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A Neuroendocrine-specific Protein Localized to the Endoplasmic Reticulum by Distal Degradation*

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Regulated endocrine-specific protein, 18-kDa (RESP18), was previously cloned from rat neurointermediate pituitary based on its coordinate regulation with proopiomelanocortin and neuroendocrine specificity. RESP18 has no homology to any known protein. Although RESP18 is translocated across microsomal membranes after *in vitro* translation, AtT-20 pituitary tumor cells, which endogenously synthesize RESP18, do not release it into the culture medium. In this work, immunostaining and subcellular fractionation have identified RESP18 as an endoplasmic reticulum (ER) protein. Biosynthetic labeling and temperature block studies of AtT-20 cells demonstrated the localization of RESP18 to the ER lumen by a unique mechanism, degradation by proteolysis in a post-ER pre-Golgi compartment. Proteases in this compartment were saturated by exogenous RESP18 overexpression in AtT-20 cells. Furthermore, a calpain protease inhibitor enhanced secretion of RESP18 from AtT-20 cells overexpressing RESP18. Saturation and inhibition of the RESP18 degrading proteases allowed RESP18 to enter secretory granules and acquire a post-translational modification, likely *O*-glycosylation; this modified 21-kDa RESP18 isoform was the only RESP18 secreted. Rat anterior pituitary extracts contain 18-kDa and *O*-glycosylated RESP18 with similar properties. Exogenous RESP18 expression in hEK-293 cells demonstrated ER localization and RESP18 metabolism similar to AtT-20 cells, indicating that the cellular machinery involved in localizing RESP18 is not specific to neuroendocrine cells. The data implicate a novel ER localization mechanism for this neuroendocrine-specific luminal ER resident.

The general functions of the endoplasmic reticulum (ER)[1] include lipid synthesis, protein synthesis, protein folding, Ca^{2+} storage, and *N*-glycosylation (1–5). Some tissues have specialized ER functions; in the liver ER, very low density lipoprotein particles are synthesized and P450 mediates chemical detoxification (6, 7). In skeletal muscle a specialized ER membrane system (sarcoplasmic reticulum) is dedicated to storing Ca^{2+} for muscle contraction (3). In B lymphocytes, the ER is the site of processing and presentation of antigens by the major histocompatibility complexes (8).

Neuroendocrine cells utilize the ER for synthesis of peptide hormone precursors destined for processing, storage in large dense core granules, and regulated secretion as peptide hormones or neurotransmitters. The neuronal ER extends into dendrites (9–12). Several studies have demonstrated unique attributes of the ER in neuroendocrine cells; the inositol 1,4,5-trisphosphate receptor, enriched in cerebellar Purkinje neurons, is immunolocalized in all portions of the cell, including dendrites, axons, and nerve terminals, and may partake in Ca^{2+} regulation and synaptic neurotransmission (10, 12–15). Purkinje neurons also express a skeletal muscle isoform of calsequestrin, an ER resident protein which buffers Ca^{2+} (11). Neuroendocrine-specific proteins A and C are cytoplasmically oriented neuroendocrine specific proteins anchored to ER membranes (16). No neuroendocrine-specific ER function has been clearly demonstrated.

RESP18 was previously cloned from a neurointermediate pituitary cDNA library based upon its regulation in parallel with proopiomelanocortin (POMC) following treatment of rats with dopaminergic drugs; several other proteins involved in the maturation of POMC, including PC1, PC2, chromogranin B, carboxypeptidase H, and peptidylglycine α-amidating monooxygenase, are regulated in a similar manner (17). The RESP18 cDNA encodes a novel 18-kDa protein with an N-terminal signal peptide. Although RESP18 biosynthesis in dexamethasone-treated AtT-20 corticotrope tumor cells approached the biosynthetic level of the major prohormone precursor, POMC, pulse-chase studies failed to reveal any processing of RESP18 beyond removal of the signal peptide, and no RESP18 or processed products were recovered from spent medium (18).

In this study, RESP18 protein was shown to be localized to the lumen of the endoplasmic reticulum in neuroendocrine cells and in stably transfected fibroblast cells expressing RESP18. Interestingly, no ER retrieval C-terminal KDEL motif (19) or membrane spanning domain with cytosolic C-terminal di-lysine ER localization motif (20) is present in RESP18. RESP18 was found to be degraded in a post-ER pre-Golgi compartment, and degradation was sensitive to calpain I and II inhibitors. Protease saturation by overexpression of RESP18 led to secretion of a 21-kDa RESP18 isoform, suggesting that RESP18 is localized to the ER lumen by degradation in a distal organelle, a unique mechanism deemed "ER localization by distal degradation."

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[1] The abbreviations used are: ER, endoplasmic reticulum; RESP18, regulated endocrine specific protein, 18-kDa; ERp72, endoplasmic reticulum 72-kDa protein; BiP, heavy chain binding protein; GRP78, glucose-regulated 78-kDa protein; TGN38, trans-Golgi network 38-kDa protein; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; ALLM, *N*-acetyl-leucyl-leucyl-methional; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; FITC, fluorescein isothiocyanate; CSFM, complete serum-free medium; CSFM-AIR, complete serum-free medium without bicarbonate buffer; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; GAM, goat-anti-mouse; GAR, goat-anti-rabbit; PAGE, polyacrylmide gel electrophoresis; POMC, proopiomelanocortin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TFMS, trifluoromethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal antisera JH1162 (RESP18) (18), JH1479 (TGN38), and JH189 (γ_3-MSH) (21) were produced in this laboratory. Polyclonal BiP/GRP78 antiserum was from Affinity BioReagents (Neshanic Station, NJ). Monoclonal β-COP antiserum was from Sigma and monoclonal BiP antiserum was from Stressgen (Victoria, Canada). Protein disulfide isomerase and ERp72 polyclonal antisera were gifts from Dr. Michael Green (Saint Louis University, MO) (22). The α-synaptotagmin antiserum was a gift from Dr. Richard Scheller (23). Goat anti-rabbit immunoglobulin linked to horseradish peroxidase was from Amersham Corp. and goat anti-mouse immunoglobulin linked to Texas Red (GAM-Texas Red) was from Jackson Immunoresearch Laboratories (West Grove, PA). Goat anti-mouse immunoglobulin linked to fluorescein isothiocyanate (GAM-FITC), goat anti-rabbit immunoglobulin linked to FITC (GAR-FITC), or Texas Red (GAR-Texas Red) were from Caltag Laboratories (San Francisco, CA). Nerve growth factor (7S) was purified as described elsewhere (24).

Generation of pCIS.RESP18 Construct—The RESP18 insert was excised from pBS.RESP18 using *Xho*I and ligated nondirectionally into pCIS.2CXXNH vector (gift from Dr. Cornelia Gorman, Genentech) prepared with *Xho*I and calf intestinal alkaline phosphatase. Plasmids containing the RESP18 insert in the correct orientation were sequenced using the dideoxy chain termination method (Sequenase system; U. S. Biochemical Corp., Cleveland, OH). Plasmids for transfection were prepared according to Quiagen maxiprep protocol (Chatsworth, CA).

Cell Culture/Transfection—AtT-20/D-16v, hEK-293, CHO, RIN/m5F, and GH_3 cells were maintained in Dulbecco's modified Eagle's medium: F-12 (Life Technologies, Inc.) containing 10% fetal clone serum (HyClone, Logan, UT) and 10% NuSerum (Collaborative Research, Bedford, MA). PC12 cells were maintained in RPMI (Life Technologies, Inc.) containing 1% fetal calf serum and treated 4 days with nerve growth factor (7 S) prior to immunostaining. Cells were fed every 2 days and passed weekly using trypsin.

The pCIS.RESP18 vector was cotransfected into hEK-293 and AtT-20 cells with pMt.neo-1 using Lipofectin, and transfected cells were selected with G418 as described previously (25). One hEK-293 and three AtT-20 G418-resistant colonies were subcloned in order to ensure clonality as evaluated by RESP18 immunostaining.

Immunostaining—Cells were plated on glass slides, some treated overnight with 50 μg/ml fibronectin (Sigma). Cells were fixed in cold 100% methanol for 5 min, blocked with 10% goat serum in PBS (50 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.4) for 20 min, and incubated with primary antiserum diluted in PBS containing 10% goat serum for 30 min at 37 °C or 2 h at 20 °C. Cells were rinsed twice for 5 min in PBS and then incubated with secondary antibody (GAR-FITC or GAM-FITC at 1:400; GAR-Texas Red or GAM-Texas Red at 1:500) in PBS containing 10% goat serum) in the dark for 1 h, washed with PBS for 5 min, and mounting under a coverslip using DABCO-PermaFluor (Lipshaw Immunon, Pittsburgh, PA). Cells were visualized and photographed with a Zeiss Axioskop microscope using a × 63 oil immersion objective (× 630 total magnification) or a Nikon Optiphot microscope equipped with a Bio-Rad MRC 600 confocal image collection apparatus using a × 60 oil immersion lens. Confocal data were collected using Kalman averaging (n = 6).

Tissue and Cell Line Extraction, Subcellular Fractionation, Sucrose Density Gradients, and Solubilization—Dissected tissue or cell lines (10% homogenate in 20 mM NaTES, 10 mM mannitol, pH 7.4, with protease inhibitors; TM buffer) were freeze/thawed three times and centrifuged at 1,000 × g for 5 min to remove debris. Supernatants were centrifuged at 436,000 × g for 15 min; pellets were resuspended in a volume of TM buffer equal to the supernatant. Protein was measured by BCA protein assay (Pierce, Rockford, IL) and analyzed by Western blot.

Differential centrifugation was as described previously with minor modifications (18). Briefly, AtT-20 or transfected AtT-20-RESP cells were grown in 100-mm plates to 80–100% confluency. Cells were harvested by scraping into wash buffer (4.5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4, 25 mM Tris-HCl, pH 7.4) and centrifuged at 600 × g for 5 min. Cells were resuspended (1.0 ml/100 μl cell pellet) in homogenization buffer (0.25 M sucrose, 1 mM $MgCl_2$, 1 mM Na_2EDTA, 10 mM HEPES, pH 7.4) containing protease inhibitors (26) and passed 6 times through a 26 gauge needle. Cells were passed 12 times through a cell cracker with a 4-mm bore and 12-μm clearance (H & Y Enterprise, Redwood City, CA). Debris was removed by centrifugation at 1,100 × g for 5 min (5K; 5000 rpm) in a Beckman TL-100 centrifuge at 4 °C. The supernatant was then centrifuged at 4,400 × g for 15 min (10K) followed by additional centrifugations for 15 min at 17,400 × g (20K), and 39,200 × g (30K).

For sucrose density gradient fractionation, each of these pellets was resuspended in 150 μl of homogenization buffer containing protease inhibitors and loaded onto a 1.9-ml sucrose step gradient prepared with layered sucrose solutions in 1 mM $MgCl_2$,1 mM Na_2EDTA, 10 mM HEPES, pH 7.4, buffer (2.5 M (0.3 ml), 2.0 M (0.2 ml), 1.6 M (0.2 ml), 1.4 M (0.2 ml), 1.2 M (0.2 ml), 1.0 M (0.2 ml), 0.8 M (0.2 ml), 0.6 M (0.2 ml), 0.4 M (0.2 ml)). Density gradients were centrifuged at 214,000 × g for 2 h, and 150-μl fractions were removed from the top of the gradient. Fractions were analyzed by Western blot with several antisera.

For solubilization of RESP18, a 10K pellet was prepared from four confluent 100-mm plates of dexamethasone-treated (1 μM for 4 days) AtT-20 cells and resuspended in 340 μl of 30 mM Tris-HCl, 1 mM EGTA, pH 7.2 (LRE) containing protease inhibitors. Aliquots of 20 μl were added to 100 μl of LRE containing 0.03 or 0.5% detergent (Triton X-100 (Pierce), deoxycholate (Sigma), CHAPS (Boehringer Mannheim), or *N*-octyl glucoside (Sigma)). Samples were frozen and thawed three times and centrifuged at 436,000 × g for 30 min to pellet the insoluble fractions. Supernatants and pellets were resuspended in LRE to an equal volume and analyzed by Western blot.

Isolation of Nuclei—Nuclei were isolated from AtT-20 cells using a modification of the method of Trapani *et al*. (27). Cells scraped from a confluent 100-mm plate were washed and resuspended in 0.5 ml of buffer A (0.3 M sucrose, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, 10 mM Tris, pH 7.5) containing protease inhibitors. Cells were lysed with 20 strokes in a 2.0-ml Dounce homogenizer. The sample was diluted with 1.0 ml of buffer A, overlaid onto a 0.65-ml sucrose cushion (2.3 M sucrose, 5 mM $MgCl_2$, 5 mM DTT, 10 mM Tris-HCl, pH 7.5) in a polyallomer tube and centrifuged 1 h at 67,200 × g at 4 °C (supernatant, 2.15 ml). The pellet (crude nuclei resuspended in 250 μl wash buffer) was either analyzed directly or washed twice by overlaying with 0.5 ml of 50% (w/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM HEPES, pH 7.5, yielding a pure nuclear pellet which was resuspended in 250 μl of the wash buffer. Nuclei appeared to be intact when viewed by light microscopy.

Pulse-Chase Metabolic Labeling and Immunoprecipitation—hEK-293 or AtT-20 cells stably transfected with the pCIS.RESP18 construct or wild type AtT-20 cells expressing endogenous RESP18 were incubated with [^{35}S]Met/[^{35}S]Cys for 15 min as described previously (18). Chase times in CSFM or CSFM-AIR medium (28) were as described in each experiment. Cells were extracted in 5 M acetic acid, 2 mg/ml bovine serum albumin, with 0.3 mg/ml phenylmethylsulfonyl fluoride (PMSF), frozen and thawed three times, and centrifuged to remove cell debris. Supernatants were lyophilized and immunoprecipitated as described previously (18). Media were centrifuged at 15,800 × g to remove debris, and supernatants were adjusted to 1% Triton X-100 and used for immunoprecipitation. The total amount of labeled protein in cells extracts was measured by trichloroacetic acid precipitation followed by scintillation counting.

Immunoprecipitation using the RESP18 antiserum directed against the N-terminal segment of RESP18 was as described elsewhere (18). Briefly, lyophilized cell extracts were resuspended in 60 μl of cold immunoprecipitation buffer (50 mM sodium phosphate, 1% Triton X-100, 10 mM mannitol, pH 7.0) containing 0.6 M KCl, 0.3 mg/ml PMSF, and a trace of phenol red, and adjusted to be above pH 7.0 with 3.0 M Tris-HCl, pH 8.0, if necessary. After 3 min of centrifugation at 15,800 × g, supernatants and media were supplemented with 250 μl of immunoprecipitation buffer containing 1 mM methionine, 0.5 mM cysteine, protease inhibitors, and 10 μl of RESP18 antiserum, and incubated at 4 °C for 8 h or overnight. Protein A-Sepharose beads were used to isolate the immune complexes as described previously (18).

Neuraminidase Treatment of RESP18 Isoforms—The higher molecular weight forms of RESP18 were investigated by digestion with neuraminidase and *O*-glycanase or treatment with trifluoromethanesulfonic acid (TFMS) (29). Rat anterior pituitary tissue was extracted in TM buffer containing 1% Triton X-100. The solubilized crude particulate fraction (10 μl) was digested with 8.3 milliunits of neuraminidase (Boehringer Mannheim) in 50 mM sodium acetate, 4 mM $CaCl_2$, 100 μg/ml bovine serum albumin, 0.3 mg/ml PMSF, pH 5.5, in a volume of 70 μl for 1 h at 37 °C. Reactions were quenched by boiling in Laemmli gel loading buffer and analyzed by Western blot. Anterior pituitary extract was also treated with *O*-glycanase (Genzyme, Cambridge, MA) or TFMS; a time course showed degradation of RESP18 with both treatments, although TFMS treatment converted some higher molecular weight RESP18 into 18-kDa RESP18 before degradation occurred (data not shown).

SDS-PAGE and Western Blots—SDS-PAGE was carried out as described previously (18). Low range prestained molecular weight markers (Amersham) were used for slab gels (16.4% acrylamide, 0.60%

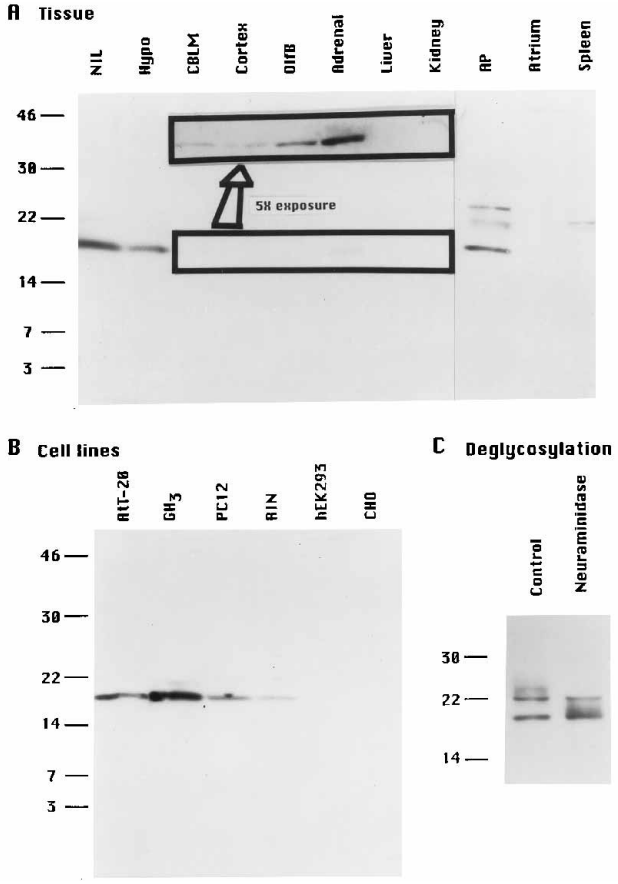

FIG. 1. **RESP18 is neuroendocrine specific.** *A* and *B*, proteins were fractionated by SDS-PAGE (16.4% acrylamide gels) and Western blot analysis was carried out using RESP18 antiserum (1:2,000). Each lane contained 40 μg of a crude particulate fraction protein (except anterior pituitary, 4 μg) prepared from the tissue or cell line indicated; the soluble fraction (not shown) contained only 24-kDa RESP18. The *inset* shows 18-kDa RESP18 from a longer exposure of the lanes indicated. Abbreviations are: *NIL*, neurointermediate pituitary lobe; *Hypo*, hypothalamus; *CBLM*, cerebellum; *OlfB*, olfactory bulb; *AP*, anterior pituitary. *C*, anterior pituitary extract was treated with neuraminidase to remove sialic acids from *O*-linked sugars as described under "Experimental Procedures."

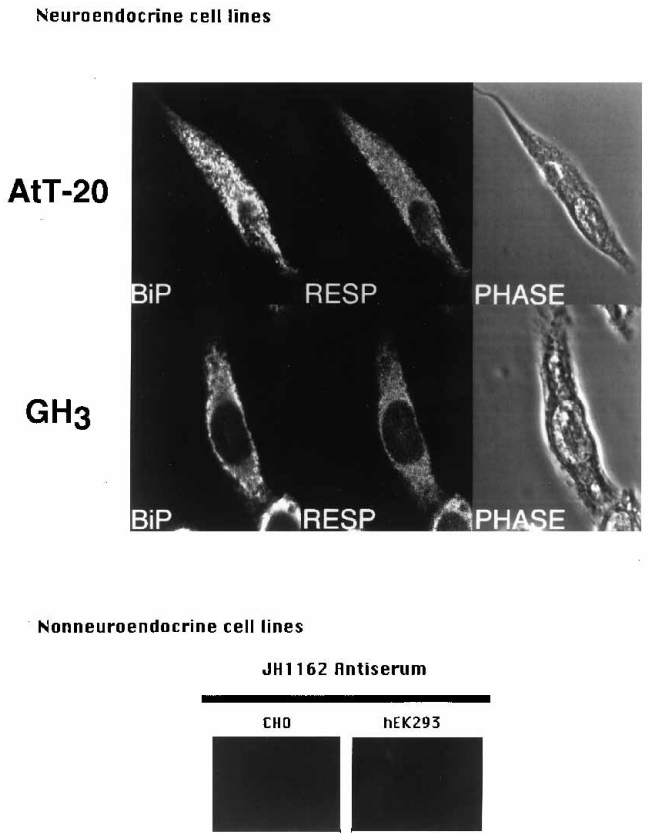

FIG. 2. **Immunocytochemical localization of RESP18 to the endoplasmic reticulum by confocal microscopy.** AtT-20 and GH_3 cells were fixed and stained with RESP18 antiserum (1:1,000) and monoclonal BiP antiserum (1:1000); primary antibodies were visualized with GAM-FITC and GAR-Texas Red using confocal microscopy. CHO and hEK-293 cells were stained with RESP18 antiserum (1:1000) and visualized with a conventional fluorescence microscope.

N,*N*′-methylenebisacrylamide); Bio-Rad high range or prestained high molecular weight markers were used for 10 and 12% acrylamide, 0.27% *N*,*N*′-methylenebisacrylamide slab gels. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) for 1500 mAmp-h. Antibodies were visualized using the ECL detection system according to the manufacturer's protocol (Amersham). For fluorography, gels were washed for two sequential 30-min periods in 30% (v/v) isopropyl alcohol, 10% (v/v) glacial acetic acid, and then in Amplify (Amersham), dried, and exposed to x-ray film. For half-life calculations fluorograms with exposure in the linear range of the film were densitized using Image 1.35 software (25).

RESULTS

RESP18 Protein in Tissues and Cell Lines—The expression of RESP18 in several rat tissues was assessed by Western blot (Fig. 1, *A* and *B*). An 18-kDa band was apparent in extracts prepared from the anterior and neurointermediate pituitary lobes, hypothalamus, and to a lesser extent the adrenal gland (Fig. 1*A*). One-tenth as much anterior pituitary protein was analyzed because this tissue contained the most RESP18. No smaller immunoreactive proteins were detected in any tissue. Longer exposure (Fig. 1*A*, *inset*) demonstrated an 18-kDa band in the cerebellum, cerebral cortex, olfactory bulb, and a barely detectable band in the kidney. Even after prolonged exposure, the RESP18 antiserum failed to detect an 18-kDa band in the liver, spleen, or atrium. Higher molecular mass bands of cross-reactive material were observed in the anterior pituitary (22 and 24 kDa) and spleen (22 kDa). RESP18 does not contain a consensus sequence for *N*-glycosylation (17). We therefore considered the possibility that RESP18 in the anterior pituitary was *O*-glycosylated. When anterior pituitary tissue extract was treated with neuraminidase, the 24-kDa RESP18 band was eliminated, and more 18-kDa RESP18 appeared; although not reduced to a single band it was clear that glycosylation of RESP18 occurred (Fig. 1*C*). RESP18 protein expression in these rat tissues mirrors RESP18 mRNA expression in the same tissues as determined by Northern blot (17).

RESP18 protein expression was also examined in several tumor cell lines. Western blot analysis of AtT-20 cells (pituitary corticotropes), GH_3 cells (pituitary somatomammotropes), PC12 cells (adrenal medulla), and RIN cells (β cells of the islets of Langerhans) revealed a single major 18-kDa protein (Fig. 1*B*). The GH_3 cell line expressed the most RESP18 and also contained an additional 19-kDa cross-reactive band. The non-neuroendocrine human embryonic kidney cells (hEK-293) and Chinese hamster ovary cells (CHO) did not express detectable levels of RESP18. These neuroendocrine cell lines appear to be valid models to study the cellular biology and biochemistry of RESP18.

RESP18 Is an Endoplasmic Reticulum Protein—The localization of RESP18 and BiP/GRP78 was examined in AtT-20 and GH_3 cells by dual label confocal microscopy (Fig. 2). RESP18 and BiP displayed similar reticular staining patterns; when examined in color, RESP18 staining (red) overlapped BiP

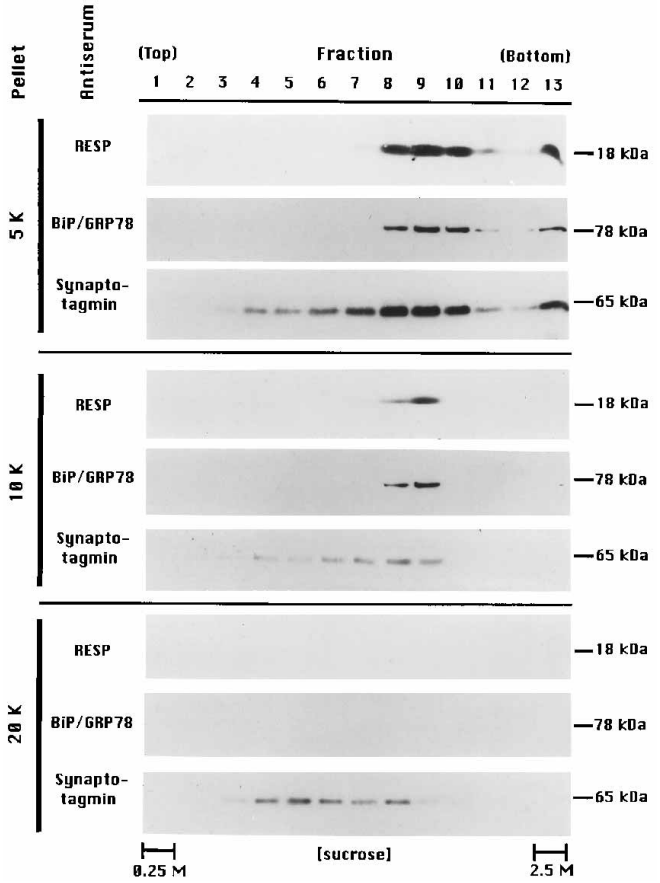

FIG. 3. **Sucrose density gradient fractionation of AtT-20 cell extracts localized RESP18 to the ER.** Differential centrifugation pellets were subjected to sucrose density centrifugation. Aliquots (50 μl) of each fraction (150 μl) were analyzed by SDS-PAGE (10 or 12% gels) and Western blot with RESP18 (1:2,000), polyclonal BiP/GRP78 (1:2,000), synaptotagmin (1:1,000), TGN38 (1:500; not shown), or γ_3MSH (1:500; not shown) antiserum. The three gradients were analyzed at the same time, and staining intensities for each antibody can be compared.

staining (green), with RESP18 staining seen in additional reticular regions of the cell relatively devoid of BiP.

Immunostaining of RIN and PC12 cell lines with RESP18 antisera exhibited a similar reticular staining pattern which extended to the cell periphery (data not shown). All four neuroendocrine cell lines were also immunostained with antisera for two additional luminal endoplasmic reticulum markers, ERp72 and protein disulfide isomerase. In all four neuroendocrine cell lines, the steady state localization of RESP18 closely resembled that of the ER markers, suggesting an ER localization for RESP18. No staining of RESP18 was observed in non-neuroendocrine CHO or hEK-293 cells.

The ER localization of RESP18 was verified by subcellular fractionation of AtT-20 cells (Fig. 3). After disruption with a cell cracker and differential centrifugation, the 5K, 10K, and 20K pellets were resuspended, fractionated on sucrose density gradients, and analyzed by Western blot. The distribution of RESP18 closely paralleled that of BiP/GRP78; RESP18 was present in fractions 8–13 of the 5K pellet and fractions 8 + 9 of the 10K pellet but was absent from the 20K pellet. Based on density, the fractions containing RESP18 would be expected to contain rough microsomes and nuclei.

RESP18 did not distribute with the secretory granule markers synaptotagmin (Fig. 3) or POMC (data not shown). The synaptotagmin antiserum detected a 65 kDa band in fractions 4–13 of the 5K pellet and fractions 4–9 of the 10K and 20K pellets; the 10K and 20K pellets contained secretory granules but very little RESP18. Synaptotagmin in the 5K pellet may be present in plasma membrane sheets. The 30K pellet contained no BiP/GRP78 or RESP18, but low density fractions were positive for synaptotagmin (data not shown). A marker of the trans-Golgi network, TGN38 (30), was enriched in lighter fractions of the 5K pellet (fractions 6–9), 10K pellet (fractions 4–8), and 20K pellet (fractions 3–9) (not shown); RESP18 was not enriched in these fractions.

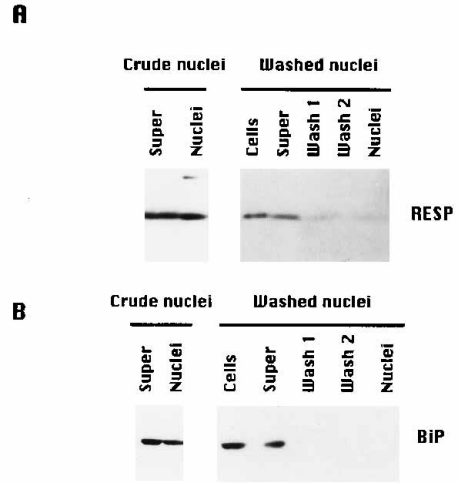

FIG. 4. **RESP18 and BiP/GRP78 are weakly associated with nuclei.** Crude or washed nuclei were isolated from AtT-20 cells as described. *Cells* (input; 10 μl of 1.5 ml), *super* (nuclear supernatant; 50 μl of 2.15 ml), *wash 1* and *2* (first or second wash of nuclear pellet; 50 μl of 0.5 ml), and *nuclei* (nuclear pellet; 25 of 250 μl) samples were analyzed by SDS-PAGE on 12% acrylamide gels and Western blot used RESP18 (*Panel A*, 1:2,000) and polyclonal BiP/GRP78 (*Panel B*, 1:2,000) antisera.

Since RESP18 and BiP/GRP78 were both detected in the densest fractions of the 5K gradient, which contains nuclei, we analyzed nuclei purified from AtT-20 cells for RESP18 and BiP/GRP78. The AtT-20 cells were treated with dexamethasone to enhance expression of RESP18, although the same result was obtained in control cells (data not shown) (18). Microscopic examination of crude and purified nuclei confirmed the presence of intact nuclei. A Western blot demonstrated that both RESP18 and BiP/GRP78 were in crude nuclei and in the cell supernatant (Fig. 4). When the crude nuclei were washed in isotonic buffer, both the ER marker, BiP/GRP78, and RESP18 were separated from the purified nuclei, suggesting that RESP18 and BiP/GRP78 were in the perinuclear region of the endoplasmic reticulum associated with nuclei after cell disruption. Immunostaining, sucrose density gradient fractionation, and analysis of isolated nuclei demonstrated a steady state endoplasmic reticulum localization of RESP18 in AtT-20 cells.

RESP18 Is Degraded in Post-ER Pre-Golgi Compartment—As observed previously, RESP18 had a half-life of 18 min in AtT-20 cells incubated at 37 °C (18). We first used reduced temperature to elucidate the cellular compartment in which RESP18 was degraded (Fig. 5*A*). Incubation at 20 °C inhibits transport of protein out of the trans-Golgi network (31, 32). In AtT-20 cells chased at 20 °C, the half-life of RESP18 was 46 min, indicating a reduction in the degradation rate. Incubation of cells at 15 °C blocks protein export from the ER (31, 33). When chased at 15 °C, RESP18 exhibited a half-life longer than the detection limit of the assay and conservatively estimated to be greater than 400 min. An Arrhenius plot was used to examine the effect of temperature on RESP18 turnover (Fig. 5*B*). The Arrhenius plot demonstrated a biphasic temperature dependence; from 37 to 20 °C a slight decrease in degradation

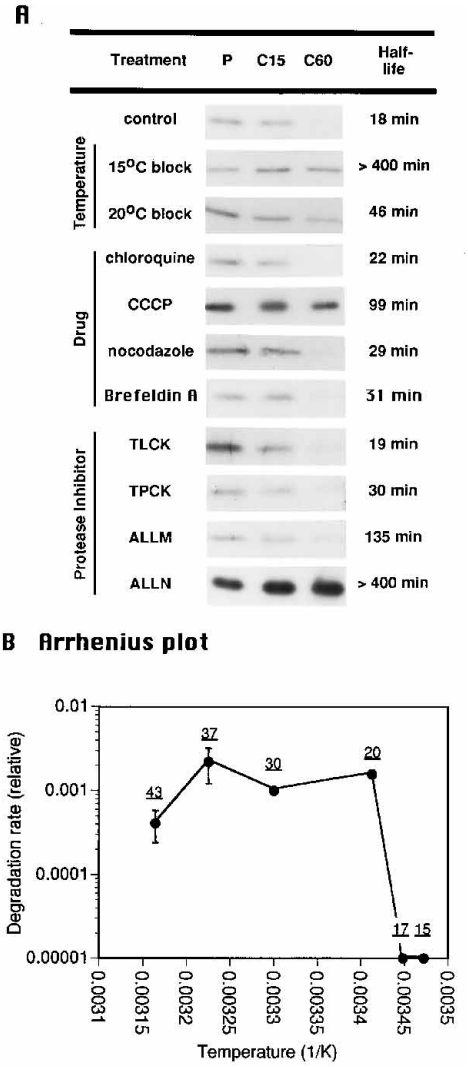

FIG. 5. **Biosynthetic labeling identified a post-ER pre-Golgi compartment as the site of RESP18 degradation.** *A*, AtT-20 cells were labeled with [^{35}S]Met/[^{35}S]Cys for 15 min and chased with CSFM containing the treatments indicated: 100 μM chloroquine, 10 μM nocodazole (also incubated 1.5 h prior to labeling); 40 μM CCCP; 2 μM TPCK; 10 μM TLCK; 100 μg/ml ALLM; 100 μg/ml ALLN. Temperature was varied only during the chase: 15, 17, 20, 30, 37, or 43 °C in CSFM-AIR. Immunoprecipitates were fractionated by SDS-PAGE followed by densitization of fluorograms. If no degradation were observed, the half-life was estimated to be >400 min. Half-lives were calculated from linear fits to logarithmic plots. All conditions were analyzed at least twice. *B*, for each temperature, the RESP18 degradation rate determined from fluorograms was normalized to the control. The temperature (°C) is *underlined* above each point.

rate was observed which we attribute to a reduction in proteolytic rate. A pronounced drop in the degradation rate was observed between 20 and 17 °C, indicating that a transport step affected in this temperature range was essential for RESP18 degradation. The temperature studies suggest that degradation of RESP18 occurs in an organelle distal to the ER and before or within the Golgi which is consistent with a detectable lag in RESP18 degradation.

We have used several drug treatments to confirm and to further localize the site of RESP18 degradation. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) is a mitochondrial uncoupler which reduces cellular levels of ATP (34) and inhibits vesicular transport (33). When AtT-20 cells were treated with CCCP during the chase, the half-life of RESP18 increased to 99 min (Fig. 5*A*). This result is consistent with the requirement of an energy-dependent step such as vesicular transport before RESP18 degradation can occur.

Chloroquine raises the alkalinity in acidic compartments and thereby inhibits the acidic proteases in lysosomes. When AtT-20 cells were pretreated and chased with a concentration of chloroquine known to alter lysosomal pH in AtT-20 cells (35, 36), the half-life of RESP18 was not significantly affected. This indicates that lysosomal proteases are unlikely to be involved in RESP18 degradation.

Nocodazole inhibits microtubule polymerization, resulting in the inhibition of retrograde transport from the Golgi to ER and protein degradation in the Golgi, and causing accumulation of protein in a post-ER pre-Golgi compartment (37, 38). Nocodazole treated AtT-20 cells stained with RESP18 antiserum exhibited a more diffuse reticular pattern when compared to untreated cells (39). Biosynthetic labeling of AtT-20 cells treated with nocodazole showed a minimal effect on RESP18 half-life, suggesting that RESP18 degradation does not occur in the Golgi or during retrograde transport from the Golgi to the ER.

Brefeldin A blocks the budding of β-COP-coated anterograde transport vesicles from the ER (40, 41) but does not affect retrograde Golgi to ER transport (42). AtT-20 cells treated with Brefeldin A and immunostained with TGN38 antiserum displayed the expected dispersion of the TGN. AtT-20 cells treated with brefeldin A displayed a minor increase of RESP18 half-life. The temperature block experiments implicated post-ER degradation, suggesting that RESP18 might be degraded in the COPII type vesicles which bud from the ER near the Golgi apparatus (43).

Protein degradation in the secretory pathway may be mediated by cysteine proteases which are active in the ER (44–46), in a post-ER pre-Golgi compartment (47–50), and in the Golgi stacks (38). Since RESP18 degradation occurs in this pathway, we tested the effect of cysteine protease inhibitors on RESP18 degradation in AtT-20 cells. Calpains are neutral cytosolic Ca^{2+} dependent cysteine proteases. Calpain I, also called μ-calpain, requires micromolar concentrations of Ca^{2+}, and calpain II, also called *m*-calpain, requires millimolar concentrations of Ca^{2+}. Calpain I and II peptide aldehyde inhibitors (ALLN and ALLM, respectively) added only during the chase dramatically increased the half-life of RESP18 in AtT-20 cells (Fig. 5*A*). ALLN completely inhibited detectable degradation, while ALLM increased the half-life of RESP18 7-fold.

The chloromethyl ketones, TLCK and TPCK, are potent inhibitors of serine proteases but can also inhibit pre-Golgi protein degradation by cysteine proteases (38, 44, 46). Pretreatment of AtT-20 cells for 30 min with TLCK or TPCK (data not shown) or addition of TPCK only during the chase (Fig. 5*A*) had a minor effect on RESP18 turnover. At concentrations similar to those used here, TPCK, TLCK, ALLN, and ALLM were not toxic to CHO cells (46) and did not affect protein synthesis in AtT-20 cells. In summary, we conclude that RESP18 is degraded by a "μ-calpain-like" cysteine protease in a post-ER pre-Golgi compartment during anterograde transport.

Saturation or Inhibition of RESP18 Degrading Protease(s) Allows Secretion of O-Glycosylated Forms of RESP18—The dramatic affect of ALLN on RESP18 stability and lack of a conventional ER localization signal led us to test the "ER localization by distal degradation" hypothesis for the ER localization of RESP18. We attempted to saturate and inhibit the proteases involved in RESP18 degradation. Rat RESP18 was stably expressed in AtT-20 cells (AtT-20-RESP). AtT-20 cells express endogenous RESP18, but levels of transfected rat RESP18 were 6-fold higher than endogenous mouse RESP18. The AtT-20-RESP cells were examined by dual-label confocal

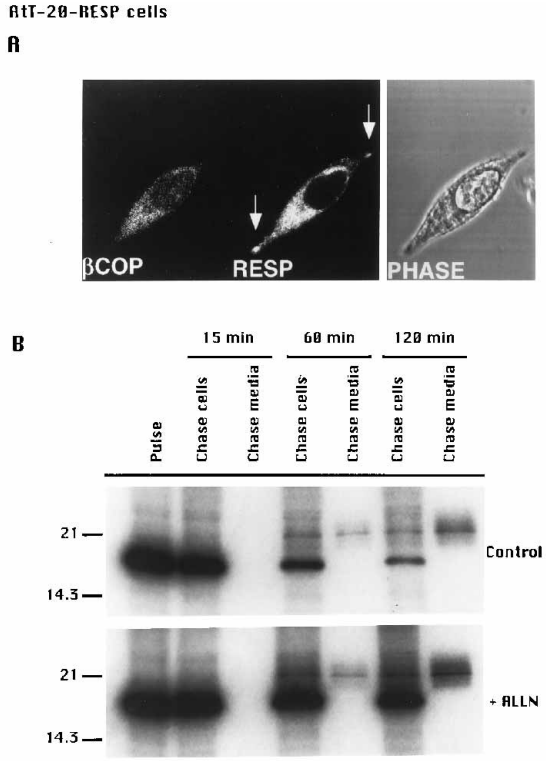

FIG. 6. **RESP18 is secreted by AtT-20-RESP cells.** *A*, AtT-20-RESP cells were immunostained with RESP18 (1:2,000) and β-COP (1:20) antisera; primary antibodies were visualized with GAR-Texas Red and GAM-FITC by confocal microscopy as described under "Experimental Procedures." *B*, biosynthetic labeling of AtT-20-RESP cells for 15 min (*pulse*), followed by chase with or without 100 μg/ml ALLN for 15, 60, or 120 min. Equal aliquots of cell extract and medium were immunoprecipitated, fractionated by SDS-PAGE, and visualized by fluorography. A long exposure is shown to accurately depict the secreted RESP18.

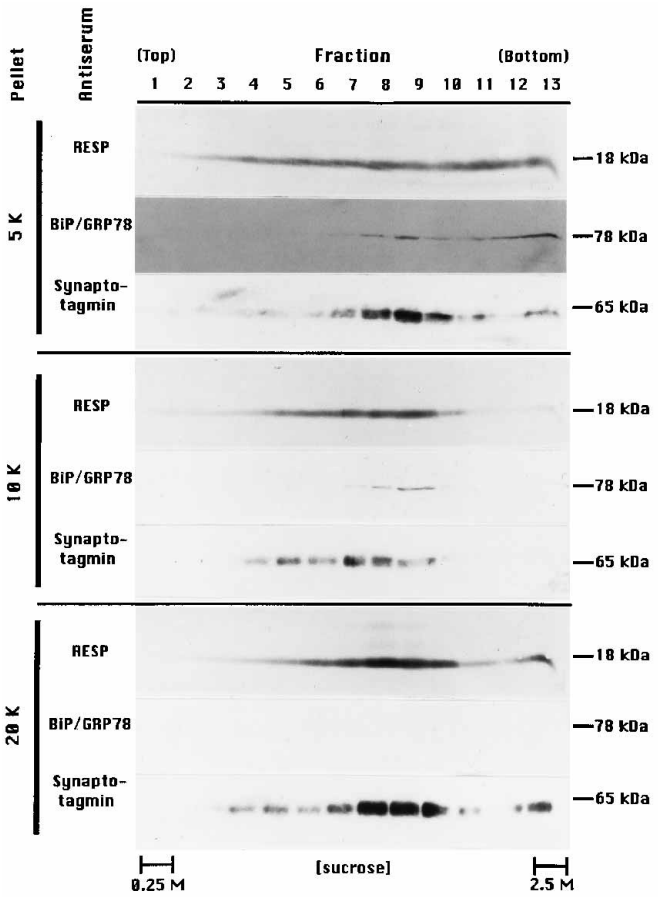

FIG. 7. **Localization of RESP18 in secretory granules in AtT-20-RESP cells.** AtT-20-RESP cells were analyzed as in Fig. 3.

microscopy to see if overexpression of RESP18 altered its localization. Staining for RESP18 showed a reticular pattern which paralleled β-COP, a marker of the ER, intermediate compartment and Golgi (51). In contrast to wild type AtT-20 cells, AtT-20-RESP cells yielded intense staining at the tips of the processes suggesting that some RESP18 traverses the Golgi (Fig. 6*A*, *arrows*); no β-COP staining was observed in the cell processes. When examined in color, RESP18 (red) almost completely overlapped β-COP (green) except in the cell processes, where only RESP18 staining was detected.

Biosynthetic labeling was used to assess the stability and routing of RESP18 in AtT-20-RESP cells (Fig. 6*B*). The half-life of RESP18 in AtT-20-RESP cells was 19 min, identical to the half-life in wild-type cells. Unlike wild-type AtT-20 cells, AtT-20-RESP cells produced a diffuse 21-kDa RESP18 band during the chase; the higher molecular weight RESP18 was first detectable in the cells after 60 min of chase (Fig. 6*B*) and may be similar to the *O*-glycosylated RESP18 observed in the anterior pituitary. The 21-kDa RESP18 isoform was also detected in the medium after 120 min of chase. Saturation of the proteases which normally degrade RESP18 in AtT-20 cells could account for the secretion of the 21-kDa RESP18. If saturation of these proteases were responsible for the small amount of RESP18 secretion observed, then inhibition of the proteases should lead to more secretion of 21-kDa RESP18.

This hypothesis was tested by including the μ-calpain inhibitor, ALLN, during the chase period (Fig. 6*B*). In AtT-20-RESP cells, 2% of the labeled RESP18 protein present after the pulse remained in cells after a 2-h chase, and a similar amount had been secreted, indicating that most of the RESP18 was degraded in the cell. When ALLN was present during the chase, 60% of labeled RESP18 remained in the cells after the 2-h chase and secretion of the 21-kDa RESP18 isoform was enhanced. When medium from ALLN-treated AtT-20-RESP cells was examined by Western blot, no BiP, ERp72, or protein disulfide isomerase (ER markers) was detected (data not shown).

The localization of RESP18 in AtT-20-RESP cells was further examined by differential centrifugation and sucrose density gradient fractionation (Fig. 7). As in wild type AtT-20 cells, AtT-20-RESP cells had RESP18 in the same fractions of the 5K and 10K pellet as the ER marker, BiP/GRP78. In contrast to wild-type AtT cells, AtT-20-RESP cells had RESP18 in the secretory granule containing fractions of the 20K and 30K pellets and the lower density sucrose fractions of the 5K and 10K pellets. The distribution of RESP18 resembled that of synaptotagmin. The BiP/GRP78 and synaptotagmin markers gave similar sucrose density gradient distributions for AtT-20 (Fig. 3) and AtT-20-RESP cells, indicating that transfection of RESP18 had no adverse effect on cells.

The immunostaining, biosynthetic labeling, and subcellular fractionation data all show that overexpression allowed RESP18 to escape the ER and become localized in the secretory granules of the cell processes. Biosynthetic labeling data demonstrate an additive effect of protease saturation and inhibition on the RESP18 degrading protease(s). Although the steady state distribution of RESP18 in AtT-20-RESP cells includes some RESP18 in secretory vesicles, biosynthetic labeling determined a half-life indistinguishable from wild type AtT-20 cells. These data suggest that very little of the newly synthesized RESP18 traverses the Golgi, even in AtT-20-RESP cells. Ap-

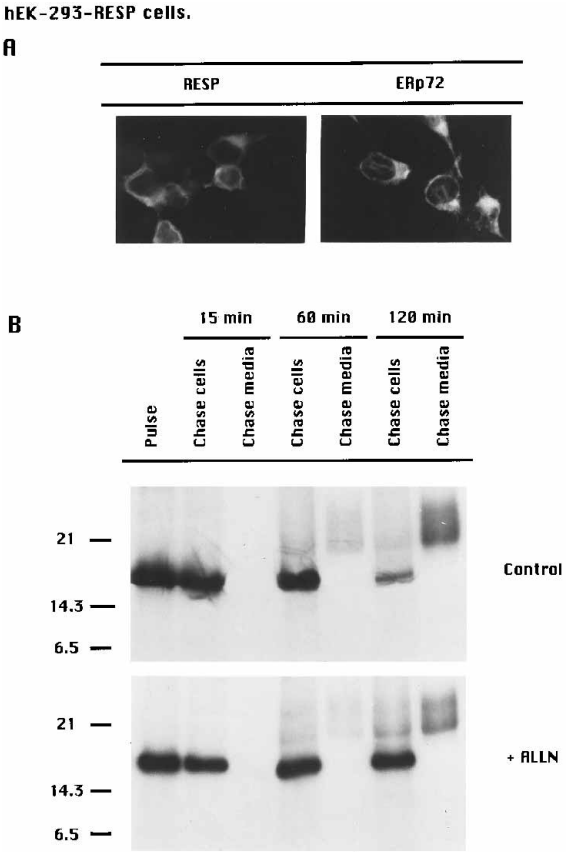

FIG. 8. **Exogenous RESP18 is an ER resident in non-neuroendocrine hEK-293 cells.** *A*, hEK-293-RESP cells were immunostained with ERp72 (1:4,000) and RESP18 (1:4,000) antisera. *B*, biosynthetic labeling of hEK-293-RESP; 15-min pulse, followed by 15-, 60-, and 120-min chase with or without 100 μg/ml ALLN as indicated.

parently the higher level of RESP18 expression does not completely saturate the relevant protease(s) and most of the RESP18 has a half-life similar to that in wild type AtT-20 cells. This interpretation is consistent with ALLN treatment of AtT-20-RESP cells, which inhibited pre-Golgi proteases and greatly lengthened the half-life of RESP18 in AtT-20-RESP cells.

The Fate of Exogenous RESP18 in Non-neuroendocrine Cells—To determine whether rapid degradation of RESP18 is limited to neuroendocrine cells, we expressed RESP18 in hEK-293 cells, non-neuroendocrine cells which do not express endogenous RESP18 (Figs. 1 and 2). Stably transfected hEK-293 cells overexpressing RESP18 (hEK-293-RESP), displayed a reticular ER immunostaining pattern extending to the cell periphery parallel to the ER marker, ERp72 (Fig. 8*A*). Although RESP18 is a neuroendocrine specific protein, the immunostaining of hEK-293-RESP cells suggests a localization which parallels neuroendocrine cells.

The metabolism of RESP18 in hEK-293 cells was examined by biosynthetic labeling (Fig. 8*B*). Following a 15-min pulse, only 18-kDa RESP18 was observed in cell extracts. RESP18 was more stable in hEK-293 cells than in wild type AtT-20 or AtT-20-RESP cells; 64% of the newly synthesized RESP18 remained after 60 min while only 14% remained in wild type AtT-20 or AtT-20-RESP cells. A diffuse 21-kDa band was observed in hEK-293-RESP cells and media after the 60- and 120-min chases and may be *O*-glycosylated RESP18 as observed in the anterior pituitary. After a 2-h chase, 41% of the labeled RESP18 remained in the cell and some degradation was apparent.

When ALLN was added during the chase, 91% of the newly synthesized RESP18 was recovered in the hEK-293-RESP

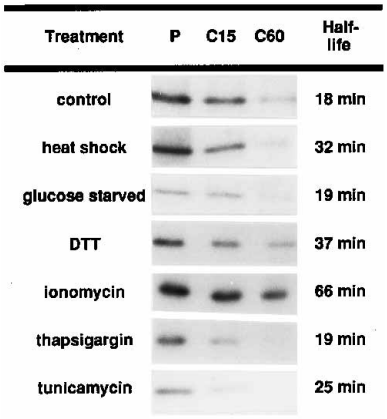

FIG. 9. **RESP18 is not a prototypical glucose regulated/heat shock protein.** AtT-20 cells were labeled for 15 min (pulse) and then chased for 15 min (*C15*) or 60 min (*C60*) with CSFM containing 10 μg/ml ionomycin, 1 μg/ml thapsigargin, 10 μg/ml tunicamycin, 2 mM DTT, or glucose-free CSFM. For some treatments drug was administered prior to the pulse; ionomycin or thapsigargin for 4 h, DTT for 2.25 h, glucose-free medium for 24 h, or heat shock for 1.5 h, and labeling medium did not contain drug. Cells were extracted and immunoprecipitated, and proteins were fractionated by SDS-PAGE and visualized by fluorography.

cells. The remaining labeled RESP18 was mostly recovered as 21-kDa RESP18 in the medium. These data suggest a similar ER localization mechanism by distal degradation in a pre-Golgi compartment. Although degradation was less efficient, non-neuroendocrine cells express proteases which degrade RESP18.

RESP18 Is a Unique Endoplasmic Reticulum Resident—Unlike RESP18, the ER resident protein ribophorin I has a half-life of 25 h (52), which may be representative of ER proteins. Endoplasmic reticulum proteins including BiP/GRP78, ERp72, protein disulfide isomerase, and endoplasmin, are protein-folding chaperones induced by the heat shock, glucose stress (53, 54), and unfolded protein responses (55). In addition, treatment with DTT to change the intracellular redox potential, tunicamycin to inhibit *N*-glycosylation of proteins, or thapsigargin to eliminate Ca^{2+} from the ER, enhances expression of these chaperones (53).

To compare RESP18 to these well characterized ER resident proteins, we have examined the effects of standard treatments on the half-life of endogenous RESP18 in AtT-20 cells (Fig. 9). The half-life of RESP18 was unaffected by glucose starvation or treatment with thapsigargin (56). In contrast, the Ca^{2+} ionophore, ionomycin, which collapses intracellular Ca^{2+} gradients and equilibrates free Ca^{2+} at the medium concentration of 1.8 mM (57, 58), lengthened the half-life of RESP18 severalfold. Heat shock, or treatment with tunicamycin or DTT, mildly increased the half-life of RESP18. These data show that RESP18 is regulated differently than ER chaperone proteins, and that drugs known to enhance protein degradation in the ER have little impact on RESP18 degradation, supporting degradation distal to the ER.

RESP18 contains no predicted membrane spanning domains. In order to determine whether the endogenous RESP18 localized in the ER of AtT-20 cells is soluble or membrane associated, we examined the solubility of RESP18 and the ER resident protein BiP/GRP78 in a microsome-enriched 10K pellet prepared from AtT-20 cells (Fig. 10). When the 10K pellet was resuspended in 30 mM Tris-HCl, 1 mM EDTA, pH 7.2 buffer, all of the RESP18 was recovered in the particulate fraction. RESP18 was not effectively solubilized when the buffer was supplemented with 0.03 or 0.5% *n*-octyl glucoside or CHAPS; these detergents solubilized BiP/GRP78 more effi-

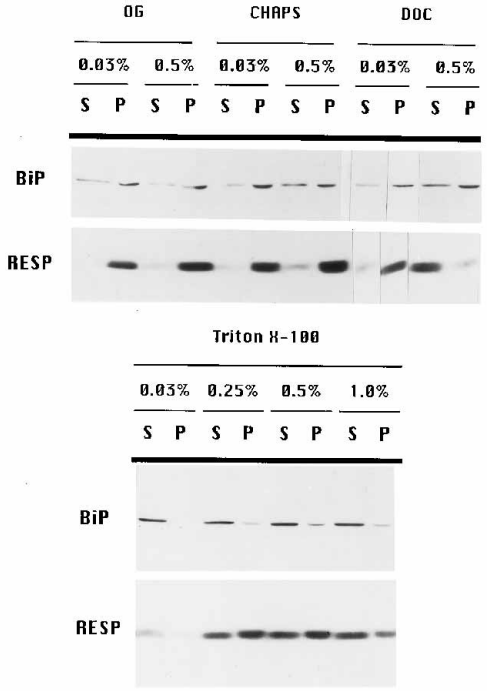

FIG. 10. **Solubilization of RESP18 and BiP/GRP78 from an enriched microsomal fraction.** A 10K fraction enriched in microsomes was prepared from AtT-20 cells and solubilized with octyl glucoside (*OG*), CHAPS, deoxycholate (*DOC*), and Triton X-100 as described. Soluble (*S*) and pellet (*P*) fractions were analyzed by Western blot with RESP18 and polyclonal BiP/GRP78 antisera as indicated.

ciently than RESP18. Buffer containing 0.5% deoxycholate solubilized almost all of the RESP18 and half of the BiP/GRP78. At low concentration of Triton X-100 (0.03%), RESP18 was degraded and BiP/GRP78 was well solubilized. High concentrations of Triton X-100 solubilized most of the BiP/GRP78 and more than half of the RESP18.

RESP18 contains two cysteines; we examined the possibility that RESP18 was disulfide linked to other proteins using nonreducing gels. RESP18 migrated at the same molecular weight on reducing and nonreducing gels, indicating that there is no covalent attachment of RESP18 to other molecules through cysteines (data not shown). We conclude that RESP18 has the solubility characteristics of a peripheral membrane protein.

DISCUSSION

RESP18 is a neuroendocrine-specific protein under dopaminergic control in melanotropes (17). We have examined the cellular biology of RESP18 to provide direction for experiments aimed at elucidating its function. A surprising result was localization of RESP18 to the ER lumen. To our knowledge RESP18 is the first neuroendocrine-specific endogenous ER luminal resident identified. This result suggests the ER may have a specialized, yet undefined function in neuroendocrine cells expressing RESP18.

Although RESP18 was identified as a luminal ER resident, it lacks an ER retrieval C-terminal KDEL sequence (19) or a predicted membrane spanning domain with a C-terminal dilysine ER localization motif (20), suggesting ER localization by a different mechanism. Several other ER resident proteins lack conventional ER localization motifs. Prolyl-4-hydroxylase α subunit (59), β-glucuronidase (60–62), and unassembled major histocompatibility complexes (63) are retained in the ER via interaction with ER residents which have a C-terminal KDEL sequence. s-Cyclophilin is retained in the ER by the C-terminal sequence VEKPFAIAKE (64). Lysyl hydroxylase is believed to be retained via electrostatic interactions with the ER membrane (65–67). Cathepsin E and α-mannosidase are localized in the ER by unknown mechanisms (68, 69).

We propose that RESP18 is localized in the ER by degradation in a distal organelle, likely in a post-ER pre-Golgi compartment. In AtT-20 cells this model is supported by the short half-life of RESP18 (18 min), a detectable lag in RESP18 degradation, *in vivo* drug and temperature effects consistent with post-ER pre-Golgi protein degradation, and the ability to saturate or inhibit proteolysis of RESP18.

More than 20 known polypeptides are degraded in a pre-Golgi compartment by a highly specific mechanism (48–50). Many of these proteins may be degraded due to slow or incomplete folding. Protein folding and pre-Golgi proteolysis may work in concert; ERp72, a molecular chaperone of the ER (70, 71), also functions as a calpain-like cysteine protease *in vitro* (72).

Analogous to the degradation of RESP18, the pre-Golgi degradation of mutant α1-antitrypsin (73), T cell receptor α subunit (47), and 2-hydroxy-3-methylglutaryl-CoA reductase (45) are not perturbed by drugs that inhibit lysosomal function or disrupt the Golgi stack; brefeldin A inhibits mutant α_1-antitrypsin degradation in the cis-Golgi network (73) and proteolysis of immunoglobulin M with mature *N*-linked sugars in the Golgi stacks (38). Degradation of RESP18 is unaffected by brefeldin A.

Although distinction between protein degradation in post-ER pre-Golgi and ER compartments is not absolute, several criteria are used to determine the location of protein degradation. ER protein degradation is generally not perturbed by a 15 °C temperature block, or drugs that deplete cellular ATP. ER protein degradation is inhibited by drugs that deplete ER calcium (74) or create a reducing environment (75) and is enhanced by drugs that inhibit *N*-glycosylation of proteins (76). ER protein degradation usually begins within 20 min of biosynthesis, and intermediates are normally not observed (44, 77). Glycoproteins degraded in the ER have only immature *N*-linked oligosaccharides and proteins degraded in the ER lack any modifications which occur in organelles distal to the ER (*i.e. O*-glycosylation, phosphorylation, sulfation, and palmitation). Proteins which are degraded in the ER include the T cell receptor subunits (75), asialoglycoprotein (77, 78), and ribophorin mutants (79). The degradation of RESP18 has none of the characteristics of ER protein degradation; a lag of about 20 min was detected for RESP18 degradation, consistent with degradation after transport from the ER.

Post-ER pre-Golgi protein degradation requires transport out of the ER, which is generally blocked at 15 °C, and is inhibited by drugs that deplete cellular ATP. RESP18 degradation in AtT-20 cells is inhibited with a 15 °C block and by CCCP, suggesting degradation in a post-ER compartment. Furthermore, an Arrhenius plot of RESP18 degradation demonstrates biphasic temperature dependence which suggests a vesicular transport step (80), as for the pre-Golgi degradation of the T cell receptor α subunit (47). A detectable lag in the degradation of many proteins, including RESP18, implicates a transport requirement for degradation (49).

The degradation of RESP18, 2-hydroxy-3-methylglutaryl-CoA reductase (45), some T cell receptor subunits (46), and protein C precursor (induced by warfarin) (81) in a pre-Golgi compartment is strongly inhibited by the two calpain inhibitors, ALLN and ALLM; both compounds are also potent inhibitors of cathepsins B, L, and H, which are cysteine proteases (82, 83). TLCK and TPCK inhibit serine proteases and to a lesser extent cysteine proteases, and had only a minor effect on the degradation of RESP18, similar to other pre-Golgi protein

degradation (38, 44, 46) and the mild inhibition of calpains by TPCK *in vitro* (84). RESP18 degradation likely involves a cysteine or "calpain-like" protease typical of protein degradation in the secretory pathway.

We have demonstrated that saturation of RESP18 degrading proteases allows accumulation of a 21-kDa RESP18 isoform in cells and secretion of this 21-kDa form; these metabolic events can be enhanced with the calpain inhibitor ALLN. Similarly, the KDEL receptor localized in the ER, intermediate compartment and Golgi complex (85), can be saturated by overexpression of proteins with the KDEL motif, resulting in secretion of the overexpressed protein or endogenous resident ER proteins (22, 86, 87).

When overexpressed in AtT-20 cells or hEK-293 cells, RESP18 was secreted as a diffuse 21-kDa band. This higher molecular weight RESP18 isoform resembles the *O*-glycosylated RESP18 detected in the anterior pituitary. If the 21-kDa RESP18 represents *O*-glycosylated RESP18, then RESP18 in wild type AtT-20 cells does not reach the compartment where *O*-glycosylation is initiated, the intermediate compartment (88) or cis-Golgi (89, 90). In addition, similar metabolism may occur *in vivo*; the high level of RESP18 expression in the anterior pituitary was accompanied by *O*-glycosylation of RESP18. The *O*-glycosylated RESP18 isoforms found in the anterior pituitary presumably function in organelles distal to the cis-Golgi network and may function extracellularly *in vivo*. Based on the specificity of *N*-acetylgalactoseaminyltransferase, which initiates *O*-glycosylation, RESP18 contains several potential *O*-glycosylation sites (91).

Although expression of RESP18 is neuroendocrine-specific, the cellular machinery resulting in the ER localization is not restricted to neuroendocrine cells. RESP18 was localized to the ER when expressed in non-neuroendocrine hEK-293 cells; an ER immunostaining pattern was observed, and RESP18 degradation was ALLN sensitive as observed in the neuroendocrine AtT-20 cell line.

In summary, RESP18 is a luminal ER protein, has a short half-life, and is likely proteolyzed during anterograde transport in a post-ER pre-Golgi compartment by cysteine or calpain-like proteases. Saturation or inhibition of these proteases led to accumulation in cells and secretion of 21-kDa RESP18, likely representing *O*-glycosylated RESP18. These data strongly suggest that RESP18 is localized to the ER by "distal degradation."

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