# 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

Kathleen S. Graham[1], Anhquen Le[1], and Richard N. Sifers[1,2,3]

[1]Department of Pathology, Section of Molecular Pathobiology

[2]Department of Cell Biology

Baylor College of Medicine

Houston, Texas 77030

[3]Corresponding author: Richard N. Sifers, Ph.D.

Department of Pathology

Baylor College of Medicine

One Baylor Plaza

Houston, Texas, 77030

(713) 798-3168

Running title: Absence of grp78/BiP induction

## SUMMARY

Greater than 85% of the transport-impaired *PiZ variant of human alpha-1-antitrypsin* (AAT[1]) *is retained within cells and subsequently* degraded within a pre-Golgi nonlysosomal compartment that is apparently separate from the endoplasmic reticulum (ER) (Le, A., Graham, K. S., and Sifers, R. N. (1990) *J. Biol. Chem., in press*). Despite this phenomenon, human patients and PiZ-bearing transgenic mice exhibit an accumulation of the undegraded protein as insoluble aggregates within distended cisternae of the hepatic ER (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 ). Immunoprecipitation of the PiZ variant from pulse-radiolabeled hepatocytes from the transgenic animals has demonstrated that a minute quantity of the newly synthesized mutant protein is apparently resistant to degradation and accumulates gradually within the particulate fraction of the cell. Although the steady-state level of the resident ER protein grp78/BiP is elevated in response to the accumulation of malfolded proteins within that subcellular compartment, this phenomenon is not ellicited by the accumulation of the insoluble PiZ variant. These results indicate that neither the accumulation of this malfolded protein within the ER nor even the distention of that subcellular compartment are sufficent to cause the up-regulation of grp78/BiP levels. The interpretation of these results with regard to the factors that regulate the levels of grp78/BiP in the ER is discussed.

## INTRODUCTION

Alpha-1-antitrypsin (AAT[1]) is synthesized predominantly by hepatocytes (1). Identical to other secretory proteins, it is synthesized at the surface of the endoplasmic reticulum and inserted into the lumen of that organelle (2). Human AAT is extremely polymorphic throughout the world population. At present, over 75 electrophoretic variants have been identified that are coded by a single gene locus (3). Several of these allelic variants are associated with serum AAT deficiency (4) which results from the hindered secretion of a mutant AAT macromolecule from hepatocytes (5).

The most prevalent variant associated with a severe deficiency of serum AAT is designated PiZ (4). A Glu to Lys substitution at position 342 of the mature PiZ variant results from a G to A transition within the human AAT structural gene (6, 7). This mutation is sufficient to prevent >85% of the newly synthesized mutant protein from undergoing secretion from cells (8-12), presumably because the macromolecule can no longer efficiently achieve its native conformation (13). The retained intracellular fraction of the PiZ variant is subjected to degradation by a pre-Golgi nonlysosomal pathway (12) which is similar to the fate of a variety of other mutant or incompletely assembled proteins that are impaired in their normal ER-to-Golgi transport (14-16). Although the retained PiZ variant is subjected to pre-Golgi nonlysosomal degradation, insoluble aggregates of the undegraded mutant protein accumulate within reminant vesicles of the hepatic rough ER of human subjects (17, 18) and transgenic mice that express the human PiZ allele (19-21). The intrahepatic accumulation of this insoluble protein can act, in part, as an etiologic agent for the development of liver disease (19, 21).

A resident ER protein designated BiP (22, 23) or grp78 (24, 25) shares extensive sequence homology with the hsp70 family of heat shock proteins that function in directing protein folding within the cell (for a review, see ref. 26). Interestingly, grp78/BiP binds

transiently to many nascent wild type transmembrane and secretory proteins (23, 27) and permanently to several mutant and incompletely assembled proteins that fail to exit the ER (23, 28). Therefore, by association, grp78/BiP has been implicated in directing the folding of proteins within the ER (26). Furthermore, although grp78/BiP is synthesized constitutively under normal conditions, its level is elevated in response to the accumulation of malfolded proteins within the ER (29-31). At present, the exact identity of "signals" that trigger this induction are not fully understood. Here we report that a minute fraction of the newly synthesized PiZ variant apparently escapes degradation by the pre-Golgi nonlysosomal degradative pathway. Unexpectedly, distention of the hepatic ER cisternae resulting from the accumulation of the PiZ variant does not cause an elevation of the steady-state levels of grp78/BiP.

## MATERIALS AND METHODS

*Chemicals and Reagents.* L-[$^{35}$S]methionine (specific activity > 800 Ci/mmol) was purchased from New England Nuclear and Protein G -Sepharose 4FF was obtained from Pharmacia. Tunicamycin (homologue A1) and glucokinase were procured from SIGMA Chemical company. All tissue culture products were purchased from GIBCO Laboratories.

*Antisera.* A species-specific IgG fraction of goat anti-human AAT was prepared as previously described (32). A monoclonal antibody preparation against immunoglobulin heavy chain binding protein (grp78/BiP) was a gift from Dr. David Bole, Yale University (23). Affinity purified goat anti-mouse albumin was purchased from Bethyl Laboratories.

*Transgenic mice and stably transfected mouse hepatoma cells.* The production of transgenic mice synthesizing the transport-impaired PiZ human AAT variant (mouse line Z11.03) was described previously (19). Primary hepatocytes from these animals were prepared and cultured as described for transgenic mice bearing the normal PiM human AAT allele (20). Production of the stably transfected clonal mouse hepatoma cell lines H1A/RSVATZ-8 (12), and H1A/N-13 (33) which express the PiZ variant and Pi $Null_{Hong\ Kong}$ variant of human AAT, respectively, is described elsewhere.

*Immunologic dot-blot analysis of human AAT .* Human AAT was detected in cell fractions by applying aliquots of protein to a nitrocellulose filter with the aid of a Bio Dot-Blot Apparatus (BioRad Laboratories). Human AAT was detected using the goat anti-human AAT as primary antibody and iodinated swine anti-goat IgG (Boehringer Mannheim) as the second antibody as

described previously (32).

*Metabolic radiolabeling and immunoprecipitation of proteins.* For pulse-chase studies, cells were subjected to methionine starvation by pre-incubation in methionine-free Dulbecco's Modified Essential Medium (DMEM) (GIBCO Laboratories) containing 1X glutamine and 0.1% fetal calf serum (GIBCO). Cells were then metabolically labeled with the same medium containing [$^{35}$S]methionine as described previously (33). The chase medium consisted of DMEM containing 1X gutamine, 0.1% fetal calf serum, and a four-fold excess of unlabeled methionine. For steady-state radiolabeling, cells were incubated in the medium described above supplemented with unlabeled methionine (3mg/l)(10). Cells were lysed (Lysis Buffer consisted of 0.05 M Tris-HCl, pH 8.0, 1% Nonident P-40, 0.15 M NaCl, and 1 mM phenylmethylsulfonyl fluoride) and proteins were immunoprecipitated using an excess of specific antisera and Protein G-Sepharose 4FF as described previously (10). However, when testing for an association between grp78/BiP and human AAT, glucose (0.1 M) and glucokinase (1 U/ml) were included in the lysis buffer to deplete the sample of ATP. In these latter experiments, all washes were performed at 4$^{0}$C. Samples were subjected to SDS-PAGE and radiolabeled proteins were detected by fluorographic enhancement of gels. Quantitation of immunoprecipitated protein was performed by scintillation counting of bands excised from gels.

*Extraction of the insoluble PiZ variant from the particulate fraction of cells.* Monolayers of cells were washed in phosphate-buffered saline (PBS). Following their lysis, cells were scraped from the dish and centrifuged at 10,000 x g for 5 minutes. The resulting cell pellet was rinsed with 0.5 ml of PBS and then vortexed for 15 seconds in 2 volumes of 2% SDS. The solution was diluted with 0.5-0.8 ml of Lysis Buffer (final SDS concentration < 0.1%), vortexed, and centrifuged as before. The entire supernatant was collected for dot-blot analysis

(see Fig. 1) or for immunoprecipitation studies as described above. This fraction represented the SDS-solubilized extract of the cellular particulate fraction.

## EXPERIMENTAL RESULTS

*Solubilization and extraction of the accumulated insoluble PiZ variant* - We (20) have shown that the transport-impaired PiZ variant of human AAT accumulates within the particulate fraction of homogenates from the livers of a transgenic mice that synthesize this protein. Because, primary hepatocyte cultures derived from these animals exhibit the identical accumulation phenomenon (Fig. 1A), this cell type was utilized as a model to determine the cause of this intracellular deposition of the mutant protein.

Because Bathurst *et al.*, (18) have shown that SDS-treatment of human liver biopsies will quantitatively extract the accumulated PiZ variant from cells, a technique was developed in order to identify the accumulated insoluble protein within primary hepatocytes from the transgenic animals. Using this technique, >90% of the insoluble PiZ variant can be solubilized from the particulate fraction of the cells that have been lysed with a nonionic detergent (Fig. 1A). Solubilization of these aggregates does not occur with non-ionic detergents such as Triton X-100 or Nonident P-40 (data not shown). Because treatment with SDS followed by its dilution to a final concentration of 1% does not interfere with the immunoprecipitation of the PiZ variant (Fig. 1B), this methodology was utilized in subsequent experiments to identify the timecourse of the intracellular deposition and accumulation of the insoluble protein within the primary hepatocytes.

*A fraction of the retained PiZ variant accumulates gradually within hepatocytes* - As demonstrated by pulse-chase analysis, the PiZ variant was synthesized as a 50 kDa species (Fig. 2). During the chase period, the apparent mass of the secreted protein increased to 56 kDa (Fig. 2) as a result of the addition of sialic acid residues to the three N-linked carbohydrate moieties present on the macromolecule (35). Although >85% of normal human AAT is secreted from cells within 45 minutes following its biosynthesis (12, 33), the secretion

of the PiZ variant is greatly impaired such that only 10-17% of the mutant protein is secreted over a three hour period (12). As espected, the retained mutant protein underwent degradation following an initial lag period of 30 minutes (Fig. 2). This was expected because a pre-degradation lag period is one characteristic of protein degradation *via* the pre-Golgi nonlysosomal pathway (12, 14-16). Examination of the SDS extract from the particulate fraction of the lysed cells failed to identify any deposition of the newly synthesized PiZ variant during the pulse or subsequent chase periods (Fig. 2). These findings suggested that a very small fraction of the newly synthesized protein was deposited within the cell.

In order to detect the apparently minute quantity of undegraded PiZ variant that is deposited intracellulary, human AAT was immunoprecipitated from monolayers of primary hepatocytes following extended pulse-labeling with [$^{35}$S]methionine for either three, six, or nine hours. A consistent, yet characteristically slow increase in the levels of the 56 kDa species present in the media during the experiment (Fig 3), indicated that the radiolabeling conditions did not interfere with protein secretion or with the viability of the cells. Quantitation of the PiZ variant immunoprecipitated from the soluble cell lysate demonstrated that the steady-state kinetics of its synthesis versus degradation was achieved between six and nine hours of radiolabeling (Fig. 3). Utilizing the extended radiolabeling conditions, the 50 kDa PiZ variant was identifiable within the particulate fraction of the radiolabeled cells (Fig. 3). Interestingly, although the retained soluble protein was subjected to degradation and reached steady-state kinetics, the gradual accumulation of the insoluble 50 kDa species (Fig. 3) suggested that a minute fraction of the newly synthesized protein is resistant to degradation.

*The steady-state level of grp78/BiP is not elevated in response to the intracellular accumulation of the PiZ variant* - It has been reported that the synthesis of malfolded proteins within the ER increases the steady-state level of grp78/BiP in yeast and mammalian cells (29-

31). Because the normally soluble PiZ variant accumulates within a subset of distended cisternae of the rough ER (17, 19, 20) as an insoluble aggregate (18, 20), it can be assumed that the protein has not folded corectly, resulting in its insolubility and subsequent deposition. Therefore, we examined whether the synthesis and accumulation of this particular malfolded protein would result in an induction of grp78/BiP. Therefore, the amount of immunoprecipitable grp78/BiP was compared between radiolabeled hepatocytes from a PiZ-bearing transgenic mouse and its non-transgenic littermate. In this analysis, a single 78 kDa protein, representing grp78/BiP, was immunoprecipitated from each cell extract (Fig. 4). However, no apparent difference existed between the steady-state level of this protein in the transgenic (lane 2) versus non-transgenic (lane 1) hepatocytes.

Recent studies have demonstrated that, similar to other liver proteins, the synthesis of human AAT is restricted to a subset of hepatocytes rather than distributed evenly throughout all the hepatocytes of the liver (for a review, see ref. 36). Therefore, it was conceivable that the absense of an induction of grp78/BiP in the primary hepatocytes from the PiZ-bearing transgenic mice might actually reflect the heterogeneity of the accumulation of the PiZ variant. Because the mutant protein has been shown to accumulate as insoluble aggregates within transfected mouse hepatoma cells (10), we repeated the aforementioned experiment using a clonal line of PiZ-bearing mouse hepatoma cells designated H1A/RSVATZ-8 (12). However, as shown in Fig. 5A, the steady-state level of grp78/BiP in the radiolabeled H1A/RSVATZ-8 cells was not elevated over that which was detected in the non-transfected mouse hepatoma cell line, Hepa1A.

Treatment of cells with tunicamycin, an inhibitor of N-linked glycosylation (37, 38), has been shown to result in the synthesis of malfolded proteins within the ER, presumably as a result of the absence of large oligosaccharide moieties that are normally present on the glycoproteins. Furthermore, this malfolding of proteins results in a concomitant induction of

grp78/BiP levels (29). Therefore, to confirm that the steady-state level of grp78/BiP is indeed inducible in H1A/RSVATZ-8 cells, equivalent monolayers were radiolabeled in the presence or absence of tunicamycin prior to the immunoprecipitation of grp78/BiP. Comparison of the steady-state level of the resident ER protein within these cells showed that it was elevated in the tunicamycin-treated cells (Fig. 5B). Quantitation of the immunoprecipitated protein demonstrated that the steady-state level of grp78/BiP increased more than two-fold in response to the treatment of H1A/RSVATZ-8 cells with this drug (quantitation not shown). These results confirmed that the regulation of grp78/BiP levels was functional in PiZ-bearing cells, and that the intracellular accumulation of the insoluble PiZ variant does not function as a sufficient to stimulte this response.

*Inability to identify an association between grp78/BiP and transport-impaired mutants of human AAT* -The unexpected finding that grp78/BiP levels are not increased in response to the intracellular accumulation of the PiZ variant led us to determine whether grp78/BiP actually participates in the folding of mutant human AAT macromolecules. To answer that question, both grp78/BiP and the PiZ variant were independently immunoprecipitated from the appropriate radiolabeled hepatoma cell line. Non-stringent conditions, including $4^0$C incubation and washes plus the depletion of ATP in the sample, were used in this analysis in order to reduce the possibility of any disruption of BiP-hAAT complexes (39). As before, a single 78 kDa protein representing grp78/BiP was immunoprecipitated from both cell lines (Fig. 6). However, using either antisera, no co-precipitating band exhibiting the an electrophoretic mobility similar to the other protein was observed (Fig. 6). This association was also examined for another transport-impaired variant of human AAT designated Pi $Null_{Hong\ Kong}$. Although none of this 45 kDa truncated variant is secreted from cells (33) and eventually undergoes

intracellular degradation (12), no apparent association with grp78/BiP was identified in this study (Fig. 6).

Although it has been reported that grp78/BiP binds permanently, not transiently, to malfolded proteins that cannot exit the ER (23, 28), we performed experiments in order to determine whether any putative transient association between grp78/BiP and the newly synthesized PiZ variant might be detectable. For this, H1A/RSVATZ-8 cells were subjected to a short five minute radiolabeling with [$^{35}$S]methionine and grp78/BiP was immunoprecipitated from cell extracts at different times during the chase period. Again, in this analysis, extremely non-stringent conditions were utilized that have been shown to optimize the stability of BiP-protein complexes (39). However, as shown in Fig. 7, no co-precipitation of grp78/BiP and the PiZ variant was detectable either immediately after the short pulse (0 chase), during the 30 minute pre-degradation lag period (15 and 30 minute chase), or after the protein had entered the degradative pathway (45 min chase). In short, using the aforementioned techniques, we were unable to identify any binding, be it transient or stable, between the PiZ variant and grp78/BiP.

## DISCUSSION

*Intracellular accumulation of the undegraded PiZ variant* - Similar to the fate of several other mutant and incompletely assembled proteins that are impaired in their ER-to-Golgi transport (14-16), the retained fraction of both the PiZ and Pi $Null_{HongKong}$ variants of human AAT are subjected to degradation within a nonlysosomal pre-Golgi compartment (12). In view of this degradative mechanism, how can the intracellular accumulation of the undegraded insoluble PiZ variant be explained? At present, two independent lines of evidence suggest that protein degradation *via* this pathway occurs in a post-ER compartment. First, the pre-Golgi nonlysosomal degradation of the newly synthesized alpha subunit of the T cell receptor complex (14), the H2 subunit of the asialoglycoprotein receptor (15), and the PiZ variant of human AAT (R. Sifers, unpublished observation) is blocked at temperatures that inhibit vesicular transport events (41-43). Second, and perhaps more convincing, is that the addition of the tetrapeptide KDEL to the carboxyl terminus of a retained truncated PiZ variant (a signal for recycling luminal proteins from a post-ER compartment back to the ER) (44, 45) prevents its degradation (12). For the KDEL sequence to exert this effect, the retained recombinant protein must exit the ER (45). Because the PiZ variant exhibits a tendency toward aggregation (40), it is conceivable that the formation of transport-incompetent aggregates of the newly synthesized protein would be unable to exit the ER *via* bulk flow for delivery to the degradative compartment. Certainly, accumulation of the undegraded protein within the distended cisternae of the ER provides additional evidence, albeit circumstantial, that its degradation occurs in another subcellular compartment.

*Accumulation of the PiZ variant does not result in an elevation of grp78/BiP levels in the ER* - The resident ER heat shock protein designated grp78/BiP functions in the assembly and folding

of a variety of proteins within the ER (27, 28, 46). One particularly interesting characteristic of this protein is that although it is expressed constitutively in cells, its levels are elevated in response to the accumulation of malfolded proteins in the ER (29-31). Therefore, it was entirely unexpected that the intracellular accumulation of the PiZ variant which leads to an enormous distention of the rough ER cisternae (17, 19, 20) failed to ellicit this response. One possible explanation for our observation is that the accumulated PiZ variant is not actually malfolded. It should be noted that in many cases, this terminology is used to describe a newly synthesized polypeptide that fails to assemble into its native oligomeric protein complex. As described in the present study, human AAT is not associated with a protein complex, but rather, exists as a monomer. Therefore, it is somewhat more difficult to substantiate that the accumulated PiZ variant is actually malfolded. One line of evidence to support the concept that the PiZ variant is hindered in its folding is the phenomenon of its retention within the ER. At present, the only seemingly common feature to the vast array of proteins that are retained within this subcellular compartment is that of malfolding. Of course, it cannot yet be concluded that this is the actual molecular "signal" that triggers protein retention. For instance, the fraction of the PiZ variant that is secreted into human plasma exhibits a somewhat reduced inhibitory capacity toward neutrophil elastase as compared to the normal PiM protein (47). Furthermore, it has been reported that even the plasma form of the PiZ variant exhibits an inherent tendency to aggregate (40). Overall, these findings suggest that the secreted form of the PiZ variant, which obviously escapes the intracellular retention machinery, exhibits a difference in its overall conformation as compared to the normal PiM protein. Finally, the most compelling evidence that the accumulated fraction of the PiZ variant exists in a malfolded state is that it accumulates as insoluble aggregates which is indicative of protein malfolding.

Another possible explanation to account for the apparent absence of an elevation in the

levels of grp78/BiP is that this response is indeed ellicited, but is masked because BiP is bound to the accumulated insoluble PiZ variant present in the particulate fraction of the cell. However, this also seems unlikely because we have been unable to detect any apparent accumulation of grp78/BiP in the particulate fraction of cells that synthesize the PiZ variant (R. Sifers, unpublished results).

*Defining the requirements for triggering an elevation of grp78/BiP levels in the ER* - At present, the exact mechanisms utilized to trigger an elevation of grp78/BiP within the ER are unknown. Treatment of cells with tunicamycin results in the synthesis and accumulation of apparently malfolded glycoproteins in the ER, along with a concomitant induction of grp78/BiP levels (29). However, it cannot be concluded whether it is the actual accumulation of these proteins or the distention of the ER that actually triggers this phenomenon. Thus, the inability of the accumulated PiZ variant to ellicit an induction of grp78/BiP can be utilized to characterize, or in this case, elliminate potential factors that might regulate the level of this resident ER protein. Our findings indicate that neither the massive accumulation of the insoluble PiZ variant within the hepatic ER of transgenic mice nor the distention of that subcellular compartment is sufficient to ellicit an elevation of grp78/BiP levels. Clearly, additional or more specific factors are required for this to occur.

As described in this study, using conditions that should optimize for the stabilization of BiP-protein complexes (39), we were unable to detect any stable or transient association between grp78/BiP and the newly synthesized PiZ variant. Presumably AAT is one of several proteins that does not utilize grp78/BiP during its folding. Moreover, to the best of our knowledge, all experiments identifying an up-regulation of grp78/BiP have either utilized cell lines that synthesize and accumulate a known recombinant "BiP-binding" protein, or cells that exhibit a mutation leading to an impairment of overall protein secretion. Our findings provide

evidence, albeit circumstantial, that the mechanism utilized for up-regulating the levels of grp78/BiP within the ER may involve the accumulation of "BiP-binding" proteins within that compartment. Presumably, the permanent binding of grp78/BiP to some accumulated proteins would significantly reduce the level of the unbound form of this macromolecule. Thus, it is likely that the reduction of unbound grp78/BiP within the ER, rather than the actual accumulation of "BiP-binding" proteins, functions as a signal to elevate its levels.

*Accumulation of the PiZ variant and liver disease* - One obvious function of degrading transport-impaired proteins is to prevent cellular damage caused by their accumulation within compartments of the secretory pathway. Clearly, the physiological importance of this degradative pathway is exhibited by the intracellular accumulation of the undegraded insoluble PiZ variant. Results from studies utilizing a transgenic mouse model (19, 21) have suggested that it is the intrahepatic accumulation of the insoluble PiZ variant that functions, in part, as an etiologic agent for the development of liver disease in these animals. However, it should be clarified that the liver disease phenotype is more likely a direct result of the cell's response to the presence of the accumulated protein which distends cisternae of the ER (19, 20). Recently Perlmutter *et al.*, (48) have reported that the steady state-level of several cytoplasmic stress proteins are elevated in peripheral blood monocytes from PiZZ individuals that exhibit liver disease. Although monocytes do synthesize and accumulate the PiZ variant, it is difficult to determine to what extent a non-liver cell type can be utilized to study the response of a hepatocyte toward the accumulated insoluble protein. Although these authors presented data to indicate that the steady-state level of ubiquitin, a cytoplasmic stress protein, was elevated in livers from these patients, no analysis was performed to examine the levels of the resident ER protein grp78/BiP in that tissue. Finally, the focus of our present study was not to characterize the liver disease phenotype in these animals, but to characterize the intracellular

deposition of the PiZ variant and examine its association with grp78/BiP. Therefore, no emphasis was made to perform our studies on transgenic animals that had developed liver disease. Thus, our findings do not necessarilly contradict those of Perlmutter *et al.*, (48). Conceivably the transgenic mouse model will be useful in future endeavors toward identifying the factors that do lead to the development of liver disease in these animals.

## ACKNOWLEDGEMENTS

K.S.G. was a participant in the Summer Medical and Research Training (SMART) program at Baylor College of Medicine. We thank Dr. David Bole for the gift of the monoclonal antibody against grp78/BiP. We wish to express our appreciation to Ms. Janet DeMayo for the breeding and maintenance of the transgenic mice. This research was supported, in part, by grants #1-89-0040 and #1-90-0044 from the Moran Foundation, and by USPHS grant RR-05425 to R.N.S.

## REFERENCES


1. Carlson, J. A., Rogers, B. R., Sifers, R. N., Hawkins, H. K., Finegold, M. J., and Woo, S. L. C. (1988) *J. Clin. Invest.* **82**, 26-36
2. Walter, P., Gilmore, R., and Blobel, G. (1984) *Cell* **38**, 5-8
3. Brantly, M., Nukiwa, Y., and Crystal, R. G. (1988) *Am. J. Med.* **84**, 13-31
4. Gadek, J. E., and Crystal, R. G. (1982) In *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., and Brown, M. S., eds) 5th Ed., pp. 1450-1467, McGraw-Hill Publications, Minneapolis, MN
5. Sifers, R. N., Finegold, M. J., and Woo, S. L. C. (1988) *Am. J. Resp. Cell Mol. Biol.* **1**, 341-345
6. 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
7. Sifers, R. N., Carlson, J. A., Clift, S. M., DeMayo, F. J., Bullock, D. W., and Woo, S. L. C. (1987) *Nucl. Acids Res.* **15**, 1459-1475
8. Perlmutter, D. H., Kay, R. M., Cole, F. S., Rossing, T. H., Van Thiel, D., and Colton, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6918-6921
9. Foreman, R. C., Judah, J. D., and Colman, A. (1984) *FEBS Lett.* **168**, 84-88
10. Sifers, R. N., Hardick, C. P., and Woo, S. L. C. (1989) *J. Biol. Chem.* **264**, 2997-3001
11. McCracken, A. A., Kruse, K. B., and Brown, J. L. (1989) *Mol. Cell Biol.* **9**, 1406-1414
12. Le, A., Graham, K. S., and Sifers, R. N. (1990) *J. Biol. Chem. (in press)*
13. Loebermann, H., Tukuoka, R., Deisenhofer, J., and Huber, R. J. (1984) *Mol. Biol.* **177**, 531-556
14. Lippincott-Schwartz, J., Bonafacino, J. S., Yuan, L. C., and Klausner, R. D. (1988) *Cell* **54**, 209-220

15. Amara, J. F., Lederkremer, G., and Lodish, H. F. (1989) *J. Cell Biol.* **109**, 3315-3324

16. Ivessa, N. E., De Lemos-Chiarandini, C., Tsao, Y. -S., Sabatini, D. D., and Kreibich, G. (1989) *J. Cell. Biol.* **109**, 207a

17. Sharp, H. L. (1971) *Hosp. Pract.* **6**, 83-96

18. Bathurst, I. C., George, P. M., Travis, J., and Carrell, R. W. (1984) *FEBS Lett.* **177**, 179-183

19. 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

20. Sifers, R. N., Rogers, B. B., Hawkins, H. K., Finegold, M. J., and Woo, S. L .C. (1989) *J. Biol. Chem.* **264**, 15696-15700

21. 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., and Sorge, J. A. (1988) *Science* **242**, 1409-1412

22. Haas, I. G., and Wabl, M. (1983) *Nature* **306**, 387-389

23. Bole, D. G., Hendershot, L. M., and Kearney, J. F. (1986) *J. Cell Biol.* **102**, 1558-1566

24. Munro, S., and Pelham, H. R. B. (1986) *Cell* **46**, 291-300

25. Lee, A. S., Delegeane, A., and Scharff, D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4922-4925

26. Rothman, J. E. (1989) *Cell* **59**, 591-601

27. Dorner, A. J., Bole, D. G., and Kaufman, R. J. (1987) *J. Cell Biol.* **105**, 2665-2674

28. Gething, M.-J., McMammon, K., and Sambrook, J. (1986) Cell 46, 939-950

29. Kozutsumi, Y., Segal, M., Normington, K., Gething, M. -J., and Sambrook, J. (1988) *Nature* **332**, 462-464

30. Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. -J., and Sambrook, J. (1989)

*Cell* **57**, 1223-1236

31. Rose, M. D., Misra, L. M., and Vogel, J. P. (1989) *Cell* **57**, 1211-1221

32. Sifers, R. N., Carlson, J. A., Clift, S. M., DeMayo, F. J., Bullock, D. W., and Woo, S. L. C., (1987) *Nucl. Acids Res.* **15**, 1459-1475

33. Sifers, R.N., Brashears-Macatee, S., Kidd, V.J., Muensch, H., and Woo, S.L.C. (1988) *J. Biol. Chem.* **263**, 7330-7335

34. Laemmli, U. K. (1970) *Nature* **227**, 680-685

35. Lodish, H. F., Kong, N., Snider,M., and Strous, G. J. A. M. (1983) *Nature* **304**, 80-83

36. Sifers, R. N., Shen, R. -F., and Woo, S. L. C. (1989) *Mol. Biol. Med.* **6**, 127-135

37. Duksin, D., and Mahoney, W. C. (1982) *J. Biol. Chem.* **257**, 3105-3109

38. Mahoney, W. C. and Duksin, D. (1980) *J. Chrom.* **198**, 506-510

39. Hurtley, S. M., Bole, D. G., Hoover-Litty, H., Helenius, A. and Copeland, C. S. (1989) *J. Cell Biol.* **108**, 2117-2126

40. Cox, D. W., Billingsley, G. D., and Callahan, J. W. (1986) *FEBS Lett.* **205**, 255-260

41. Saraste, J., and Kuismanen, E. (1984) *Cell* **38**, 535-549

42. Saraste, J., Palade, G. E., and Farquar, M. G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6425-6429

43. Tartakoff, A. M. (1986) *EMBO J.* **5**, 1477-1482

44. Munro, S., and Pelham, H. R. B. (1987) *Cell* **48**, 899-907

45. Pelham, H. R. B. (1988) *EMBO J.* **7**, 913-918

46. Lodish, H. F. (1988) *J. Biol. Chem.* **263**, 2107-2110

47. Ogushi, F. , Fells, G. A., Hubbard, R. G., Strauss, S. D., and Crystal, R. G. (1987) *J. Clin. Invest.* **80**, 1366-1374

48. Perlmutter, D. H., Schlesinger, M. J., Pierce, J. A., Punsal, P. I., and Schwartz, A. L. (1989) *J. Clin. Invest.* **84**, 1555-1561

## ABBREVIATIONS

[1]The abbreviations used are: AAT, alpha-1-antitrypsin; ER, endoplasmic reticulum; SDS, sodium dodocyl sulfate; PAGE, polyacrylamide gel electrophoresis; grp78, 78 kDa glucose regulated protein; BiP, immunoglobulin heavy chain binding protein

## FIGURE LEGENDS

FIG. 1. **Extraction of the insoluble PiZ variant from hepatocytes of transgenic mice.** A. For this experiment, a monolayer (100mm-diameter dish) of primary hepatocytes obtained from a PiZ-bearing transgenic mouse (line Z11.03) was lysed with Lysis Buffer containing the nonionic detergent NP40 (*Materials and Methods*), collected by scraping, and centrifuged at 10,000 x g for 5 minutes. The soluble and particulate fraction of the cell lysate were heated with disruption buffer for five minutes at 85°C and equivalent fractions of each sample was subjected to an immuno-dot blot analysis for the detection of human AAT (*Materials and Methods*). Treatment of the cell pellet with an equal volume of 2% SDS allows for >90 % of the insoluble protein to be extracted into the supernatant (*Sup.*). B. The secreted PiZ variant obtained from pulse labeling a 100mm-diameter dish of primary hepatocytes with [$^{35}$S]methionine (300 uCi in 3 ml of medium) for three hours was vortexed for 30 seconds in either 2% SDS or Lysis Buffer. The samples were diluted with Lysis Buffer to a final concentration of 0.1% SDS. Human AAT was immunoprecipitated from equal volumes of each sample, fractionated by SDS-PAGE, and detected by fluorography.

FIG. 2. **Fate of the newly synthesized human PiZ variant in hepatocytes from transgenic mice.** Equal monolayers (60mm-diameter dishes) of primary hepatocytes from a PiZ-bearing transgenic mouse were pulse-labeled for 10 minutes with [$^{35}$S]methionine (100 uCi/ml of medium) and chased for three hours in media containing a 4-fold excess of unlabeled methionine. Human AAT was immunoprecipitated from the soluble cell lysate, the media, and the SDS-solubilized cell lysate pellet from an individual dish of cells at various timepoints. The immunoprecipitates were subjected to SDS-PAGE (8% acrylamide gel) and radiolabeled bands were detected by fluorography. The apparent molecular mass of each protein is designated. An

artifact is present in the 0.5 hr lane of the media samples and does not represent human AAT.

FIG. 3. **Timecourse of the intracellular accumulation of the insoluble PiZ variant.** Three equivalent monolayers of primary hepatocytes from a PiZ-bearing transgenic mouse were pulse-radiolabeled with media containing [$^{35}$S]methionine (600 uCi/3ml of medium/dish). Human AAT was immunoprecipitated from the soluble cell lysate (*s*), the media (*Med.*), and the SDS-solubilized cell lysate pellet (*i*) from an individual dish at three, six, or nine hours of labeling. The immunoprecipitates were subjected to SDS-PAGE (8% acrylamide gel) and radiolabeled bands were detected by fluorography. Quantitation of the excised bands via scintillation counting is shown at the bottom of the figure. The apparent molecular mass of each protein is designated.

FIG. 4. **Steady-state level of grp78/BiP in primary hepatocytes from transgenic mice.** Individual 60mm-diameter dishes of primary hepatocytes from a PiZ-bearing mouse (*lane 2*) and its non-transgenic littermate (*lane1*) were pulse-radiolabeled with media containing [$^{35}$S]methionine (100 uCi/ml of medium) for three hours. Mouse albumin (*lower panel*) was immunoprecipitated from an aliquot of the medium and grp78/BiP (*upper panel*) was immunoprecipitated from the cell extract from each dish. The immunoprecipitates were subjected to SDS-PAGE (8% acrylamide gel) and radiolabeled bands were detected by fluorography. Mouse albumin (*mAlb*); grp78/BiP (*BiP*).

FIG. 5. **Steady-state level of grp78/BiP in a clonal line of stably-transfected PiZ-bearing mouse hepatoma cells.** A. Monolayers (100mm-diameter dishes) of the mouse hepatoma cell line Hepa 1A (*lane 1*), and a clonal line of PiZ-bearing hepatoma cells designated H1A/RSVATZ-8 cells (*lane 2*) were were pulse-radiolabeled with media containing

[$^{35}$S]methionine (600 uCi/3ml of medium/dish) for three hours. Mouse albumin (*lower panel*) was immunoprecipitated from an aliquot of the media and grp78/BiP (*upper panel*) was immunoprecipitated from the cell extract from each dish. The immunoprecipitates were subjected to SDS-PAGE (8% acrylamide gel) and radiolabeled bands were detected by fluorography. The apparent molecular mass of each protein is designated. A contaminating band of a slightly lower molecular mass is shown. Mouse albumin (*mAlb*); grp78/BiP (*BiP*). B. Identical monolayers of H1A/RSVATZ-8 cells were pre-incubated for one hour at 37$^{0}$C in regular growth medium containing 50 mM Hepes-HCl, pH 7.4, with or without tunicamycin (homologue A; 10 ug/ml). Cells were then radiolabeled for four hours in pulse-labeling media (*Materials and Methods*) containing 50 mM Hepes-HCl, pH 7.4, with or without tunicamycin as before. Grp78/BiP (*upper panel*) was immunoprecipitated from the cell extract and mouse albumin (*lower panel*) was immunoprecipitated from an aliquot of the media. Immunoprecipitates were subjected to SDS-PAGE (10-15% gradient acrylamide gel) and radiolabeled bands were detected by fluorography. The apparent molecular mass of each protein is designated. Mouse albumin (*mAlb*); grp78/BiP (*BiP*).

FIG. 6. **Test for the co-immunoprecipitation of grp78/BiP with retained human AAT variants at steady-state conditions.** Identical monolayers (100mm-diameter dishes) of H1A/RSVATZ-8 cells and H1A/N-13 cells were were pulse-radiolabeled in steady-state radiolabeling medium (*Materials and Methods*) containing [$^{35}$S]methionine (600 uCi/3ml of medium/dish) for three hours. Grp78/BiP (*lane 1*) and human AAT (*lane 2*) were immunoprecipitated from the soluble cell extracts under non-stringent conditions (*Materials and Methods*). The immunoprecipitates were subjected to SDS-PAGE (8% acrylamide gel) and radiolabeled bands were detected by fluorography. The apparent molecular mass of each protein

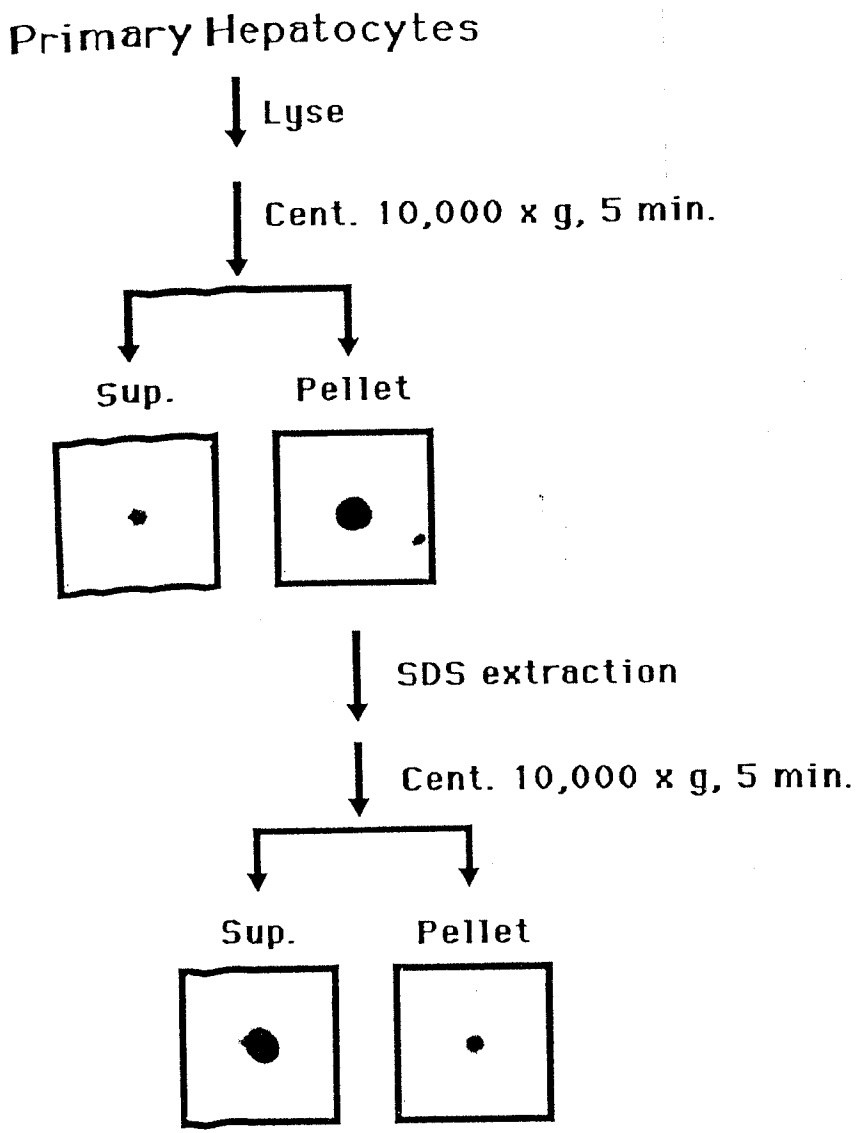
is designated. Anti-BiP (*a-BiP*); anti-human AAT (*a-hAAT*). An asterick (*) denotes a contaminating band that is sometimes present in the immunoprecipitated material.

FIG. 7. **Test for the identification of a transient association between grp78/BiP and the newly synthesized PiZ variant.** Identical monolayers (110mm-diameter dishes) of H1A/RSVATZ-8 cells were pulse-radiolabeled for 5 minutes with media containing [$^{35}$S]methionine (200 uCi/ml of medium) and chased in media containing a 4-fold excess of unlabeled methionine. Cells were harvested at the designated timepoint and human AAT (*hAAT*) or grp78/BiP (*BiP*) was immunoprecipitated under non-stringent conditions (*Materials and Methods*) from equal halves of the soluble cell lysate. The immunoprecipitates were subjected to SDS-PAGE (8% acrylamide gel) and radiolabeled bands were detected by fluorography. The apparent molecular mass of each protein is designated.

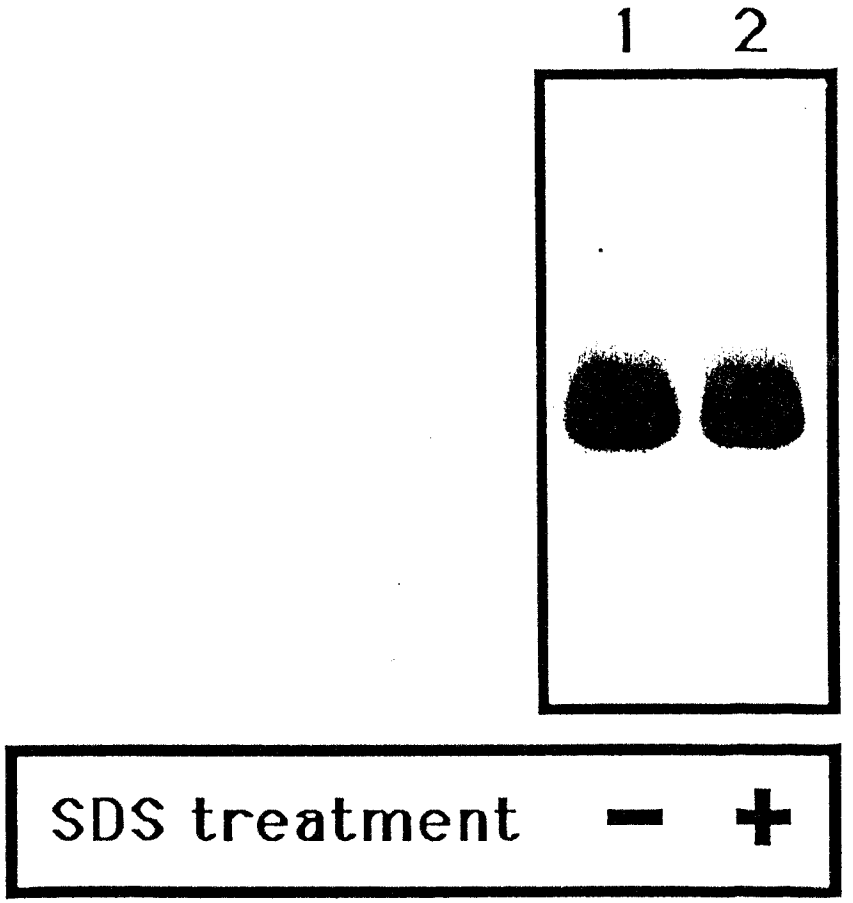
Fig. 1A

Fig. 1B

A




B



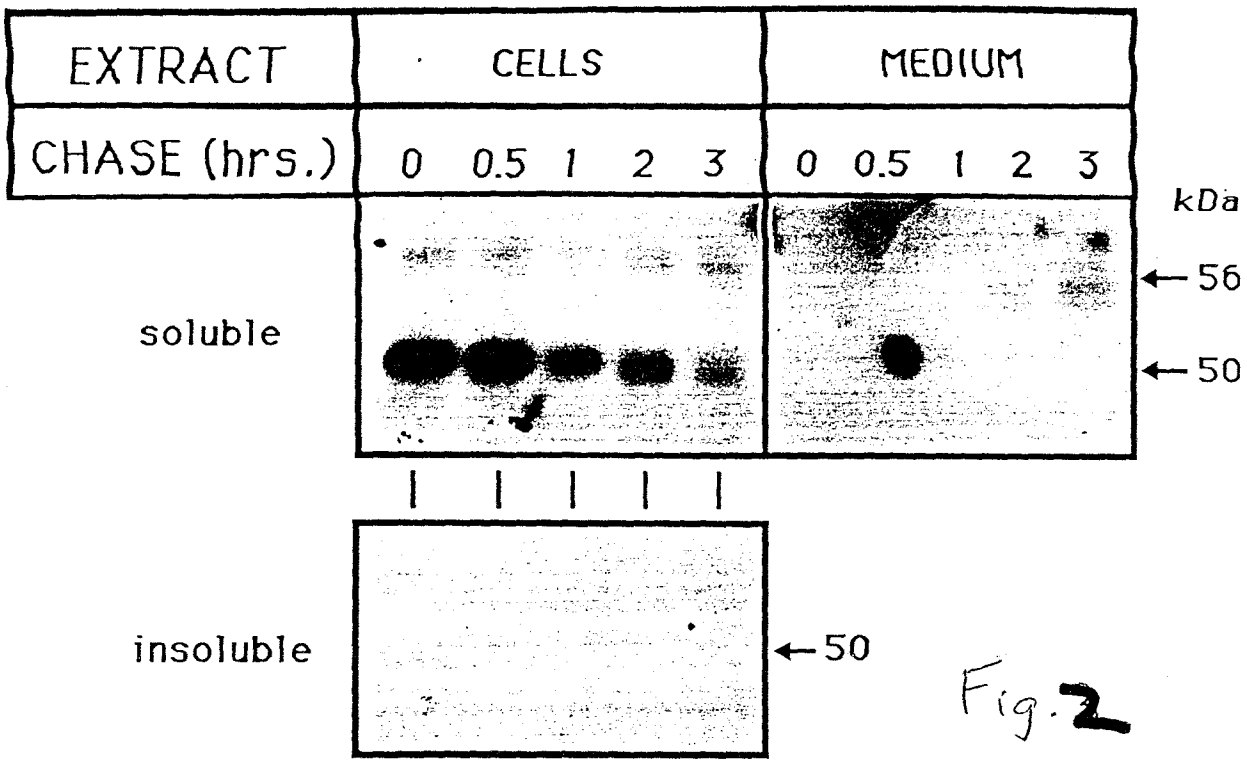

EXTRACT
CELLS
MEDIUM
CHASE (hrs.)
0 0.5 1 2 3
0 0.5 1 2 3
kDa
soluble
56
50
insoluble
50

Fig. 2

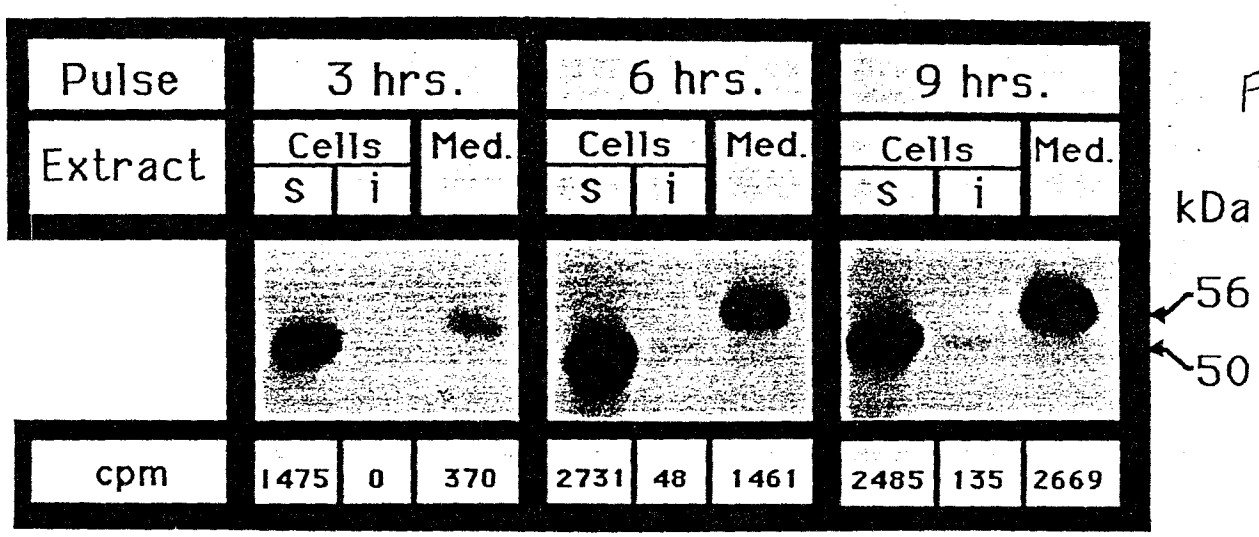

Pulse
3 hrs.
6 hrs.
9 hrs.
Extract
Cells
Med.
S
i
kDa
56
50
cpm
1475 0 370
2731 48 1461
2485 135 2669

Fig. 3

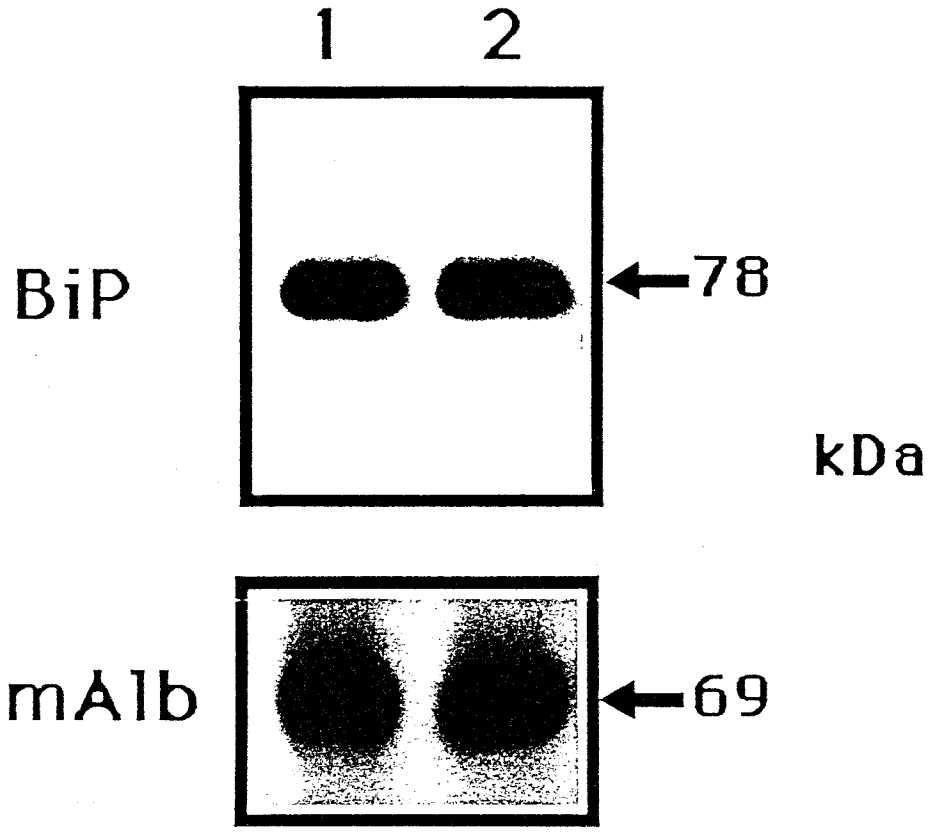



Fig. 4

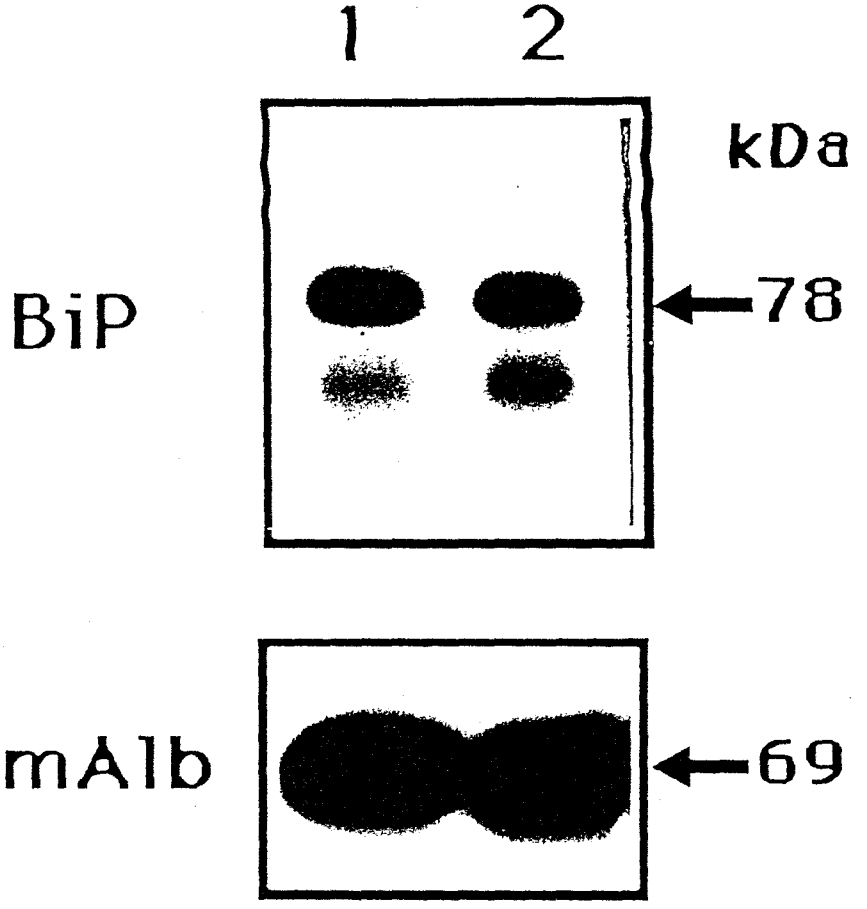



Fig. 5A

B

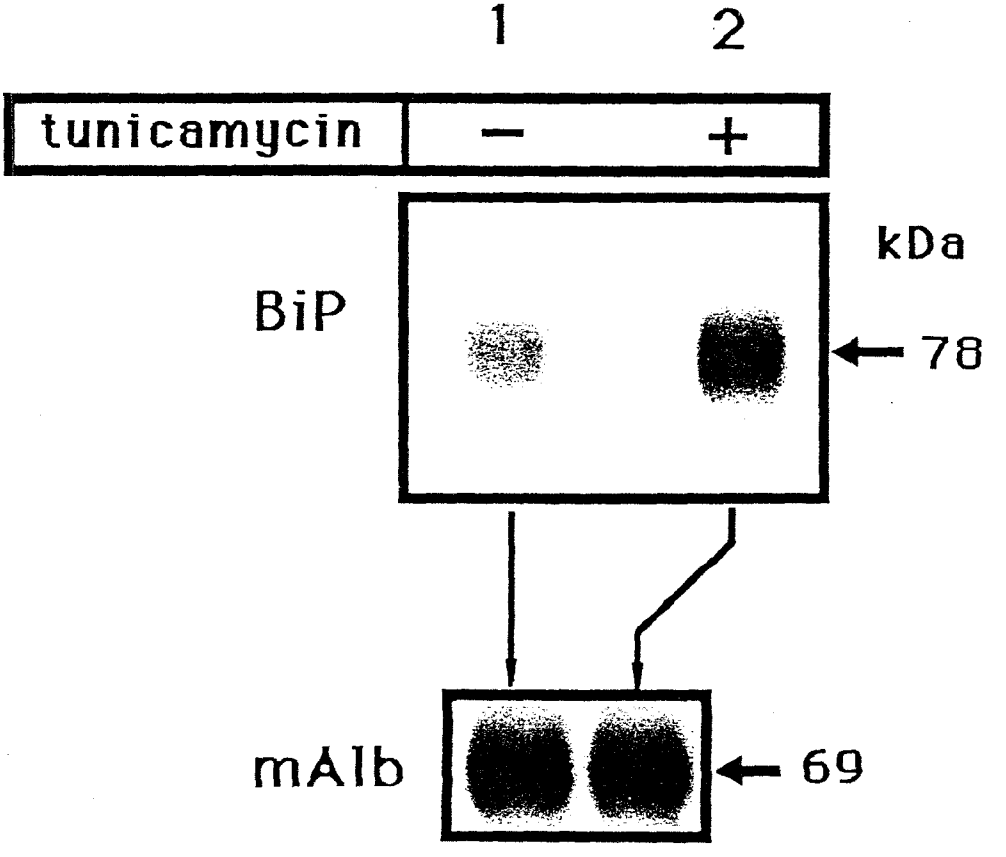



Fig. 5B

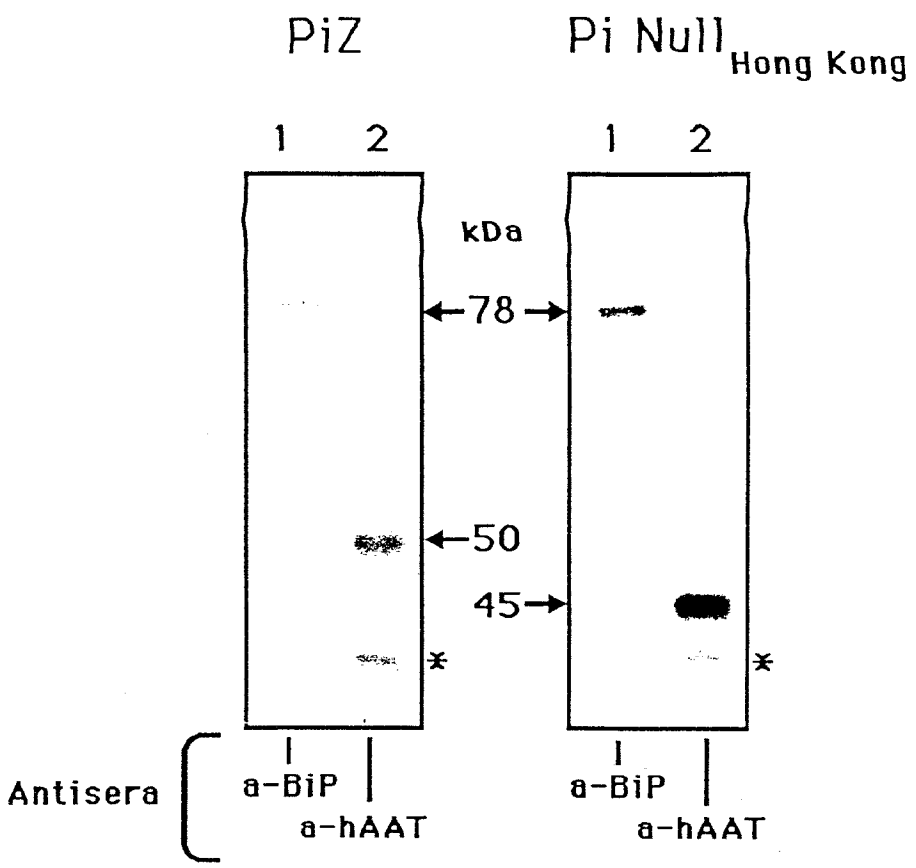

PiZ
Pi Null Hong Kong
1
2
1
2
kDa
78
50
45
*
*
Antisera
a-BiP
a-hAAT
a-BiP
a-hAAT

Fig. 6

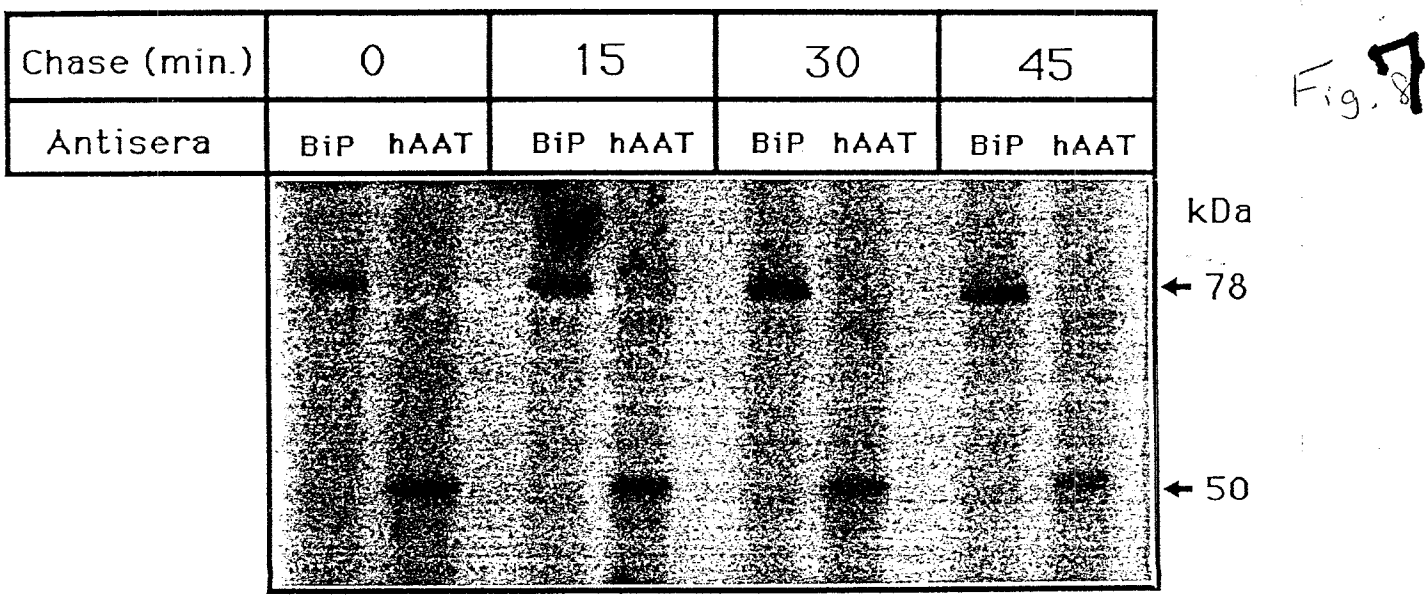

Chase (min.)
0
15
30
45
Antisera
BiP hAAT
BiP hAAT
BiP hAAT
BiP hAAT
kDa
78
50

Fig. 7