has been spent. Therefore, this project is no longer active.

III. PUBLICATIONS AND PRESENTATIONS RESULTING FROM THIS FUNDING

A. PRESENTATIONS

Le, A., Graham, K.S., and Sifers, R.N. (1989). The PiZ variant of human alpha-1-antitrypsin undergoes intracellular degradation within a pre-Golgi compartment. *J. Cell Biol.* 109, 188a. (Given as an oral presentation at the 1989 Annual Cell Biology Meeting in Houston).

B. PUBLICATIONS

Le, A., Graham, K.S., and Sifers, R.N. (1990). Intracellular degradation of the transport-impaired PiZ human alpha-1-antitrypsin variant: biochemical mapping of the degradative event among compartments of the secretory pathway. *J. Biol. Chem.* 265, 14001-14007.

Graham, K.S., Le, A., and Sifers, R.N. (1990). Accumulation of the insoluble PiZ variant of human alpha-1-antitrypsin within the hepatic endoplasmic reticulum does not elevate the steady-state level of grp78/BiP. *J. Biol. Chem.* (in press).

Copies of the above manuscripts/articles are included.

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Intracellular Degradation of the Transport-impaired Human PiZ α_1-Antitrypsin Variant

BIOCHEMICAL MAPPING OF THE DEGRADATIVE EVENT AMONG COMPARTMENTS OF THE SECRETORY PATHWAY*

(Received for publication, February 8, 1990)

Anhquyen Le‡, Kathleen S. Graham‡§, 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

The naturally occurring PiZ and Pi Null$_{Hong\ Kong}$ variants of the human secretory protein α_1-antitrypsin (AAT) are retained within an early compartment of the secretory pathway. Intracellular degradation of these transport-impaired secretory proteins is initiated 30–45 min following their synthesis and translocation into the endoplasmic reticulum (ER). Interestingly, the overall rate of degradation of the retained mutant protein is significantly accelerated when all subcellular compartments are buffered at pH 6. In contrast, degradation is virtually abolished when intravesicular compartments are buffered at pH 8. However, despite this pH sensitivity neither lysosomotrophic amines, leupeptin, or leucine methyl ester have an apparent effect on the intracellular removal of the PiZ variant. The inability of a variety of inhibitors of ER-to-Golgi protein trafficking to hinder the degradative process suggests that degradation of the PiZ variant occurs prior to its delivery to the Golgi complex. To biochemically map the subcellular site of the degradation of the retained mutant protein, a recombinant truncated PiZ variant containing the tetrapeptide KDEL at its carboxyl terminus (a signal for sorting luminal proteins from a post-ER compartment back to the ER) was expressed in cells. Attachment of this ER-recycling signal to the recombinant protein prevented its intracellular degradation. These findings indicate that degradation of the PiZ variant occurs following its export from the ER.

The secretion of proteins from eukaryotic cells occurs via their bulk flow through an ordered series of membrane-bound subcellular compartments (1, 2). Proteins enter this secretory pathway during their biosynthesis via translocation into the endoplasmic reticulum (ER).[1] During transit through the ER, proteins fold into their correct conformation and may undergo a variety of covalent modifications (3, 4). During early stages of this pathway, molecular "decisions" are made to determine the fate of each newly synthesized protein. For example, many soluble luminal ER proteins exhibit the tetrapeptide sequence Lys-Asp-Glu-Leu (KDEL) at their carboxyl terminus which functions as a signal for their retrieval from a post-ER "salvage" compartment back to the ER (5, 6). An additional regulatory mechanism involves the subcellular retention and subsequent degradation of many misfolded proteins and incompletely assembled protein complexes at an early stage of the secretory pathway (7–10).

Human α_1-antitrypsin (AAT) is a major hepatic secretory protein (11). It functions as the predominant serine protease inhibitor in human sera (12). Human AAT is synthesized as a single polypeptide chain that is modified by the covalent addition of three *N*-linked oligosaccharides during its transit through the ER (13). In addition to the "trimming" of terminal sugars from the oligosaccharide moieties (3, 13), the removal of an amino-terminal signal peptide (14) and the proper folding of the macromolecule must occur (2) prior to its entrance into transition vesicles *en route* to the Golgi complex (15).

Human AAT is a very polymorphic protein. At present, over 75 electrophoretic variants have been identified (16). The entire amino acid sequence of normal human AAT, designated PiM, is known (14) and several variants have been identified that contain mutations that result in the deficiency or complete absence of this protein from sera. In many cases, this deficiency results from the retention and degradation of a mutant AAT variant within cells (for a review, see Ref. 17). In the present study, we have analyzed the fate of the retained fraction of two transport-impaired variants of human AAT. The PiZ variant contains a Glu to Lys substitution at residue 342 (18, 19) that hinders its secretion from cells (20–23). A frameshift mutation in the human AAT allele designated Pi Null$_{Hong\ Kong}$ results in the synthesis of a truncated protein that is devoid of an active inhibitory site. Secretion of the Pi Null$_{Hong\ Kong}$ variant is completely abolished (24). Here we report that the retained fraction of these transport-impaired secretory proteins are removed from the cell via a pathway that is apparently unaffected by inhibitors of lysosomal protein degradation and inhibitors of ER-to-Golgi intracellular protein transport. A degradative pathway bearing similar kinetic and biochemical characteristics has recently been identified in the removal of unassembled subunits of several multimeric membrane proteins which include chains of the T cell receptor (TCR) (9), a truncated form of ribophorin I (25), and the H2 subunit of the asialoglycoprotein receptor (10).

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§ A 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. Tel.: 713-798-3168.

[1] The abbreviations used are: ER, endoplasmic reticulum; AAT, α_1-antitrypsin; endo H, endo-β-*N*-acetylglucosaminidase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis. TCR, T cell receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Finally, the intracellular stability of a recombinant truncated PiZ variant exhibiting KDEL as the carboxyl-terminal amino acids has indicated that degradation of the retained PiZ variant occurs in a post-ER compartment.

MATERIALS AND METHODS

Chemicals and Reagents—All DNA restriction and modifying enzymes were purchased from either Pharmacia LKB Biotechnology Inc. or Boehringer Mannheim. Chemicals utilized for the inhibition of lysosomal proteolytic activity or for the inhibition of *ER-to-Golgi* vesicular protein trafficking were purchased from Sigma. L-[^{35}S] Methionine (specific activity > 800 Ci/mmol) was purchased from Du Pont-New England Nuclear. Protein G-Sepharose 4FF was obtained from Pharmacia. All tissue culture products were procured from GIBCO.

Construction of a Human AAT Expression Vector—The bacterial chloramphenicol acetyltransferase (CAT) gene was removed from pRSVCAT (26) following digestion with *Hin*dIII plus *Bal*I. A genetically engineered human AAT cDNA containing a Glu^{342}-Lys^{342} substitution (22) was blunt-end ligated to the linearized plasmid and designated pRSVATZ. All methodology used in the preparation of this DNA construct was identical to the production of the human α_1-antitrypsin expression vector controlled by the simian virus promoter as described previously (22). Transcription of DNA coding for human AAT was regulated by the Rous sarcoma virus long terminal repeat element (26) which resulted in high levels of protein synthesis.

Cultured Cells—The mouse hepatoma cell line Hepa 1a was stably transfected with pRSVATZ by co-transfection with pSV$_2$NEO according to a technique described by Chu and Sharp (27). Growth of transfected cells with the antibiotic G418 allowed for the isolation of a single clonal cell line synthesizing the highest level of the PiZ variant as determined by pulse labeling/immunoprecipitation analyses. This clonal cell line was designated H1A/RSVATZ-8 and was used for experimentation. The previously developed stably transfected mouse hepatoma cell lines H1A/M15 and H1A/N13 (24) were used for analysis of the intracellular fate of the normal (Pi M) and Pi Null$_{\text{Hong Kong}}$ variant of human α_1-antitrypsin, respectively.

Manipulating the pH of All Subcellular Compartments—The intravesicular pH of all subcellular compartments was "clamped" by a technique described by Sandvig *et al.* (28). Briefly, pulse-labeled cells were incubated at 37 °C in a solution containing 0.14 M KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 50 μM nigericin (a K^+/H^+ exchanger), 100 μM valinomycin (a K^+ ionophore), and 40 mM HEPES-HCl in order to regulate the pH of all intravesicular cellular compartments. Because this treatment resulted in some cellular toxicity as judged by trypan blue exclusion experiments, cells present in the media during later chase periods were collected and combined with cells from the monolayer for final analyses.

Construction of Recombinant DNA Coding for the Synthesis of a Truncated PiZ Variant Containing a Signal That Functions in the Recycling of Luminal Proteins to the ER—A genetically engineered PiZ human AAT cDNA (22) was ligated to the *Eco*RI site of pUC18 and designated phATZc/pUC18. The recombinant plasmid was digested with *Ava*I which cut once at the pUC18 polylinker plus once in the cDNA coding for amino acids Pro^{362} and Glu^{363} of the PiZ variant (14). Two sets of oligonucleotides were synthesized (Genetic Designs, Houston, TX) and annealed that coded for the synthesis of either Ala-Lys-Asp-Glu-Leu (AKDEL) or a control sequence Ala-Lys-Asp-Ala-Ser (AKDAS) followed by a nonsense codon (Fig. 6). These were directionally ligated to the *Ava*I-digested phATZc/pUC18 as a result of nonidentical *Ava*I-compatible ends on the annealed oligonucleotides. A 1.2-kilobase DNA fragment coding for either the recombinant truncated PiZ-AKDEL or truncated PiZ-AKDAS protein was generated by digestion of the plasmid with *Eco*RI plus *Sal*I (Fig. 6) and blunt-end ligated to the *Hin*dIII/*Bal*I-digested pRSVCAT described above. The resulting DNA clones were designated pRSVATZ(*Ava*I)-AKDEL and pRSVATZ(*Ava*I)-AKDAS, as appropriate. The efficiency of transfection of Hepa 1A cells by these DNA was quantitated by co-transfection with pCMV-β-Gal (29). β-Galactosidase activity was assayed at 37 °C in 0.1 ml of a solution containing 0.1 M Tris-HCl (pH 7.5), 5 μl of cell extract, and 1 mg/ml *p*-nitrophenyl-β-galactoside (Sigma). β-Galactosidase activity was measured as an increase of absorbance at 420 nm caused by the production of *p*-nitrophenol.

Metabolic Labeling and Immunoprecipitation of Human AAT—For pulse-chase studies, cells were grown in 60-mm diameter dishes and metabolically labeled with 100–200 μCi of L-[^{35}S]methionine/dish. *Human AAT was quantitatively immunoprecipitated from the soluble* cell extract and media of individual dishes using an excess of specific antisera and protein G-Sepharose 4FF as described previously (22). In some cases, immunocomplexes were digested overnight with endoglycosidase H as described previously (22, 30). Immunocomplexes were subjected to SDS-PAGE (8% polyacrylamide) and radiolabeled human AAT was detected by fluorographic enhancement of the gels. *Quantitation of immunoprecipitated protein was performed by* scintillation counting of bands excised from gels. Methodology for the *treatment of cells with particular drugs prior to and during experi*ments is described in the legend to Table I.

RESULTS

Intracellular Degradation of Retained Human AAT Variants Is Initiated following a Lag Period—The fate of normal human AAT (PiM) and the retained fraction of the PiZ and truncated Pi Null$_{\text{Hong Kong}}$ variants was analyzed by pulse-chase analysis in stably transfected mouse hepatoma cells. As expected, >90% of the PiM protein was secreted from these cells within 30 min following synthesis (Fig. 1). The apparent higher molecular mass of the secreted protein (56 kDa) results from the addition of charged sialic acid residues to oligosacharides during the transit of the protein through a later compartment of the Golgi complex (24). In contrast, however, all of the newly synthesized PiZ variant was retained within cells during the same period (Fig. 1). Following an initial lag, a gradual loss of the intracellular PiZ variant was observed. Overexposure of the film allowed for the detection of a small amount of the 56-kDa form of the protein present in the medium (Fig. 1). Scintillation counting of the excised radiolabeled bands demonstrated that only 10–15% of the newly synthesized PiZ variant was actually secreted during the 3-h chase (quantitation not shown). These findings suggested that the majority of the retained protein underwent degradation. To verify this assumption, the fate of the truncated Pi Null$_{\text{Hong Kong}}$ variant was also examined. No radiolabeled proteins were detected in the media from these cells, even after 3 h of incubation (Fig. 1). This was expected because previous studies have demonstrated that the entire fraction of the Pi Null$_{\text{Hong Kong}}$ variant is retained within cells (24). Identical to the intracellular fate of the retained PiZ variant, degradation of the truncated Pi Null$_{\text{Hong Kong}}$ variant was not immediate but rather was initiated following a similar lag period (Fig. 1). For either protein, the duration of the lag was normally 30–45 min. As detected

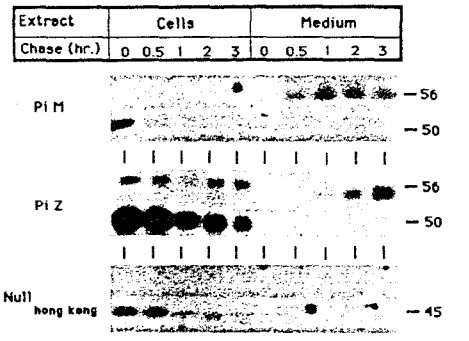

FIG. 1. **Retained human AAT variants undergo degradation.** Mouse hepatoma cells stably transfected with either the normal human AAT gene (*Pi M*) (cell line H1A/M15), the Pi Z variant (cell line H1A/RSVATZ-8), or the Pi Null$_{\text{Hong Kong}}$ variant (cell line H1A/N13) were subjected to pulse-chase studies as described under "Materials and Methods." At various timepoints during the chase period, human AAT was quantitatively immunoprecipitated from cell extracts and medium from each dish. Immunocomplexes were fractionated by SDS-PAGE (8% polyacrylamide) and radiolabeled human AAT was detected by fluorography. An overexposed film is shown in *panel PiZ*. The apparent molecular mass of each protein is shown.

TABLE I

Effect of the inhibition of lysosomal proteolytic activity and ER-to-Golgi protein transport on the degradation of the retained PiZ variant

Monolayers of H1A/RSVATZ-8 cells were preincubated in regular growth medium containing the indicated drug prior to pulse-chase experiments as described under "Materials and Methods." Each drug was present in the medium throughout the entire experiment unless otherwise indicated. Immunoprecipitated human AAT was detected by fluorography following SDS-PAGE and quantitated by scintillation counting of the excised radiolabeled bands.

Treatment[a]	% inhibition of degradation[d]
Control	0
Inhibitors of lysosomal degradation or autophagy	
NH_4Cl (2–50 mM)	10
Chloroquine (0.1 mM)	8
Leupeptin (0.1 mg/ml)	7
Leucine methyl ester (5–20 mM)[b]	11
Colchicine (10^{-5} M)	7
Inhibitors of ER-to-Golgi protein traffic[c]	
Monensin (2 μM)	18
Nigericin (50 μM)	11
CCCP (0.2 μM)	13

[a] The preincubation of cells was for 60 min prior to radiolabeling, unless otherwise indicated.

[b] Preincubation was for 30 min.

[c] Under these conditions, the amount of the PiM protein secreted into the medium following a 2-h chase was <5% of that secreted by control cells.

[d] The percent inhibition of degradation over a 3-h period is compared to control cells.

by SDS-PAGE, a gradual reduction of the apparent molecular mass of both the retained PiZ and Pi $Null_{Hong\ Kong}$ variants was observed during the middle and later stages of this process (Fig. 1). It should be noted that although the PiZ variant can be detected accumulating within the ER as an insoluble aggregate in cells (30), its disappearance from cells as shown here, results entirely from its degradation, rather than from its insolubility. Preliminary studies using primary hepatocytes from PiZ-bearing transgenic mice have shown that only a small fraction of the PiZ variant is deposited intracellularly and accumulates gradually over a long period of time.[2] This conclusion is supported by the observation that the kinetics of the intracellular degradation of the Pi $Null_{Hong\ Kong}$ variant, which does not form an insoluble aggregate within cells (31), is identical to that of the PiZ variant (Fig. 1). Therefore, the actual subcellular deposition of the protein is not demonstrable in these pulse-chase studies.

Inhibitors of Lysosomal Proteolytic Activity Exhibit No Apparent Effect on the Degradation of the Retained PiZ Variant—It is well established that the incubation of cells with weak bases results in raising the pH of all acidic subcellular compartments, including lysosomes (32, 33). Because the optimal activity of many lysosomal proteases requires a very acidic environment (33), we analyzed the degradation of the retained PiZ variant in cells that were pre-incubated in media containing concentrations of NH_4Cl or chloroquine that were sufficient to raise lysosomal pH (33). As shown in Table I, incubation of cells with either of these weak bases had no significant effect on the degradation of the retained PiZ variant (Table I).

Certainly, increasing intralysosomal pH serves as a less than conclusive method to verify whether lysosomal proteases are involved in the degradation of the retained PiZ variant. Therefore, cells were treated with medium containing leupeptin which inhibits the proteolytic activity of lysosomal cathepsins B, H, L, N, S, and T (33, 34). Furthermore, as an additional test, cells were incubated in medium containing leucine methyl ester under conditions that have been reported to irreversibly inhibit total lysosomal degradative function in hepatocytes (33, 35). However, treatment with leupeptin, primaquine, or leucine methyl ester had no apparent influence on the degradation of the retained protein (Table I). It should be noted that even when higher concentrations of drugs were used resulting in a partial inhibition of protein synthesis, degradation of the small amount of protein that was synthesized exhibited the normal kinetics of degradation (data not shown). Finally, we tested whether media containing colchicine would interfere in the degradation of the retained PiZ variant. The inability of this drug to effect the removal of the retained protein (Table I) suggested that lysosomal autophagy (36) has little influence on this degradative process.

Duration of the Pre-degradation Lag Period Exhibits a pH-sensitive Component—Although raising the pH of acidic subcellular compartments had little influence on the degradation of the retained PiZ variant (Table I), we tested whether altering the pH of all intravesicular compartments would affect this process. Utilizing a technique described by Sandvig *et al.* (28), the pH of all subcellular compartments was controlled by chasing pulse-labeled cells in a buffered isotonic KCl solution containing nigericin (a K^+/H^+ exchanger) and valinomycin (a K^+ ionophore). Under these conditions the pH of all intravesicular compartments is identical to the pH of the extracellular milieu (9, 28). Application of this technique did not significantly alter the kinetics of degradation of the PiZ variant when pulse-labeled H1A/RSVATZ-8 cells were chased in this isotonic solution buffered at pH 7 (Fig. 2*A*). Although a slightly longer lag period of 50–60 min was exhibited (Fig. 2*A*), this was not necessarily a direct result of the procedure because slight variations in the duration of the lag period have been observed in control dishes of other experiments (data not shown). In contrast to these results, degradation of the retained protein was greatly inhibited when cells were chased at pH 8 (Fig. 2*A*). Conversely, the apparent rate of degradation of the retained PiZ variant was greatly accelerated when cells were chased at pH 6 (Fig. 2*A*). Quantitation of these findings has demonstrated that under these latter conditions there is a significant reduction in the duration of the pre-degradation lag period (Fig. 3). Concomitant with these findings is the observation that the entire population of the retained PiZ variant exhibits a distinct apparent size reduction following 30 min of chase at pH 6 (Fig. 2*A*). These findings are in contrast to the gradual apparent size reduction of both the retained PiZ and Pi $Null_{Hong\ Kong}$ variants that is normally exhibited during the middle and later stages of the degradative process (Fig. 1).

To determine whether this pH sensitivity is specific to the degradation of the retained PiZ variant, rather than actually reflecting the activation of a nonspecific degradative mechanism, H1A/M15 cells were subjected to the same procedure. As expected because of the presence of nigericin in the chase buffer, the PiM protein was not secreted from cells (Fig. 2*B*). However, unlike the PiZ variant, the PiM protein remained stable for 3 h when chased at either pH 7 or 6 (Fig. 2*B*). The stability of the PiM protein under these conditions suggests that the increased rate of degradation of the PiZ variant at pH 6 did not reflect the action of nonspecific proteolytic activity. Because this technique was somewhat toxic to the cells, additional experiments were performed whereby the intravesicular compartments of pulse-labeled H1A/RSVATZ-8 cells were regulated by chasing them in media containing 0.2 μM carbonyl cyanide *m*-chlorophenylhydrazone and buff-

[2] R. Sifers, unpublished data.

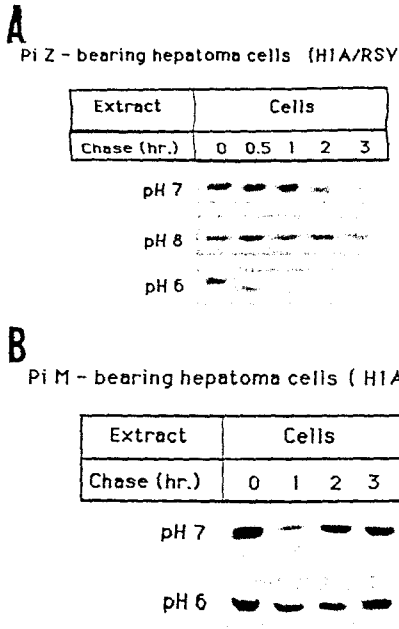

FIG. 2. **The pre-degradation lag period is sensitive to changes in intravesicular pH.** Confluent dishes of H1A/RSVATZ-8 cells (*A*) and H1A/M15 cells (*B*) were pulse-labeled with 100–200 μCi of [^{35}S]methionine for 10 min and chased at 37 °C in a solution containing 0.14 M KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 50 μM nigericin (a K^+/H^+ exchanger), 100 μM valinomycin (a K^+ ionophore), and 40 mM HEPES-HCl to regulate the pH of all intravesicular compartments (see Materials and Methods). Under these conditions, protein secretion was blocked (data not shown). At various timepoints, human AAT was immunoprecipitated from the cell extract and medium from each dish. Immunoprecipitates were subjected to SDS-PAGE (8% polyacrylamide) and human AAT was detected by fluorography. The intracellular 50-kDa human AAT band is shown in each panel. The weak intensity of the band shown in *B* at pH 7 resulted from an accidental spill of the sample.

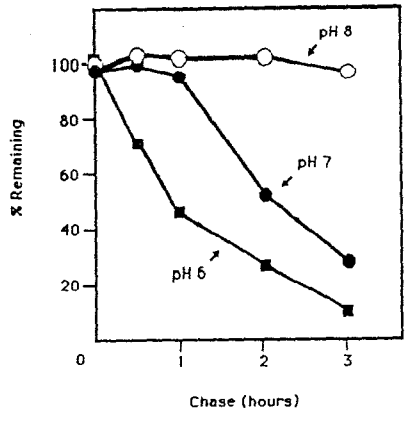

FIG. 3. **Quantitation of the pH-sensitive kinetics of degradation of the retained PiZ variant.** Quantitation of the pH-sensitive degradation of the retained PiZ variant was performed by scintillation counting of the bands excised from the gel shown in Fig. 2*A*. The percent of immunoprecipitable protein is shown as a function of time during the chase period at various intravesicular pH.

ered with 0.1 M HEPES as described by Lippincott-Schwartz *et al.* (9). Similar results were observed using this methodology (data not shown). Overall, these data indicate that the initiation of the intracellular degradation of the retained PiZ variant exhibits a pH-sensitive component. Our aforementioned findings that neither lysosomotrophic amines nor specific inhibitors of lysosomal proteolytic activity have an affect on the degradation of the retained PiZ variant suggests that the pH-sensitive component of this degradative pathway is nonlysosomal.

Asparagine-linked Oligosaccharides of Retained Human AAT Variants Do Not Acquire Resistance to Digestion by Endoglycosidase H—The ER is considered to function as the compartment for the retention of transport-impaired human AAT variants (17). However, considering the presence of the lag period plus its apparent pH sensitivity, it is conceivable that the retained PiZ variant could be sorted to a post-ER compartment for the actual degradative event. It has been established that *N*-linked oligosacharides of glycoproteins in the ER exhibit sensitivity to digestion by endoglycosidase H (endo H) (37). The action of glycolytic enzymes present in the medial stacks of the Golgi complex alter the structure of these oligosaccharides such that they no longer serve as substrates for endo H (37). Thus, we examined the endo H sensitivity of the oligosaccharides of the retained PiZ and Pi $Null_{Hong\ Kong}$ variants at 1 h following their synthesis which was approximately equivalent to their intracellular half-lives. Neither protein exhibited oligosaccharide moieties that were resistant to digestion by this enzyme (Fig. 4).

Inhibition of ER-to-Golgi Vesicular Transport Has No Affect on the Degradation of the Retained PiZ Variant—Of course, it is entirely possible that the oligosaccharide moieties of a misfolded protein might be sterically hindered for recognition by glycolytic enzymes present in the Golgi. In such a case the endo H sensitivity of oligosaccharides would not serve as an accurate method to eliminate the possibility of ER-to-Golgi transport of the retained proteins prior to degradation. Thus, as a more accurate method to test this possibility, degradation of the PiZ variant was examined following the treatment of cells with media containing the carboxylic ionophore monensin which is a proven inhibitor of ER-to-Golgi vesicular transport (9, 38). Addition of 2 μM monensin to the medium of H1A/M15 cells completely eliminated the secretion of the normal PiM human AAT over a 3-h period (Fig. 5*A*), and its oligosaccharides remained sensitive to digestion with endo H (data not shown). However, treatment of H1A/RSVATZ-8 cells with monensin had no effect on the kinetics of the degradation of the retained PiZ variant (Fig. 5*B*, Table I). These results suggest that the degradation of the retained PiZ variant occurs in a pre-Golgi compartment.

To further test this hypothesis, H1A/RSVATZ-8 cells were incubated in regular growth media containing either the K^+/H^+ exchanger nigericin or the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone, both of which inhibit ER-to-Golgi vesicular transport (39). However, neither of these drugs significantly altered the kinetics of the degradation of the PiZ variant at concentrations that prevented the secretion the PiM protein (Table I). In each case, the intracellular half-life of the retained PiZ variant has differed no more than 20% of normal. Furthermore, a lag was always observed prior to the onset of degradation (data not shown).

Attachment of KDEL, But Not KDAS, Prevents the Intra-

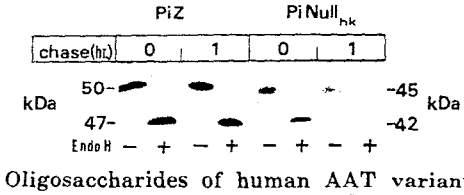

FIG. 4. **Oligosaccharides of human AAT variants retain sensitivity to digestion with endoglycosidase H.** Immunocomplexes from the 0 and 1-h chase periods from H1A/RSVATZ-8 cells and H1A/N-13 cells were subjected to digestion with endoglycosidase H. Samples were fractionated by SDS-PAGE (8% polyacrylamide) and radiolabeled proteins were detected by fluorography. The apparent molecular mass of each protein is shown.

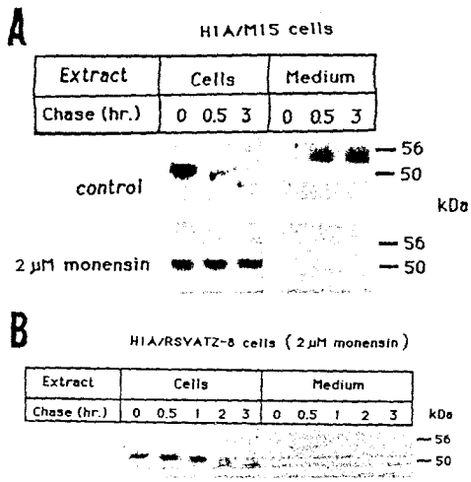

FIG. 5. **Treatment of cells with monensin has no effect on the degradation of the retained PiZ variant.** Confluent monolayers of H1A/M15 cells (*A*) and H1A/RSVATZ-8 cells (*B*) were preincubated for 60 min in regular growth medium containing 2 μM monensin. Cells were then subjected to pulse labeling and chased as described under "Materials and Methods" except that monensin was present in all media. At various timepoints, human AAT was immunoprecipitated from the cell extract and medium from each dish. Immunoprecipitates were subjected to SDS-PAGE (8% polyacrylamide) and human AAT was detected by fluorography. The apparent molecular mass of each proteins is indicated.

cellular Degradation of a Transport-impaired Truncated PiZ Variant—To determine whether the ER functions as the compartment of pre-Golgi degradation, we took advantage of the recent findings of Pelham (6) showing that the carboxyl-terminal sequence Lys-Asp-Glu-Leu (KDEL) functions as a signal that is necessary and sufficient to sort lumenal ER proteins from soluble secretory proteins in a biochemically distinct post-ER salvage compartment. Recombinant DNA constructs designated pRSVATZ(*Ava*I)-AKDEL and pRSVATZ(*Ava*I)-AKDAS were prepared that code for the synthesis of a truncated PiZ variant containing either AKDEL or AKDAS as the carboxyl-terminal amino acids, respectively (Fig. 6*A*). Truncated recombinant proteins bearing the Glu^{342} to Lys^{342} substitution were utilized in this study because all naturally occurring truncated human AAT variants exhibit an impaired intracellular transport (17). The DNA construct coding for the synthesis of a recombinant protein bearing Lys-Asp-Ala-Ser (KDAS) as the carboxyl-terminal amino acids was utilized as a negative control in this experiment because this tetrapeptide does not function in the recycling of luminal ER proteins (5).

Both DNA constructs were independently used in the transfection of the mouse hepatoma cell line Hepa 1a which had been used for all of the analyses of the degradation of the retained PiZ variant. The transfection efficiency for either DNA construct was analyzed as described under "Materials and Methods." Transfected cells were radiolabeled with [^{35}S] methionine for 6 h in order to detect the transiently expressed proteins. Using antisera to human AAT, a radiolabeled protein of the predicted size (49 kDa) was immunoprecipitated from the cell extract of cells transfected with either DNA construct (Fig. 6*B*). A very faint diffuse band of 55 kDa was present in the media of cells transfected with pRSVATZ(*Ava*I)-AKDAS DNA (Fig. 6*B*). This indicated that a small amount of the recombinant truncated PiZ-AKDAS protein was secreted during the long radiolabeling period. In contrast, as a typical result of the recycling of a soluble protein to the ER, no immunoprecipitable AAT was present in the media

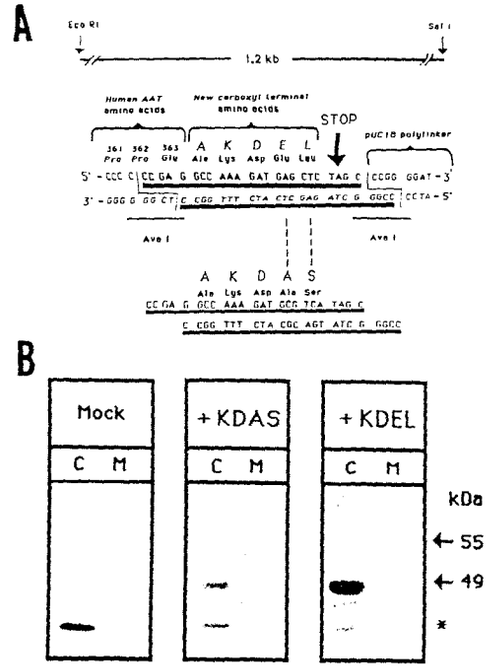

FIG. 6. **Analysis of the intracellular fate of recombinant truncated PiZ AAT variants containing the amino acids AKDEL or AKDAS at their carboxyl terminus.** *A*, two sets of annealed oligonucleotides (*bold type* and *underlined*) were used to construct recombinant DNA clones designated pRSVATZ(*Ava*I)-AKDAS and pRSVATZ(*Ava*I)-AKDEL that would code for truncated human AAT variants containing either AKDEL (*top set*) or AKDAS (*bottom set*) as the carboxyl amino acids, respectively. Nonidentical *Ava*I restriction sites at the termini of the annealed oligonucleotides used for their directional ligation are depicted. Differences in the sequence of the two sets of oligonucleotides are marked by *vertical dashed lines*. Flanking DNA sequences coding for amino acids 361–363 of human AAT plus nucleotides representing the polylinker of pU18 are indicated. The 1.2-kilobase DNA fragment used for ligation of this DNA to *HindIII/BalI*-digested pRSVCAT for the construction of pRSVATZ(*Ava*I)-AKDEL and pRSVATZ(*Ava*I)-AKDAS are shown (see "Materials and Methods"). *B*, Hepa 1a cells were mock-transfected (*Mock*) or transfected with CMV-β-Gal plus either pRSVATZ(*Ava*I)-KDAS (*+KDAS*) or pRSVATZ(*Ava*I)-KDEL (*+KDEL*) (see "Materials and Methods"). β-Galactosidase activity was used to normalize transfection efficiencies prior to immunoprecipitation. In this experiment, the transfection efficiency was identical for both DNA constructs. Human AAT was immunoprecipitated from the cell extracts and medium of Hepa 1a cells pulse-labeled with [^{35}S]methionine (200 μCi/ml medium) for 6 h. Immunoprecipitates were fractionated by SDS-PAGE (8% polyacrylamide) and radiolabeled bands were detected by fluorographic enhancement. The apparent molecular mass of the radiolabeled proteins is indicated. *C*, cell extract; *M*, medium. An *asterisk* shows a contaminating radiolabeled band that was present in the cell extracts from this experiment.

from cells that had been transfected with pRSVATZ(*Ava*I)-AKDEL (Fig. 6*B*). Comparison of the intensity of the immunoprecipitated cellular proteins showed an abundant excess of the truncated PiZ-AKDEL protein as compared to the steady state level of the truncated PiZ-AKDAS (Fig. 6*B*). Quantitation of the excised radiolabeled bands demonstrated a 9-fold excess in the steady state level of the protein when AKDEL, rather than AKDAS, were the carboxyl-terminal amino acids (quantitation not shown).

Two independent experiments were performed to determine whether the difference in the intracellular levels of the two recombinant proteins resulted from a difference in their degradation. Cells were transfected with either DNA construct, radiolabeled for six hours as described above, and then chased for either 0, 1, or 3 h prior to the immunoprecipitation of

human AAT from the cell extracts. As expected, a small amount of the secreted 55k-Da form of the recombinant truncated PiZ-AKDAS protein was detectable in the medium following the prolonged radiolabeling (Fig. 7). However, none was detected in the media at 1 or 3 h of chase. This suggests that a small amount of this recombinant protein is secreted from cells, which is similar to that observed for the naturally-occurring PiZ variant. The gradual disappearance of the truncated PiZ-AKDAS protein from the cell extract (Fig. 7) indicates that it is retained within cells and undergoes degradation. In contrast, the truncated PiZ-AKDEL protein was completely retained within cells (Fig. 7). Furthermore, its stability within cells during the 3-h chase (Fig. 7) demonstrates that it does not undergo degradation. The ability of KDEL, but not KDAS, to prevent the degradation of the recombinant truncated PiZ variant indicates that this ER-recycling signal is "dominant" to that of any signal for degradation.

DISCUSSION

Intracellular Degradation of the Retained PiZ Variant Is pH-sensitive—Interestingly, only a small fraction of the PiZ variant of human AAT can successfully exit the cell. The remainder of the newly synthesized protein is retained intracellulary and undergoes degradation. Results from subsequent experiments in our laboratory have demonstrated that the Pi $Null_{Hong\ Kong}$ variant exhibits the identical biochemical and kinetic characteristics of degradation as observed for the PiZ protein. Because the Pi $Null_{Hong\ Kong}$ variant is a truncated protein devoid of its active inhibitory region (24), none of the characteristics of the degradative pathway described in this report can be attributed to the protease inhibitory capacity of AAT.

In an attempt to characterize the subcellular compartment where the mutant proteins are degraded, we first tested whether inhibitors of lysosomal proteases would interfere in the degradative process. In the present study, degradation of the PiZ variant was not affected by leupeptin, or by leucine methyl ester, a potent irreversible inhibitor of lysosomal proteolytic activity in hepatocytes (34). Furthermore, the use of lysosomotrophic amines had no demonstrable effect on the degradative process. Overall, these findings would seem to suggest that degradation of the retained PiZ variant is independent of lysosomal proteases. This conclusion has also been made for the degradation of unassembled subunits of the TCR complex (9, 40) and for the H2 subunit of the asialoglycoprotein receptor (10) that are impaired in their transport to the Golgi complex. However, it should be noted that the acceleration of the degradation of the retained PiZ variant and unassembled subunits of the TCR complex (9) at acidic intravesicular pH is contrary to this conclusion. Our findings do not clearly differentiate whether it is the reduction of the pre-degradation lag period or an activation of the degradative machinery that results in the overall increased rate of degradation of the PiZ variant. Therefore, in light of the pH sensitivity phenomenon, it is still quite difficult to exlude the possibility that lysosomes are not somehow involved in this degradative pathway. Thus, although no evidence presently exists for the involvement of a lysosomal component associated with this degradative pathway, this possibility cannot yet be totally eliminated.

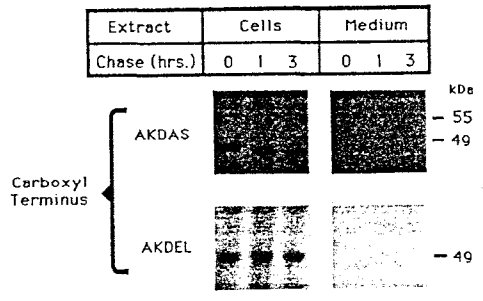

FIG. 7. **Addition of the KDEL signal prevents the intracellular degradation of a recombinant truncated PiZ variant.** In two independent experiments, Hepa 1a cells were transfected with the pRSVATZ(*Ava*I)-KDAS or pRSVATZ(*Ava*I)-KDEL DNA constructs as described under "Materials and Methods." Cells in 60-mm diameter dishes were pulse-labeled for 6 h with [^{35}S]methionine (200 μCi/ml medium). Radiolabeled cells and media were either harvested immediately or chased in media containing unlabeled methionine for the designated period. Human AAT was immunoprecipitated from the cell extracts and media and subsequently fractionated by SDS-PAGE (8% polyacrylamide). Radiolabeled bands were detected by fluorographic enhancement of the dried gel. The apparent molecular mass of the radiolabeled proteins is indicated.

Evidence That Degradation of the PiZ Variant Occurs in a Post-ER Subcellular Compartment—Results from oligosaccharide modification experiments plus the observation that a variety of inhibitors of ER-to-Golgi transport fail to interupt the degradation of the retained PiZ variant has suggested that the degradative event occurs prior to delivery of the protein to the Golgi complex. The presence of a lag period prior to the onset of degradation led us to examine whether the PiZ variant might exit the ER during this period prior to its degradation. We took advantage of the fact that human AAT is a soluble protein. Pelham (6) has demonstrated that the carboxyl-terminal sequence Lys-Asp-Glu-Leu (KDEL) functions as a signal that is necessary and sufficient to sort luminal ER proteins from soluble secretory proteins in a biochemically distinct post-ER subcellular compartment ("salvage compartment"). Using anti-idiotype antibody methodology, an intracellular receptor for the KDEL sequence has been identified (41). As described in the present study, for the KDEL signal to have an effect on the degradation of the PiZ variant, the retained protein must exit the ER. Therefore, the stability and subsequent intracellular accumulation of the truncated PiZ-AKDEL recombinant protein indicates that the PiZ variant must pass through the salvage compartment prior to its degradation. In light of these findings, it is reasonable to assume that the compartment of degradation follows the salvage compartment along the post-ER secretory pathway. Because the ER-to-Golgi movement of secretory proteins is managed via bulk flow (1), even if one would argue that a "degradation signal" might target the PiZ variant for degradation, it is difficult to imagine how the addition of KDEL, but not KDAS, could prevent degradation unless the degradative site follows the salvage compartment along the secretory pathway. Indeed, a degradation "signal" must exist because the extended cellular retention of the normal PiM protein was insufficient to trigger its degradation by this pathway.

Evidence for the Presence of a Common Degradative Pathway for Removing Proteins That Are Impaired in Their Transport to the Golgi Complex—Recent findings have demonstrated that the degradation of the retained H2 subunit of the asialoglycoprotein receptor (10), unassembled TCR chains (9), and a truncated form of ribophorin I (25) exhibits biochemical and kinetic characteristics identical to that reported here for the PiZ variant. For example, the degradation of all these proteins is initiated following a lag period and occurs in a pre-Golgi compartment. Furthermore, degradation of the retained H2 subunit of the asialoglycoprotein receptor (10) and unassembled TCR chains (9) exhibits a temperature

sensitivity that is reminiscent of all vesicular transport events (42–44). These data were the first to suggest that the degradation of a protein by this particular mechanism might actually occur following its transport out of the ER. Finally, the characteristic size reduction associated with the degradation of the PiZ variant has also been identified in the intracellular removal of the H2 subunit of the asialoglycoprotein receptor. A transient 35-kDa polypeptide produced by a specific endoproteolytic cleavage of this protein occurs during its degradation (10). Overall, these findings indicate that a single degradative mechanism is apparently able to remove several independent unrelated proteins from the secretory pathway if they fail to function in ER-to-Golgi transport. Degradation of a soluble mutant secretory protein as shown in the present study indicates that the targetting of proteins for degradation by this pathway does not require a membrane-associated domain. Further studies will undoubtedly demonstrate the range of proteins that can serve as substrates for this degradative pathway.

It is likely that several degradative mechanisms are utilized for the removal of misfolded proteins from the early stages of the secretory pathway. The observation that the recombinant truncated PiZ-AKDEL protein is diverted from its degradative compartment suggests that when a native soluble resident ER protein containing the KDEL signal is misfolded, it most likely undergoes degradation by an alternative mechanism. The exact identity of signals, whether stable or transient, that target proteins for degradation within the secretory pathway remain to be identified.

Acknowledgment—We express our appreciation to Dr. Jennifer Lippincott-Schwartz for her helpful suggestions.

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J. Cell Biol.
Vol. 109, p 188a, 1989

1020 *Inhibition of EGF-Stimulated Tyrosine Phosphorylation of PLCγ and Phosphoinositide Hydrolysis by Activation of Protein Kinase C.* W.R.Huckle, J.R.Hepler, S.G.Rhee, T.K.Harden, and H.S.Earp, Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC 27599.

We have shown that protein kinase C (PKC) modulates the activation of phosphoinositide (PtdIns)-specific phospholipase C (PLC) by the EGF receptor (EGF-R; *J. Biol. Chem (1988) 263:7610*). Recently, others have reported EGF-dependent tyrosine phosphorylation of the γ-isoform of PLC. We now assess the coupling between EGF-stimulated increases in PLCγ phosphotyrosine ([P-Tyr]) content (determined by anti-[P-Tyr] immunoblotting of anti-PLCγ immunoprecipitates) and [^{3}H]inositol phosphate (InsP) accumulation in [^{3}H]inositol-loaded WB liver epithelial cells. No [P-Tyr]PLCγ was found in the absence of EGF treatment. Appearance of [P-Tyr]PLCγ occurred at all concentrations of EGF that provoked PtdIns hydrolysis and preceded InsP production. Termination of EGF-stimulated InsP accumulation was accompained by return of [P-Tyr]PLCγ to near-basal levels. In contrast, Angiotensin II, another agent that stimulates InsP production in WB cells, did not produce measurable PLCγ phosphorylation. Activation of PKC with phorbol 12-myristate 13-acetate (PMA) inhibited (IC_{50}=3-10 nM) both EGF-dependent PtdIns hydrolysis and PLCγ phosphorylation by >90%. Depletion of PKC by prolonged PMA treatment, which potentiates EGF-stimulated PtdIns hydrolysis, also potentiated EGF-dependent increases in [P-Tyr]-PLCγ. Five other [P-Tyr]-containing proteins, including EGF-R, were found in anti-PLCγ precipitates from EGF-treated cells. These results are consistent with a role for tyrosine phosphorylation in the regulation of PLCγ activity by EGF. Since EGF promotes PtdIns hydrolysis and release of diacylglycerol, the inhibitory effects of PMA and the potentiating effects of PKC depletion suggest that activation of PKC may be responsible, in part, for the rapid termination of these EGF actions. Our results also reveal the existence of a multimeric EGF-R/substrate complex.

Minisymposium 12: Intracellular Protein Degradation (1021-1025)

1021 The 26S ATP/Ubiquitin-Dependent Protease, R. Hough, G. Pratt, and M. Rechsteiner, Department of Biochemistry, University of Utah, Salt Lake City, UT 84132.

Ubiquitin (Ub), the most highly conserved protein in eucaryotes, can be covalently attached to a variety of cellular proteins. Some, but not all, of the modified proteins are then degraded in an ATP-dependent reaction by an enormous 26S multiprotein complex. Using Ub-lysozyme conjugates as substrate we have purified the 26S protease complex and shown that it contains subunits ranging in size from 21,000-110,000 daltons. The largest polypeptide chain in the complex is the major substrate for casein kinase II in reticulocyte lysate. Using crosslinking procedures we have identified 2 subunits that bind ATP and have tentatively identified a subunit that binds Ub. Our purification also yields a smaller 20S protease that contains a subset of the chains present in the larger complex. The smaller protease does not degrade Ub-lysozyme conjugates, nor is it stimulated by ATP. It will, however, degrade a variety of fluorogenic peptides and, under certain conditions, intact proteins. Models will be presented on the possible relationship between the 2 proteases and on potential mechanisms by which the larger protease degrades ubiquitinated substrates.

1022 The Proteolytic Recognition Signal of the Ubiquitin-Dependent Pathway and Its Function in S. cerevisiae, V. Chau, D. Finley, L. Gregori, and P. Boucher, Department of Pharmacology, Wayne State University School of Medicine, Detroit, MI. 48201, and Department of Mol. Cell. Physiology, Harvard Medical School, Boston, MA (Spon. by M. Bagchi).

Proteins to be degraded by the ubiquitin-dependent pathway are first linked with ubiquitin in the form of a specific multiubiquitin chain. In this chain, the carboxyl terminus of one ubiquitin is linked to the internal Lys^{48} of an adjacent ubiquitin. The formation of this chain on substrate proteins, but not the linkage of monoubiquitin moieties, can be blocked specifically by replacing ubiquitin with a mutant (Ub-R48) in which Lys^{48} has been converted to an Arg residue. This blockage reveals the chain's function as the proteolytic recognition signal, perhaps as the binding site for the downstream protease of this pathway. The overexpression of Ub-R48 in the yeast S. cerevisiae has also been used to monitor the degradation of specific proteins in vivo. The degradation of abnormal canavanine-containing proteins, as well as specific short-lived proteins, is retarded by the overexpression of Ub-R48. Ub-R48 appears to function fully in non-proteolytic processes, but when the expression of wild type ubiquitin genes is completely inhibited, the expression of Ub-R48 alone is insufficient to maintain cell growth. This result indicates that ubiquitin-dependent protein degradation is essential in yeast.

1023 Characterization of Neutral and Acidic Proteases in Endosomal Vesicles. J.S. Blum, M.L. Fiani and P.D. Stahl, Department of Cell Biology, Washington University School of Medicine, St. Louis, MO 63110. (Spon. by R.W. Mercer).

Receptor-ligand complexes are internalized into the endocytic pathway where susceptible ligands are rapidly proteolyzed. The macrophage-specific mannose receptor was used to target proteins into endosomal vesicles to facilitate the study of proteases in this compartment. Studies using rabbit alveolar macrophages have shown that the aspartyl protease cathepsin D is present in endosomes. Using the mouse J774 cell line we have found that both cathepsin D as well as cysteine proteases cleave proteins inside endocytic vesicles. Bovine serum albumin bearing mannose residues is delivered into early endosomes and cleaved only after vesicle acidification. The plant toxin ricin A chain also binds the mannose receptor and is transported into endosomes as shown by Percoll density gradient separation. Proteolysis of ricin A chain is observed in endosomes under conditions where the intravesicular pH is neutral or acidic. The enzymes responsible for the cleavage of ricin A chain are not found on the plasma membrane but are present in early endosomes (2 and 5 min after uptake). Calpain inhibitors and several other inhibitors of cysteine proteases block the proteolysis of ricin A chain in endosomes at neutral pH. Both cysteine proteases and cathepsin D cleave ricin A chain as the intravesicular pH drops. Pepstatin A, an inhibitor of cathepsin D has been shown to antagonize ricin A chain cytotoxity in macrophages. Thus, endosomal proteases may be important in the activation or translocation of this toxin. Changes in endosomal pH appear to be important in regulating the action of specific proteases localized in these vesicles.

1024 The PiZ Variant of Human Alpha-1-Antitrypsin Undergoes Intracellular Degradation Within a Pre-Golgi Compartment. A. Le, K.S. Graham, and R.N. Sifers, Departments of Pathology and Cell Biology, Section of Molecular Pathobiology, Baylor College of Medicine, Houston, Texas, 77030, (Spon. S.L.C. Woo.)

Alpha-1-antitrypsin (AAT) is a major hepatic secretory protein. Its primary physiological role is to protect elastin fibers in the alveoli of the lung from degradation by excess levels of neutrophil elastase. The hindered secretion of the PiZ variant of human AAT results from its inefficient export from the hepatic endoplasmic reticulum (ER) where it accumulates as an insoluble aggregate within a subset of ER cisternae. Interestingly, pulse-chase analyses of primary cultures of hepatocytes from PiZ-bearing transgenic mice and in stably transfected mouse hepatoma cells has demonstrated that the newly synthesized PiZ variant is soluble, exhibits endoglycosidase H-sensitive oligosaccharides, and undergoes intracellular degradation. Kinetically, this degradative process is characterized by a 30 minute lag period, followed by a linear rate of degradation. Degradation of the PiZ variant is unaffected by pre-treatment of cells with lysosomotrophic amines such as chloroquine and ammonium chloride. Furthermore, leupeptin, an inhibitor of lysosomal cathepsin B does not affect this degradative process, indicating that lysosomes are not involved. Overall, these results suggest that the intracellular degradation of the PiZ variant occurs within a pre-Golgi compartment that might be contiguous with the ER. Conceivably, only a small fraction of the newly synthesized PiZ variant becomes insoluble within the ER and is resistant to degradation.