FINAL REPORT for Moran Foundation Project (1-92-0060)

Date: 6/1/94

Project Title: "Mechanistic Biochemistry of Chaperone-associated Protein Folding"

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

PROGRESS REPORT:

A 90 kDa protein co-immunoprecipitates with a secretion-impaired variant of human alpha1-antitrypsin. The naturally-occurring null$_{Hong\ Kong}$ variant of human alpha$_1$-antitrypsin is truncated at its carboxyl terminus which predicts misfolding of the polypeptide following its biosynthesis. The null$_{Hong\ Kong}$ variant is retained and degraded in a pre-Golgi compartment of stably transfected murine hepatoma cells. Because retention of unassembled and misfolded polypeptides in the endoplasmic reticulum (ER) is often accompanied by their association with a molecular chaperone, we sought to identify cellular proteins that might be stably associated with the retained null$_{Hong\ Kong}$ variant. We used long-term metabolic radiolabeling of cells with [^{35}S]methionine in combination with a low stringency immunoprecipitation technique and identified the specific co-immunoprecipitation of a radiolabeled 90 kDa protein, which we designated p90.

Evidence for a hydrophobic interaction between co-precipitating p90 and the misfolded alpha1-antitrypsin polypeptide. To test for a hydrophobic interaction between calnexin and the null$_{Hong\ Kong}$ variant, immunoprecipitates were washed with a variety of relatively mild detergents. Significantly, of those tested only deoxycholate was able to dissociate the complex in a specific manner. This suggests that calnexin probably binds an exposed hydrophobic region of the misfolded polypeptide.

The 90 kDa protein is identical to the molecular chaperone calnexin. Several criteria including mobility in SDS-PAGE, absense of asparagine-linked oligosaccharides, and immunoreactivity of the deoxycholate-dissociated protein with peptide-specific antiserum indicated that p90 is identical to calnexin, a calcium-binding phosphoprotein of the ER membrane.

Bound calnexin is phosphorylated . Performing immunoprecipitation experiments with [^{32}P] demonstrated that co-precipitating calnexin is phosphorlyated. This demonstrates that it is the phosphorylated form of this molecular chaperone that actually binds the retained misfolded null$_{Hong\ Kong}$ variant.

Binding of calnexin to the misfolded human alpha1-antitrypsin variant requires calcium. Results from co-immunoprecipitation analyses and velocity sedimentation experiments have verified that chelation of calcium ions *in vitro* results in the dissociation of calnexin from the null$_{Hong\ Kong}$ variant. These data indicate that calcium might play a significant role in forming or even maintaining the alpha1-antitrypsin - calnexin complex within the cell.

Calnexin binds the misfolded alpha1-antitrypsin polypeptide via a stoichiometric association. A gift of antiserum against calnexin was used to immunoprecipitate the protein from cells. Approximately 20-30 % of the retained null(HK) variant polypeptides co-precipitated with calnexin. Using velocity sedimentation experiments, 20-30% of the retained null(HK) variant polypeptides sedimented in association with calnexin, in the form of a 7.6 S complex. Metabolic incorporation of [^{35}S]methionine into both the null$_{\text{Hong Kong}}$ variant and co-precipitating calnexin indicated that the two proteins are combined in a 1:1 stoichiometric association. Overall, these experiments verified that 20-30 % of the retained misfolded null$_{\text{Hong Kong}}$ variant polypeptides are bound stoichiometrically to the molecular chaperone calnexin.

PRESENTATIONS AND PUBLICATIONS:

Presentation:
Le, A., and Sifers, R. N., Association between calnexin and a secretion-incompetent variant of human alpha1-antitrypsin. Presentation at the Gordon Conference on Molecular Membrane Biology, 6/93, (*no published abstract*).

Publication:
Le, A., Steiner, J. L., Ferrell, G. A., Shaker, J. C., and Sifers, R. N. (1994). Association between calnexin and a secretion-incompetent variant of human alpha$_1$-antitrypsin. *J. Biol. Chem.* 269, 7514-7519.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 10, Issue of March 11, pp. 7514–7519, 1994
Printed in U.S.A.

Association between Calnexin and a Secretion-incompetent Variant of Human α_1-Antitrypsin*

(Received for publication, July 23, 1993, and in revised form, October 21, 1993)

Anhquyen Le‡, Julie L. Steiner§¶, Gail A. Ferrell§¶, Jennifer C. Shaker§¶, 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 null$_{Hong\ Kong}$ variant of human α_1-antitrypsin is truncated at its carboxyl terminus, and is retained and degraded in a pre-Golgi compartment of stably transfected murine hepatoma cells (Le, A., Graham, K. S., and Sifers, R. N. (1990) *J. Biol. Chem.* 265, 14001–14007). Long-term metabolic radiolabeling with [^{35}S]methionine or [^{32}P]orthophosphate in combination with low stringency immunoprecipitation of the null$_{Hong\ Kong}$ variant has resulted in the co-precipitation of a radiolabeled 90-kDa protein designated p90. Several criteria, including mobility in SDS-polyacrylamide gel electrophoresis, absence of asparagine-linked oligosaccharides, and immunoreactivity with peptide-specific antiserum, have indicated that co-precipitating p90 is identical to calnexin, a calcium-binding phosphoprotein of the endoplasmic reticulum membrane (Wada, I. W., Rindress, P. H., Ou, W.-J., Doherty, J. J., Louvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. M. (1991) *J. Biol. Chem.* 266, 19599–19610). Finally, results from co-immunoprecipitation analyses and velocity sedimentation experiments have verified that approximately 30% of the retained null$_{Hong\ Kong}$ variant polypeptides are associated with calnexin in a 1:1 molar ratio and can be dissociated with either deoxycholate or chelation of calcium ions at 37 °C. Overall, these findings may extend our current understanding of the molecular pathogenesis of serum α_1-antitrypsin deficiency.

In eukaryotic cells, "secretory proteins" enter the exocytic pathway as the nascent polypeptides are translocated across the membrane of the rough endoplasmic reticulum (ER)[1] (1). Present evidence suggests that these soluble polypeptides exit the ER via their bulk flow (2) into nonselective carrier vesicles that bud from the smooth region of the organelle (3). Significantly, export of many newly synthesized soluble and integral membrane proteins from the ER requires that they must first fold or assemble into their native conformation, and failure to fulfill this requirement can often result in their retention and subsequent degradation in the cell (for reviews, see Refs. 4 and 5). An important problem in cell biology is to identify and characterize the various cellular components responsible for this intracellular retention mechanism.

Human α_1-antitrypsin (AAT) inhibits the hydrolytic activity of serine proteinases (6, 7) and is a major constituent of human serum. The protein is secreted predominantly from hepatocytes and exists as a monomeric glycoprotein of 394 amino acids (8). The primary structure of human AAT has been deduced by nucleotide sequence analysis (9, 10) and by direct sequencing of the protein (11). The macromolecule exhibits extensive polymorphism throughout the world population, and several allelic variants are associated with a decreased concentration of the proteinase inhibitor in serum (12, 13). Significantly, this phenomenon is often caused by the intracellular retention of a mutant variant AAT polypeptide within the hepatic ER (14). A primary physiologic role of human AAT is to protect lung elastin fibers from degradation by neutrophil elastase, and its deficiency or absence from serum can result in an uninterrupted degradation of elastin fibers which has been implicated in emphysema (15, 16).

A TC dinucleotide deletion within the naturally occurring null$_{Hong\ Kong}$ variant allele predicts the premature termination of the encoded polypeptide at 333 amino acids (17). The entire cohort of newly synthesized null$_{Hong\ Kong}$ variant polypeptides are retained in the ER of stably transfected murine hepatoma cells (17) and are subsequently degraded in a nonlysosomal pre-Golgi compartment (18). Because retention of unassembled and misfolded polypeptides in the ER is often accompanied by their association with a molecular chaperone (4, 19), we have sought to identify cellular proteins that are stably associated with the retained null$_{Hong\ Kong}$ variant. Using long-term metabolic radiolabeling of cells, low stringency immunoprecipitation, and velocity sedimentation analyses, we have identified a stable stoichiometric complex consisting of the null$_{Hong\ Kong}$ variant and calnexin, a calcium-binding phosphoprotein of the ER membrane (20).

MATERIALS AND METHODS

Chemicals and Reagents—Routine growth media for mammalian tissue culture, fetal bovine serum, and methionine-deficient Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies, Inc. Phosphate-deficient DMEM, all phosphatase inhibitors, and Tran^{35}S-label (specific activity > 1000 Ci/mmol) were purchased from ICN Biomedicals. [^{32}P]Orthophosphoric acid (specific activity > 8000 Ci/mmol) and ^{125}I-protein A (specific activity, 70–100 μCi/μg protein) were purchased from DuPont NEN. Protein G-Sepharose 4FF was purchased from Pharmacia LKB Biotechnology Inc. Recombinant endoglycosidase H (cloned from *Streptomyces plicatus*) and all biological detergents were purchased from Boehringer Mannheim. EDTA, EGTA, and all buffers and salts were purchased from Sigma.

Antisera—An immunoglobulin fraction of goat anti-human AAT was purchased from Organon Teknika-Cappel. Rabbit polyclonal antisera against amino acid residues 30–45 of canine calnexin (anti-calnexin

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

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

‖ To whom all correspondence should be addressed: Baylor College of Medicine, Dept. of Pathology, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-3169; Fax: 713-798-5838.

[1] The abbreviations used are: ER, endoplasmic reticulum; AAT, α_1-antitrypsin; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis.

peptide 1) or against residues 487–505 in the cytoplasmic tail of the molecule (anti-calnexin peptide 3) were gifts from Dr. John J. M. Bergeron, McGill University. Rabbit polyclonal antiserum to grp94 was a generous gift from Dr. Michael Green, Saint Louis University.

Stably Transfected Murine Hepatoma Cells—Preparation of clonal lines of stably transfected murine hepatoma cells (line Hepa 1a) synthesizing either the $null_{Hong\ Kong}$ variant (cell line H1A/N13) or the normal human PiM(Val213) variant (cell line H1A/M15) was described previously (17).

Metabolic Radiolabeling of Proteins—For metabolic radiolabeling of methionine residues, 100-mm diameter dishes of confluent cells were incubated in 3 ml of methionine-deficient DMEM supplemented with 1 × glutamine, 1% fetal calf serum, 3 mg/liter unlabeled L-methionine, and 100–300 μCi of Tran^{35}S-label. Metabolic radiolabeling of phosphorylated proteins was accomplished by first washing confluent cell monolayers with 37 °C phosphate-deficient DMEM, followed by incubation in 3 ml of that medium supplemented with 1 × glutamine, 1% fetal calf serum, and 70–180 μCi of [^{32}P]orthophosphoric acid.

Low Stringency Immunoprecipitation of Proteins—Radiolabeled cell monolayers were washed with ice-cold phosphate-buffered saline, set on an ice cold metal surface, and lysed at 4 °C by scraping with a spatula in 0.5–1 ml of 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl (Buffer S) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% Nonidet P-40. Alternative detergents were utilized for cell lysis in some experiments as described in the appropriate figure legends. For analysis of phosphorylated proteins, radiolabeled cells were lysed as described above with ice-cold 0.1 M $NaPO_4$, pH 7.4, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. Following centrifugation of lysed cells at 10,000 × *g* for 3 min, the supernatant was subjected to immunoprecipitation at low stringency conditions. This included a 2-h incubation with continuous gentle mixing of the soluble cell lysate in Buffer S with an appropriate antibody pre-immobilized to protein G-Sepharose 4FF beads. The pre-immobilization procedure was performed by incubating the antibody and protein G-Sepharose for 30 min at 23 °C. An identical incubation of the immobilized material with bovine serum albumin (20 mg/ml) was then performed to block nonspecific binding sites. Following the 2-h incubation, beads were washed with four changes of ice-cold cell lysis buffer containing 0.5% Nonidet P-40 plus four changes of the same solution containing 0.5 M NaCl. Each wash included a mechanical agitation for 1 min prior to centrifugation and removal of the supernatant. Washed immunocomplexes were heated for 10 min at 75 °C in disruption buffer containing 2% sodium dodecyl sulfate. Eluted proteins were separated from the beads by centrifugation and then fractionated by SDS-PAGE. Radiolabeled proteins were detected by fluorographic enhancement of the vacuum-dried gel, as described previously (18).

Detergent Sensitivity Analysis—$Null_{Hong\ Kong}$ variant polypeptides were immunoprecipitated from [^{35}S]methionine-radiolabeled H1A/N13 cells and washed at low stringency conditions as described above. The immobilized material was then washed four times with ice-cold Buffer S to remove all detergent from the beads. The washed beads were aliquoted into several fresh tubes and each was incubated with gentle mixing for 5 min at 4 °C with 1 ml of a selected detergent in Buffer S. Beads were collected by centrifugation, washed once with ice-cold Buffer S, and bound proteins were eluted with disruption buffer and fractionated by SDS-PAGE. Radiolabeled proteins were detected by fluorography.

Western Blot Analysis—The $null_{Hong\ Kong}$ variant was immunoprecipitated at low stringency conditions from several dishes of nonradiolabeled cells that had been lysed with 0.5% Nonidet P-40, as described above. Beads were washed with selected detergents, and dissociated proteins were collected in the supernatant following centrifugation. Samples were concentrated to approximately 50 μl with Centricon-30 ultrafiltration units (Amicon), and proteins were subjected to SDS-PAGE. Fractionated proteins were electrophoretically transferred to nitrocellulose paper, and nonspecific protein binding sites were blocked with Tris-buffered saline, pH 7.4, containing 0.05% sodium azide and 5% skim milk, as described previously (9). Blocked filters were then probed overnight by incubation at 23 °C with the desired antibody, followed by washing, and then incubation with ^{125}I-protein A (5 × 10^5 cpm/ml) for 3 h. Filters were washed, and radioactive signals were detected by autoradiography.

Sedimentation Velocity Centrifugation—H1A/N13 cells were metabolically radiolabeled with [^{35}S]methionine for 24 h. Cell monolayers were lysed at 4 °C with a selected detergent, and the soluble supernatant was subjected to velocity sedimentation in linear 5–20% sucrose gradients as described previously (21), except that 0.1% sodium cholate was included in the gradient to provide an environment of detergent monomers. The $null_{Hong\ Kong}$ variant was immunoprecipitated at low stringency conditions from individual gradient fractions and then subjected to SDS-PAGE. Radiolabeled proteins were detected by fluorography and quantitated by scintillation counting of excised gel pieces. In all experiments, reported values are expressed following subtraction of "background" radioactivity from an identical procedure performed with radiolabeled Hepa 1A cells.

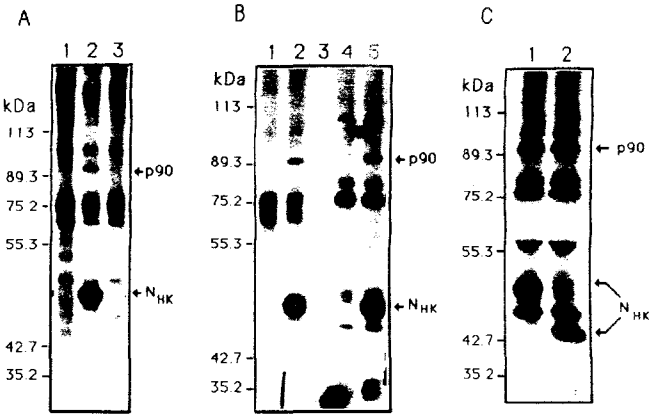

FIG. 1. **A nonglycosylated 90-kDa protein co-immunoprecipitates with the $null_{Hong\ Kong}$ variant.** *A*, nonreducing SDS-PAGE of proteins immunoprecipitated with anti-human AAT at low stringency conditions from Hepa 1a (*lane 1*) and H1A/N13 (*lane 2*) cells metabolically radiolabeled with [^{35}S]methionine for 5 h. *Lane 3*, result of preblocking the antibody with human serum AAT prior to immunoprecipitation from a radiolabeled H1A/N13 cell lysate. *B*, proteins immunoprecipitated from radiolabeled Hepa 1a (*lanes 1* and *4*) and H1A/N13 (*lanes 2* and *5*) cells fractionated by SDS-PAGE without (*lanes 1* and *2*) and with (*lanes 4* and *5*) disulfide-reducing agents. *Lane 3* is a blank. *C*, immunoprecipitated proteins from radiolabeled H1A/N13 cells subjected to either mock-digestion (*lane 1*) or digestion overnight with endoglycosidase H (*lane 2*). Migration of molecular weight standards, the $null_{Hong\ Kong}$ variant (N_{HK}), and p90 (*p90*) in SDS-PAGE is shown with *horizontal arrows*.

RESULTS

Co-immunoprecipitation of a Nonglycosylated 90-kDa Protein—Murine hepatoma cells that stably synthesize the $null_{Hong\ Kong}$ variant (cell line H1A/N13) were metabolically radiolabeled for 5 h in medium containing [^{35}S]methionine. Cells were lysed with 0.5% Nonidet P-40, and the 45-kDa $null_{Hong\ Kong}$ variant was immunoprecipitated at low stringency conditions from the soluble cell lysate (Fig. 1*A*, *lane 2*). Although several radiolabeled proteins of varying masses were identified in the immunoprecipitated material, a single radiolabeled 90-kDa protein, designated p90, was present only in the immunoprecipitate from stably transfected cells (*lane 2*), but not in the immunoprecipitate from nontransfected cells (*lane 1*) or when the cell lysate had been incubated with an antibody that had been preblocked with human serum AAT (*lane 3*).

Whereas reduction of disulfide bonds with β-mercaptoethanol altered the mobility in SDS-PAGE of several nonspecifically bound proteins (Fig. 1*B*, compare *lanes 1* and *4*), neither the intensity or mobility of p90 was affected significantly (compare *lanes 2* and *5*). This result confirmed that p90 did not consist of a disulfide-linked homodimer of the 45-kDa $null_{Hong\ Kong}$ variant. Furthermore, mobility of the immunoprecipitated $null_{Hong\ Kong}$ variant was increased in SDS-PAGE following its deglycosylation by endoglycosidase H (Fig. 1*C*, *lane 2*). However, this treatment did not alter the mobility of co-precipitating p90 (*lane 2*) and indicated that the protein does not contain asparagine-linked oligosaccharide moieties.

Specific Dissociation of p90 with Deoxycholate—Retention of misfolded and unassembled proteins within the ER is often accompanied by their hydrophobic interaction with one or more molecular chaperones (19). Thus, considering that p90 might associate with the $null_{Hong\ Kong}$ variant through hydrophobic

interactions, we examined the potential ability of several detergents to dissociate the two macromolecules. For this, $null_{Hong\ Kong}$ variant polypeptides were immunoprecipitated at low stringency conditions from [^{35}S]methionine-radiolabeled H1A/N13 cells lysed with Nonidet P-40. Aliquots of the immunocomplexes were then incubated at 4 °C with one of several detergents prior to subjecting them to SDS-PAGE and fluorography (see "Materials and Methods"). As expected, incubation of the immunoprecipitated material with the harsh ionic detergent lithium dodecyl sulfate dissociated p90 and all nonspecifically bound proteins, plus partially disrupted binding of the $null_{Hong\ Kong}$ variant to the immobilized antibody (compare *lanes 1* and *10*). Significantly, of the detergents tested only incubation with sodium deoxycholate specifically and quantitatively dissociated p90 from the immunoprecipitated $null_{Hong\ Kong}$ variant (*lane 8*).

Recognition of p90 by Antiserum against Calnexin—The identity of co-precipitating p90 was accomplished by combining the specificity of its deoxycholate-mediated dissociation from the immunoprecipitated $null_{Hong\ Kong}$ variant with its specific immunologic cross-reactivity. For this, retained $null_{Hong\ Kong}$ variant polypeptides were first immunoprecipitated at low stringency conditions from several dishes of nonradiolabeled H1A/N13 cells following their lysis with Nonidet P-40. The immunoprecipitated material was then washed with sodium deoxycholate as described above. Eluted proteins were concentrated by ultrafiltration, fractionated by SDS-PAGE, and electrophoretically transferred to nitrocellulose. Because of the relative migration of co-precipitating p90 in SDS-PAGE and the intracellular location of the retained $null_{Hong\ Kong}$ variant, filters were first probed with antiserum against the ER-resident protein grp94 (22), but no immunoreactivity was detected (data not shown). However, in a subsequent experiment, a single immunoreactive 90-kDa protein was identified when the filter was probed with a polyclonal peptide-specific antiserum raised against amino acids 30–45 of canine calnexin (Fig. 2*B*, *lane 3*). Moreover, migration of the immunoreactive protein in SDS-PAGE was identical to that of endogenous murine calnexin present in a crude Hepa 1A cell extract (*lane 5*). Furthermore, no cross-reactive proteins were detected in a sodium cholate wash of the p90-$null_{Hong\ Kong}$ complex (*lane 4*), a deoxycholate wash of an immunocomplex generated from nontransfected Hepa 1A cells (*lane 1*), or from a deoxycholate wash of a mock-immunoprecipitate from H1A/N13 cells (*lane 2*). Overall, these data confirmed that the detergent-mediated dissociation of the immunoreactive 90-kDa protein was identical to that of p90, and that p90 shared an antigenic epitope with murine calnexin.

Metabolic Phosphorylation of p90—Because the calnexin polypeptide can undergo phosphorylation *in vitro* (23, 24), we asked whether co-precipitating p90 would incorporate radiolabeled phosphate during metabolic radiolabeling of cells. For this, dishes of cells were incubated for 5 h in medium containing either [^{35}S]methionine or [^{32}P]orthophosphate, and soluble cell lysates were prepared with 0.5% Nonidet P-40. As shown in Fig. 3*A*, whereas the immunoprecipitated $null_{Hong\ Kong}$ variant was detected in H1A/N13 cells radiolabeled with [^{35}S]methionine (*lane 1*), it was not identified in cells radiolabeled with [^{32}P]orthophosphate (*lane 3*). This result was expected because the human AAT polypeptide is not a substrate for phosphorylation. However, four phosphorylated proteins did apparently co-precipitate with the $null_{Hong\ Kong}$ variant in the ^{32}P-radiolabeled H1A/N13 cells (*lane 3*), but three of these were also present in the immunocomplex generated from Hepa 1A cells (*lane 4, asterisks*) which indicated that their association with the beads was of a nonspecific nature. Significantly, migration of the remaining ^{32}P-radiolabeled 90-kDa protein (*lane 3*) was identical to that of co-precipitating p90 in [^{35}S]methionine-radiolabeled cells (*lane 1*) and murine calnexin immunoprecipitated from cells radiolabeled with either reagent (*lanes 2* and *5*). Moreover, digestion of ^{32}P-radiolabeled p90 and ^{32}P-radiolabeled calnexin with V8 proteinase resulted in the generation of identical peptide maps (data not shown).

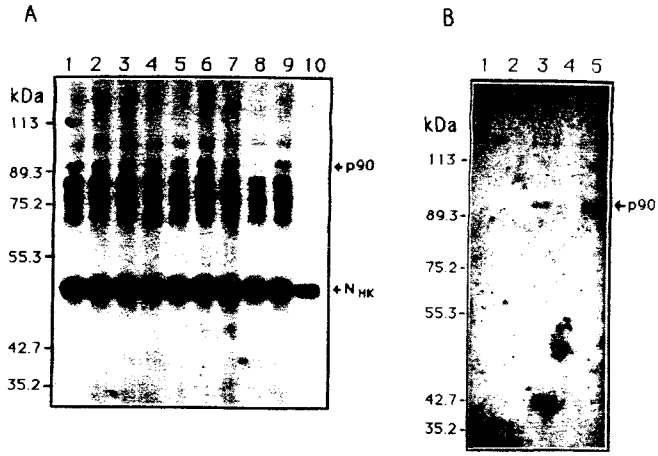

FIG. 2. **Recognition of detergent-dissociated p90 with anti-calnexin peptide 1 antiserum.** *A*, stability of the p90-$null_{Hong\ Kong}$ variant complex in various detergents. Aliquots of the immunoprecipitated p90-$null_{Hong\ Kong}$ variant complex from [^{35}S]methionine-radiolabeled H1A/N13 cells were incubated as described under "Materials and Methods" with either no detergent (*lane 1*) or 0.5% of the following detergents: Nonidet P-40 (*lane 2*), Triton X-100 (*lane 3*), Tween 20 (*lane 4*), octyl-β-glucoside (*lane 5*), digitonin (*lane 6*), sodium cholate (*lane 7*), sodium deoxycholate (*lane 8*), CHAPS (*lane 9*), or lithium dodecyl sulfate (*lane 10*). *B*, immunoreactivity of detergent-dissociated p90 with anti-calnexin peptide 1 antiserum. Detergent washes, as described above, were performed on immunocomplexes prepared from nonradiolabeled cells. Western blotting of the released proteins was performed with anti-calnexin peptide 1 antiserum (see "Materials and Methods"). *Lane 1*, deoxycholate wash of immunocomplex from Hepa1a cells; *lane 2*, deoxycholate wash of a mock-immunoprecipitation from H1A/N13 cells; *lane 3*, deoxycholate wash of immunocomplex from H1A/N13 cells; *lane 4*, cholate wash of an immunocomplex from H1A/N13 cells; *lane 5*, crude extract from Hepa 1a cells. Migration of molecular weight standards, the $null_{Hong\ Kong}$ variant (N_{HK}), and p90 (*p90*) in SDS-PAGE is shown with *horizontal arrows*.

Chelation of Calcium Ions Dissociates p90—Because calnexin is a calcium-binding protein (20, 23, 24), we asked whether the availability of free calcium ions in a cell lysate would affect the stability of the p90-$null_{Hong\ Kong}$ variant complex. To address this question, [^{35}S]methionine-radiolabeled H1A/N13 cells were lysed with Nonidet P-40, and aliquots of the soluble lysate were incubated in the presence of divalent cation chelators prior to low stringency immunoprecipitation of the $null_{Hong\ Kong}$ variant. As shown in Fig. 4, incubation of cell lysates in the presence of either 2 mM EDTA (*lane 5*) or 2 mM EGTA (*lane 6*) at 4 °C did not prevent the co-precipitation of p90 with the $null_{Hong\ Kong}$ variant as compared with that of a nontreated control sample (*lane 1*). In contrast, incubation with 2 mM EDTA at 37 °C for 10 min completely abrogated the co-precipitation of p90 (*lane 3*), and the elevated temperature alone was not sufficient to destabilize the complex (*lane 2*). Ability of 2 mM EGTA to dissociate the complex at the elevated temperature (*lane 4*) confirmed that chelation of calcium ions was responsible for dissociation of p90 from the $null_{Hong\ Kong}$ variant.

Stoichiometric Association between p90 and a Subset of Retained $Null_{Hong\ Kong}$ Variant Polypeptides—To determine the actual percent of retained $null_{Hong\ Kong}$ variant polypeptides that associated with p90, [^{35}S]methionine-radiolabeled H1A/N13 cells were lysed with Nonidet P-40 and small aliquots of the soluble cell lysate were incubated with an excess of immu-

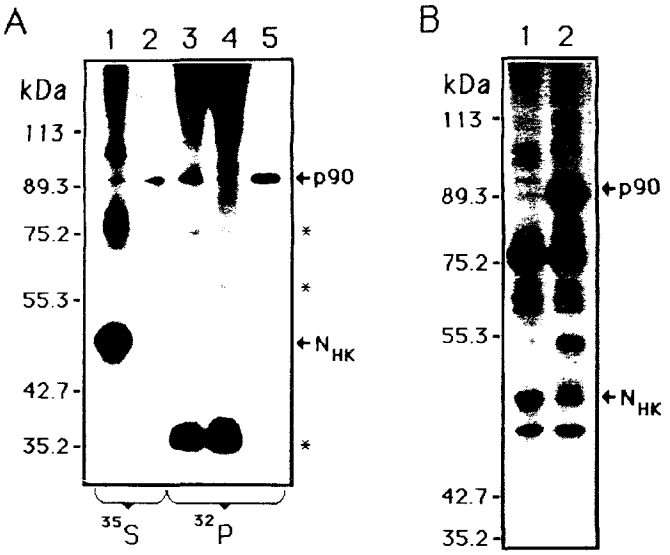

FIG. 3. **Metabolic phosphorylation of p90 and co-precipitation of the null$_{Hong\ Kong}$ variant with antiserum to calnexin.** *A*, metabolic phosphorylation of co-immunoprecipitating p90. H1A/N13 cells (*lanes 1, 2,* and *3*) and Hepa 1a cells (*lanes 4* and *5*) were radiolabeled for 5 h with [^{35}S]methionine (^{35}S) or [^{32}P]orthophosphate (^{32}P). Cells were lysed with 0.5% Nonidet P-40 and subjected to low stringency immunoprecipitation with either a polyclonal antibody to human AAT (*lanes 1, 3,* and *4*) or anti-calnexin peptide 3 antiserum (*lanes 2* and *5*) at more stringent conditions. *Asterisks* denote prominant "background" bands. *B*, co-immunoprecipitation of the null$_{Hong\ Kong}$ variant with antiserum to calnexin. [^{35}S]Methionine-radiolabeled H1A/N13 cells were lysed with 0.5% Nonidet P-40, and identical aliquots were subjected to low stringency immunoprecipitation with a polyclonal antibody to human AAT (*lane 1*) or with anti-calnexin peptide 3 antiserum (*lane 2*). Migration of molecular weight standards, the null$_{Hong\ Kong}$ variant (N_{HK}), and p90 (*p90*) in SDS-PAGE is shown with *horizontal arrows*.

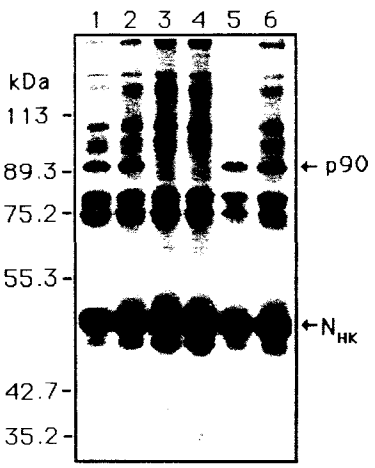

FIG. 4. **Calcium chelators dissociate p90.** H1A/N13 cells were radiolabeled with [^{35}S]methionine for 5 h and lysed with Nonidet P-40. Soluble lysates were incubated for 10 min at various conditions prior to low stringency immunoprecipitation of human AAT. *Lane 1*, 4 °C with no additions; *lane 2*, 37 °C with no additions; *lane 3*, 37 °C with 2 mM EDTA; *lane 4*, 37 °C with 2 mM EGTA; *lane 5*, 4 °C with 2 mM EDTA; *lane 6*, 4 °C with 2 mM EGTA. Migration of molecular weight standards, the null$_{Hong\ Kong}$ variant (N_{HK}), and p90 (*p90*) in SDS-PAGE is shown with *horizontal arrows*.

noprecipitating antiserum raised against residues 487–505 in the cytoplasmic tail of canine calnexin (anti-calnexin peptide 3) (see "Materials and Methods"). Quantitative immunoprecipitation of murine calnexin at low stringency conditions resulted in the co-precipitation of a radiolabeled 45-kDa protein (Fig. 3*B*, *lane 2*) that was not detected when cell lysates were incubated in the absence of antiserum to calnexin, and additional antiserum did not increase the amount immunoprecipitable calnexin (data not shown). Quantitative analysis indicated that approximately 25–30% of the entire population of retained radiolabeled null$_{Hong\ Kong}$ variant polypeptides co-precipitated with murine calnexin (compare *lanes 1* and *2*).

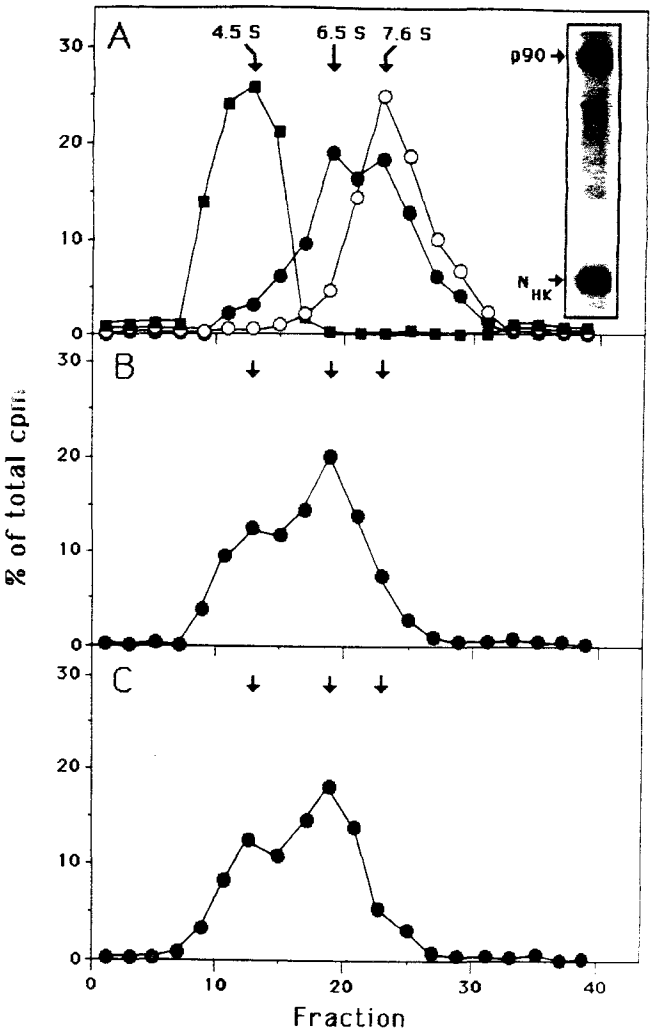

FIG. 5. **Velocity sedimentation of the retained null$_{Hong\ Kong}$ variant polypeptides.** H1A/N13 cells were radiolabeled for 24 h with [^{35}S]methionine, and soluble cell lysates were treated as indicated prior to velocity sedimentation in linear 5–20% sucrose gradients. Human AAT was then immunoprecipitated at low stringency conditions from odd-numbered gradient fractions. The immunoprecipitated null$_{Hong\ Kong}$ variant and co-precipitating p90 were quantitated by scintillation counting of excised gel pieces. *A*, sedimentation profile of the null$_{Hong\ Kong}$ variant (*closed circles*) and co-immunoprecipitating p90 (*open circles*) from H1A/N13 cells lysed with 0.5% Nonidet P-40. Sedimentation of the secreted normal M(Val213) variant from H1A/M15 cells (*solid squares*). The *inset* shows a fluorogram of the immunoprecipitated null$_{Hong\ Kong}$ variant and co-precipitating p90 from fraction 27 of the gradient. *B*, sedimentation profile of the retained null$_{Hong\ Kong}$ variant from H1A/N13 cells lysed with 1% sodium deoxycholate (*closed circles*). *C*, sedimentation profile of the null$_{Hong\ Kong}$ variant (*closed circles*) from a 1% Nonidet P-40 cell lysate preincubated for 10 min at 37 °C in the presence of 2 mM EGTA. All data are expressed as percent of total immunoprecipitating radioactivity of the appropriate protein. Calculated Svedberg units (S) are shown for individual peaks.

H1A/N13 cells were then radiolabeled for 24 h with [^{35}S]methionine and Nonidet P-40-soluble cell lysates were subjected to velocity centrifugation in linear sucrose gradients. The retained null$_{Hong\ Kong}$ variant polypeptides sedimented as two poorly resolved 6.5 and 7.6 S species (Fig. 5*A*), and both were larger than the 4.5 S secreted normal human PiM(Val213) monomer (Fig. 5*A*). Furthermore, at this longer radiolabeling period an abundant amount of a 90-kDa protein co-precipitated with the null$_{Hong\ Kong}$ variant polypeptides (Fig. 5*A*, *inset*) and

was restricted to the 7.6 S species (Fig. 5*A*, *graph*). This 90-kDa protein was dissociated from the variant polypeptides and subsequently immunoprecipitated with anti-calnexin peptide 3 antiserum, which confirmed its identity as p90/calnexin (not shown). Quantitation of immunoprecipitated proteins from fraction 27 (Fig. 5*A*, *inset*), which should contain primarily the 7.6 S species, demonstrated that co-precipitating p90 exhibited 1.6-fold more radiolabel than did the immunoprecipitated null$_{\text{Hong Kong}}$ variant (quantitation not shown).

In subsequent experiments, a specific loss of the 7.6 S species was observed when radiolabeled H1A/N13 cells were either lysed with deoxycholate (Fig. 5*B*) or lysed with Nonidet P-40 and then incubated for 10 min at 37 °C with 2 mM EGTA (Fig. 5*C*). Sedimentation of the null$_{\text{Hong Kong}}$ variant was not significantly altered when cell lysates were incubated at 37 °C in the absence of EGTA (data not shown). Importantly, loss of the 7.6 S species resulted in the concomitant and quantitative appearance of a 4.5 S species of AAT, and no co-immunoprecipitating 90-kDa protein was identified in either this or the remaining 6.5 S species (Figs. 5, *B* and *C*). Moreover, the newly appearing 4.5 S species sedimented in a manner identical to that of the secreted normal human AAT polypeptide (Fig. 5*A*). Quantitation of the 4.5 S peak from either the deoxycholate lysis or EGTA treatment indicated that it accounted for 25–30% of the total population of retained null$_{\text{Hong Kong}}$ variant polypeptides.

DISCUSSION

Calnexin (or p88 and IP90) is an abundant calcium-binding phosphoprotein of the ER membrane (20, 23, 24). Its role as a molecular chaperone has been implicated for integral membrane proteins, such as the histocompatibility class I molecule (25–27) and the T- and B-cell antigen receptor (28). Initially, interaction of calnexin with soluble secretory proteins was not anticipated (25). However, following the submission of this article, Ou *et al* (29) reported a transient association between calnexin and a variety of newly synthesized secretory glycoproteins, including AAT, in the human hepatoma cell line HepG2.

Brodbeck and Brown (30) have shown that removal of only 4 amino acids from the carboxyl terminus of the human AAT polypeptide totally abrogates its secretion from cells. Significantly, truncation of carboxyl-terminal amino acids of the naturally occurring null$_{\text{Hong Kong}}$ variant polypeptide not only prevents its secretion (17), but also predicts the disruption of β-pleated sheets B and C that are present in the normally folded macromolecule (31). Because secretory glycoproteins synthesized in the presence of azetidine-2-carboxylic acid, which should cause them to misfold, stabilizes their association with calnexin in HepG2 cells (29), it would be predicted that the misfolded null$_{\text{Hong Kong}}$ variant might associate with this molecular chaperone (29). Several criteria, including mobility in SDS-PAGE, absence of asparagine-linked oligosaccharides, and immunoreactivity with peptide-specific antiserum, have indicated that p90 which co-precipitates with a subset of the retained null$_{\text{Hong Kong}}$ variant is identical to calnexin.

In the present study, results from co-immunoprecipitation analyses and velocity sedimentation experiments were in agreement that approximately 25–30% of the retained null$_{\text{Hong Kong}}$ variant polypeptides are associated with calnexin. These data suggest that calnexin does not dissociate from the variant polypeptides during sedimentation velocity centrifugation. Because the number of methionine residues in the reported sequence of canine calnexin (20) is exactly twice that of the null$_{\text{Hong Kong}}$ variant (17), association of a 2-fold excess of radiolabel should be associated with calnexin as compared with the variant polypeptide if the molecules are bound stoichiometrically. Indeed, a 1.6-fold excess of radioactivity was associated with calnexin that co-precipitated with the null$_{\text{Hong Kong}}$ variant present in the 7.6 S complex. Considering the potential contamination of the calnexin-null$_{\text{Hong Kong}}$ variant complex with AAT polypeptides from the 6.5 S species plus the slow turnover of the molecular chaperone (29), we suspect that the two proteins are associated in a 1:1 molar ratio.

Dissociation of the calnexin-null$_{\text{Hong Kong}}$ variant complex with deoxycholate suggests that their interaction might result from the exposure of hydrophobic regions at the surface of the misfolded variant polypeptide. However, the specificity of this detergent for inducing the dissociation event is not yet understood. Because EGTA caused the dissociation of the complex it is likely that the availability of calcium ions plays a role in the interaction between the two molecules. In this regard, Wada *et al.* (20) have reported that several proteins, including pgp35/SSRα, co-purify with calnexin isolated from canine pancreas microsomes. Although we have not detected additional proteins that specifically co-precipitate with the null$_{\text{Hong Kong}}$ variant in our experiments, it is conceivable that these are actually present but are poorly radiolabeled because of their low rate of turnover in the cell. Finally, protein aggregation favors an association with BiP/grp78, but this molecular chaperone has not been observed to associate with the null$_{\text{Hong Kong}}$ variant (32). Considering this latter observation, it is premature to conclude that the abundant 6.5 S species of human AAT which is devoid of calnexin results merely from the aggregation of the misfolded macromolecules.

Recently, a novel mechanism coined "loop-sheet" polymerization has been implicated in the intracellular accumulation of the naturally occurring Z variant (33, 34) and S$_{\text{Iiyama}}$ (35) variant of human AAT. Truncation of the null$_{\text{Hong Kong}}$ variant eliminates the reactive center loop of AAT which is utilized in this aggregation pathway (33). Whereas calnexin can immobilize 30% of the retained null$_{\text{Hong Kong}}$ variant molecules to the membrane of the ER and thus prevent their secretion, the actual role that this molecular chaperone exerts on the intracellular retention of the misfolded polypeptide will be the subject of future investigations. Nonetheless, identification of the calnexin-null$_{\text{Hong Kong}}$ variant complex may extend our current understanding of the molecular pathogenesis of serum α_1-antitrypsin deficiency.

Acknowledgments—We thank Dr. David Williams for suggesting that p90 might be identical to calnexin. We also express our appreciation to Dr. John Bergeron for the gifts of peptide-specific antisera against canine calnexin.

REFERENCES

1. Walter, P., Gilmore, R., and Blobel, G. (1984) *Cell* **38,** 5–8
2. Wieland, F. T., Gleason, M. L., Serafini, T. A., and Rothman, J. E. (1987) *Cell* **50,** 289–300
3. Lodish, H. F., Kong, N., Hirani, S., and Rasmussen, J. (1987) *J. Cell Biol.* **104,** 221–230
4. Rothman, J. E. (1987) *Cell* **50,** 521–522
5. Klausner, R. D., and Sitia, R. (1990) *Cell* **62,** 611–614
6. Hance, A. J., and Crystal, R. G. (1975) *Am. Rev. Respir. Dis.* **112,** 657–661
7. Gadek, J. E., Fells, G. A., and Zimmerman, R. L. (1981) *J. Clin. Invest.* **68,** 889–898
8. Carlson, J. A., Rogers, B. B., Sifers, R. N., Hawkins, H. K., Finegold, M. J., and Woo, S. L. C. (1988) *J. Clin. Invest.* **82,** 26–36
9. Sifers, R. N., Carlson, J. A., Clift, S. M., DeMayo, F. J., Bullock, D. W., and Woo, S. L. C. (1987) *Nucleic Acids Res.* **15,** 1459–1475
10. Long, G. L., Chandra, T., and Woo, S. L. C. (1984) *Biochemistry* **23,** 4828–4837
11. Carrell, R. W., and Boswell, D. R. (1986) in *Proteinase Inhibitors* (Barrett, A. J., and Salvesen, G., eds) pp. 403–420, Elsevier Science Publishers B. V., Amsterdam
12. Brantly, M., Nukiwa, Y., and Crystal, R. G. (1989) *Am. J. Med.* **84,** 13–31
13. Fabbretti, G., Sergi, C., Consales, G., Faa, G., Brisigotti, M., Romeo, G., and Callea, F. (1992) *Liver* **12,** 296–301
14. Sifers, R. N., Finegold, M. J., and Woo, S. L. C. (1989) *Am. J. Respir. Cell Mol Biol.* **1,** 341–345
15. Senior, R. M., Tegner, H., Kuhn, C., Ohlsson, K. (1977) *Am. Rev. Respir. Dis.* **116,** 469–475
16. Janoff, A., Sloan, B., and Weinbaum, G. (1977) *Am. Rev. Resp. Dis.* **115,** 461–468
17. Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muensch, H., and Woo, S. L. C. (1988) *J. Biol. Chem.* **263,** 7330–7335

18. Le, A., Graham, K. S., and Sifers, R. N. (1990) *J. Biol. Chem.* **265,** 14001–14007
19. Gething, M.-J., and Sambrook, J. (1992) *Nature* **355,** 33–45
20. Wada, I., Rindress, D., Cameron, P. H., O., W.-J., Doherty, J. J., Louvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. M. (1991) *J. Biol. Chem.* **266,** 19599–19610
21. Le, A., Ferrell, G. A., Dishon, D. S., Le, Q.-Q. A, and Sifers, R. N. (1992) *J. Biol. Chem.* **267,** 1072–1080
22. Mazzarella, R. A., and Green, M. (1987) *J. Biol. Chem.* **262,** 8875–8883
23. Cala, S. E., Ulbright, C., Kelley, J. S., and Jones, L. R. (1993) *J. Biol. Chem.* **268,** 2969–2975
24. Ou, W. J., Thomas, D. Y., Bell, A. W., and Bergeron, J. J. M. (1992) *J. Biol. Chem.* **267,** 23789–23796
25. Ahluwalia, N., Bergeron, J. J. M., Wada, I., Degen, E., and Williams, D. B. (1992) *J. Biol. Chem.* **267,** 10914–10919
26. Degen, E., and Williams, D. B (1991) *J. Cell Biol.* **112,** 1099–1115
27. Degen, E., Cohen-Doyle, M. F., and Williams, D. B. (1992) *J. Exp. Med.* **175,** 1653–1661
28. Hochstenbach, F., David, V., Watkins, S., and Brenner, M. B. (1992) *Proc. Natl. Sci. U. S. A.* **89,** 4734–4738
29. Ou, W.-J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. M. (1993) *Nature* **364,** 771–776
30. Brodbeck, R. M., and Brown, J. L. (1992) *J. Biol. Chem.* **267,** 294–297
31. Loebermann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) *J. Mol. Biol.* **177,** 531–556
32. Graham, K. S., Le, A., and Sifers, R. N. (1990) *J. Biol. Chem.* **265,** 20463–20468
33. Lomas, D. A., Evans, D. L., Finch, J. T., and Carrell, R. W. (1992) *Nature* **357,** 605–607
34. Sifers, R. N. (1992) *Nature* **357,** 541–542
35. Lomas, D. A., Finch, J. T., Seyama, K., Nukiwa, T., and Carrell, R. W. (1993) *J. Biol. Chem.* **268,** 15333–15335