Expression of pro-Muclin in pancreatic AR42J cells induces functional regulated secretory granules

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De Lisle, Robert C., Oxana Norkina, Eileen Roach, and Donna Ziemer. Expression of pro-Muclin in pancreatic AR42J cells induces functional regulated secretory granules. Am J Physiol Cell Physiol 289: C1169–C1178, 2005. First published June 29, 2005; doi:10.1152/ajpcell.00099.2005.—It is not clear how protein cargo is sorted to and retained in forming regulated secretory granules (RSG). Here, the sulfated mucin-type glycoprotein pro-Muclin was tested for its ability to induce RSG in the poorly differentiated rat pancreatic cell line AR42J. AR42J cells express RSG content proteins, but they fail to make granules. Adenovirus-pro-Muclin-infected AR42J cells store amylase, accumulate RSG, and respond to hormonal stimulation by secreting the stored protein. Expression of pro-Muclin combined with the inducing effect of dexamethasone resulted in a significant enhancement of the efficiency of regulated secretion. The effect of pro-Muclin was a strong decrease in constitutive secretion compared with dexamethasone-induction alone. A pro-Muclin construct missing the cytosolic tail domain was less effective at improving the efficiency of regulated secretion compared with the full-length construct. Increased expression of cargo (using adenovirus amylase) also modestly enhanced regulated secretion, indicating that part of pro-Muclin’s effect may be due to increased expression of cargo protein. Overall, the data show that pro-Muclin acts as a sorting receptor that can induce RSG, and that its cytosolic tail is important in this process.

Regulated secretion; protein sorting

Whereas all eukaryotic cells have a constitutive secretory pathway for delivery of proteins to the plasma membrane and secretion from the cell, some have a specialized system for storage and regulated release of proteins (3, 23). Cells that have a regulated pathway include neurons, endocrine cells, exocrine cells, and the sperm and egg germ cells. A major mechanism for protein sorting into the regulated pathway is the propensity of cargo proteins to co-aggregate at the mildly acidic pH and high Ca\(^{2+}\) levels of the trans-Golgi network (TGN) and secretory granules (9). How co-aggregation in the TGN leads to formation of a regulated secretory granule is unknown.

By analogy to the sorting of lysosomal hydrolases to endosomes/lysosomes by the mannose 6-phosphate receptor (19), and protein traffic from the endoplasmic reticulum to the Golgi mediated by the p24 family of proteins (30), it has been proposed that Golgi cargo receptors are important in sorting of regulated proteins to secretory granules (11). Such receptors would be membrane proteins, or, at least, be membrane-associated. They are predicted to bind to the protein aggregates in the TGN lumen, thereby anchoring the cargo to the TGN membrane. This somehow identifies that patch of TGN membrane for the assembly of the other proteins and lipids needed to make a regulated secretory granule (RSG) [e.g., rabs, N-ethylmaleimide-sensitive factor attachment for protein receptor (vesicular soluble N-ethylmaleimide sensitive factor receptors (v-SNAREs)), and cholesterol-glycolipid rich rafts] (35).

The best characterized Golgi cargo receptor is carboxypeptidase E (CPE), which is expressed in neuronal and endocrine cells and has been shown to affect protein sorting to the regulated pathway (11). Cells or animals deficient in CPE show defects in sorting of neuropeptides/hormones to RSG (reviewed in Ref. 35). A complication of these studies is that CPE is a processing enzyme that normally modifies the cargo itself. In addition, the loss of CPE in knockout mice also decreases expression of other cargo-processing enzymes, the prohormone convertases. It is difficult to tell whether the effects of mutant or absent CPE are due to loss of the cargo receptor function or to an inability of unprocessed cargo to properly aggregate (35, 38). Nevertheless, the concept of TGN cargo receptors is attractive because of its potential to explain cargo selection and recruitment of accessory membrane and cytosolic proteins needed to make a functional RSG.

Muclin was discovered as an abundant component of the exocrine pancreatic RSG, the zymogen granule (12). Muclin was later shown to be derived from a precursor, a type I membrane protein called pro-Muclin, encoded by the dmbt1 gene (GenBank accession no. NM_007769) (16). As it passes through the secretory pathway, pro-Muclin becomes N- and O-glycosylated, and its O-linked sugars are sulfated in the TGN. After exiting the Golgi, pro-Muclin is cleaved to produce mature Muclin, which remains in the zymogen granule. The other product of pro-Muclin cleavage is an 80-kDa glycoprotein called apactin, which contains the transmembrane domain. Apactin is efficiently removed from the maturing RSG, and it is targeted to the actin cytoskeleton-rich apical plasma membrane (37). It was shown that purified Muclin directly associates with several of the zymogen granule content proteins at mildly acidic pH levels found in the TGN/RSG (4). It was further demonstrated in that work that the sulfated, O-linked oligosaccharides are the primary structure that mediate this pH-dependent interaction.

From these observations, it was proposed that pro-Muclin acts as a Golgi cargo receptor, which binds the RSG content proteins as they aggregate in the acidic environment of the TGN, and links the aggregate to the TGN membrane. The experiments in this article demonstrate that expression of pro-Muclin in a poorly differentiated pancreatic cell line is sufficient to induce functional RSGs. Furthermore, the efficiency of regulated secretion induced by pro-Muclin is synergized by concomitant treatment of the cells with the glucocor-
ticoid analog dexamethasone. The experiments also test the importance of the cytosolic tail and transmembrane domains of pro-Muclin and show that the cytosolic tail is important to pro-Muclin’s role in the regulated pathway.

MATERIALS AND METHODS

Plasmid constructs and recombinant replication-deficient adenovirus. The pAd-Easy adenovirus system, which uses a cytomegalovirus (CMV) promoter, was used according to the supplier’s instructions (QBiogene, Carlsbad, CA) (Fig. 1A). GFP was amplified by PCR using primers that included SexAI restriction sites (forward primer: 5'-GCG TAC CTG TTA GCA GGC AGG AGC TGT TCA C-3'; reverse primer: 5'-GGC TAC CAG TGT CTA GTC GCG ACT TGT ACA GCT C-3'). The GFP PCR product was digested with SexAI and ligated into full length pro-Muclin (GenBank accession no. U37438, in the Bluescript plasmid; Stratagene, La Jolla, CA) at the single SexAI site, which places the GFP tag in frame in the second CRP domain of the protein (Fig. 1A) (4). The GFP-pro-Muclin was subcloned into pCI-Neo (Invitrogen) using EcoRV and NotI restriction sites (called pCI-GFP-pro-Muclin). Next, GFP-pro-Muclin was excised using SalI/PJMI, which left behind the −400 bp 3'UTR of the cDNA. The pShuttle-CMV plasmid (QBioGene) was digested with HindIII/NotI and the GFP-pro-Muclin fragment was ligated into the plasmid using an adaptor (annealed and phosphorylated 5'-GGG GGG-3' and 5'-AGC TCC CCC CTG T-3' oligonucleotides). This pSh-CMV-GFP-pro-Muclin was linearized with PmeI and co-transformed with pAd-Easy into BJ15183 Escherichia coli cells for recombination.

Recombinant adenoviruses were also prepared that express pro-Muclin with the cytosolic tail deleted (Ad-Δ-Tail) and with the transmembrane and cytosolic tail regions deleted (Ad-Δ-TMD). The Δ-TMD construct was prepared by digesting pCI-GFP-pro-Muclin with NotI and BsiWI to remove the 3'-end of the cDNA and then ligating in a NotI- and BsiWI-digested PCR product to replace a portion of the ZP domain removed by the restriction digestion. This PCR was performed using the forward primer: 5'-CCG CGT ACG GGT AGC TCA GTC CCA AT' -3; and the reverse primer: 5'-AAC AAA TCG CGG CCG CCT TAC TGG GAG CTT GCT GGC TTC TCC AC' -3. To prepare the Δ-Tail construct, PCR was used to amplify the entire end of the ZP domain plus the transmembrane domain (forward primer: 5'-CCG CGT ACG GGT AGC TCA GTC CCA AT' -3; reverse primer: 5'-AAC AAA TCG CGG CCG CCT TAC TGG GAG CTT GCT GGC TTC TCC AC' -3). Then, Δ-TMD and Δ-Tail were inserted into the Bluescript vector (Subcloning, Carlsbad, CA) (Fig. 1B) as described above. Ad-Δ-TMD and Ad-Δ-Tail were used to produce replication-deficient adenovirus (QBiogene). For the Δ-TMD construct, GFP was inserted into the pCI-Neo plasmid (QBiogene) using linearized pShuttle-CMV plasmid as template. The Δ-TMD plasmid was then co-transformed with pAd-Easy into BJ15183 Escherichia coli cells for recombination.

Recombinant adenoviruses were also prepared that express full length pro-Muclin with GFP fused into the cytosolic domain (GFP-pro-Muclin). Expression cassettes were designed that contained the CMV promoter, GFP and pro-Muclin (Fig. 1C). The GFP expression cassette included a secretory GFP cassette (sGFP) and an sGFP-transmembrane domain-cytosolic tail cassette (sGFP-TMD-Tail) (16) were used to prepare adenoviruses. Adenoviral titers were determined using the TCD50 techniques according to the supplier’s protocol (QBioGene).

Cell culture. Rat pancreatic AR42J cells were purchased from the American Type Culture Collection (CRL-1492) and were maintained in Kaighn’s modified Ham’s F-12 medium (Sigma, St. Louis, MO) with 20% fetal bovine serum (FBS) and penicillin and streptomycin (34) in a 5% CO2 atmosphere. Cells were used between passages 25 and 71. AR42J cells were seeded at 1.25 × 105 cells/well in 24-well plates 1 day before infection with the replication-deficient adenovirus. The medium was removed, and adenovirus was added (0.2 ml/well; all adenoviruses were adjusted to 2 × 106 plaque forming units/ml, resulting in a final multiplicity of infection of ~150) and incubated for 90 min at 37°C, followed by addition of 0.8 ml Kaighn’s medium with 10% FBS. When dexamethasone (Sigma) was used, it was added at this time at a final concentration of 100 nM. Cells were used 48 h later for analysis.

Amylase storage and release. Amylase storage in AR42J cells was expressed as amylase activity in the cell pellet per microgram DNA. DNA was measured according to the method of Cesareano et al. (5). Amylase release was measured according to Ref. 27. Cells were washed twice in HEPES-buffered Ringer’s solution supplemented with 0.5% bovine serum albumin. The cells were preincubated for 15

Fig. 1. Green fluorescent protein (GFP)-pro-Muclin expression cassette and characterization of Ad-GFP-pro-Muclin infection of AR42J cells. A: the protein has a signal peptide (SP), followed by tandem repeats of scavenger receptor cysteine rich (SRCR) domains (red boxes) interspersed with CRP domains (blue boxes), followed by five complement subcomponent C1r/C1s, sea urchin protein Vegf, and BMP-1 (CUB) domains (dark green boxes) and another SRCR domain, a zona pellucida (ZP) domain (yellow box), a transmembrane domain (TMD), and finally a short cytosolic domain (C-Tail). pro-Muclin is predicted to be cleaved after the fourth CUB domain (16). The cDNA for pro-Muclin was modified to insert GFP into the second CRP domain as indicated. Two truncation mutants were prepared that lack the C-Tail or the TMD and C-Tail domains (see text for details). These pro-Muclin constructs were used to generate replication-deficient adenovirus for high level protein expression in cultured cells. B: cells were infected with replication-defective adenovirus and were fixed 48 h later. Cells were also stained with a SPOT II digital camera on a Nikon Eclipse TE300 fluorescence microscope with a ×20 objective (numerical aperture (NA) = 0.45). GFP fluorescence was recorded using a fluorescence filter set and cells were also imaged by phase-contrast illumination. Virtually 100% of the cells express GFP-pro-Muclin. The fluorescent image was adjusted to maximize the dynamic range using the “levels” function of Adobe PhotoShop. C: Ad-GFP-pro-Muclin infected cells were fixed and immunolabeled for amylase (sheep anti-amylase and donkey anti-sheep Texas red conjugates) and pro-Muclin (rabbit anti-Muclin plus donkey anti-rabbit FITC) (a–d). e: Ad-GFP-infected cells were imaged for amylase and GFP fluorescence. Samples were coveredslipped with SlowFade (Molecular Probes) and imaged on a Nikon Eclipse TE300 fluorescence microscope with a ×100 oil immersion objective (NA = 1.4) using appropriate fluorescence filters. Digital images were adjusted to maximize the dynamic range using the levels function of Adobe PhotoShop.
min at 37°C, after which time aliquots of the media were collected. The cells were incubated a further 40 min at 37°C in the absence or presence of the cholecystokinin analog caerulein (100 nM final) to stimulate secretion. The cells and the media were saved for amylase activity determination. Amylase release was calculated as media activity released during the 40 min period minus that released during the 15-min preincubation period and expressed as percentage of the total activity. Amylase enzyme activity was measured in a microtiter format at 405 nm with the use of 4,6-ethyldiene(glucose)-p-nitrophe-nyl-glucose-α,β-maltotetraside, which liberates p-nitrophenol when hydrolyzed (no. 85305, Raichem, San Diego, CA).

Isolation of secretory granules from AR42J cells. Cells were washed and resuspended in 2 ml 0.3 M sucrose, 10 mM MOPS, pH 7.0, 0.1 mM MgSO₄, and protease inhibitors, and pressurized to 500 psi in a nitrogen cavitation bomb (Parr, Moline, IL) for 5 min on ice. The bomb was depressurized and the disrupted cells were centrifuged at 450 x 15 min to pellet nuclei and unbroken cells. The postnuclear supernatant was mixed with Percoll (Sigma; 40% final), 0.25 M

A Schematic Model of GFP-pro-Mucln

B Infection of AR42J Cells with Ad-GFP-pro-Mucln

C Expression of GFP-pro-Mucln Increases Amylase Content of AR42J Cells

D Localization of Amylase with Mucln or GFP
sucrose, 50 mM MES, pH 5.5, 0.2 mM EGTA, 0.1 mM MgSO₄, and protease inhibitors. The samples were centrifuged at 100,000 g × 15 min to form the gradient. Gradients were fractionated with an AutoDensi Flow II (Labconco, Kansas City, MO).

Morphological analysis. For ultrastructural analysis, cells or subcellular fractions were fixed in 4% paraformaldehyde, 1.6% glutaraldehyde, and embedded in an epoxy resin, and sectioned for standard transmission electron microscopy. Electron microscope images were photographed at ×7,200 magnification, and the negatives were digitized using a flatbed scanner (Hewlett-Packard, Palo Alto, CA). The percentage of the cell cytoplasm (excluding nuclear areas) occupied by zymogen granules was measured using the “magic wand” tool of Photoshop software (Adobe, San Jose, CA). Granules were defined as being electron dense and circular or elliptical in shape. Analysis was performed by an individual unaware of the identity of the samples.

For GFP fluorescence and indirect immunofluorescence, cells were fixed in 2% paraformaldehyde containing 0.1% saponin (Sigma) to permeabilize cell membranes. Cells were immunostained with anti-amylase (sheep polyclonal, 1:500 dilution; The Binding Site, San Diego, CA) plus anti-sheep Ig coupled to Texas red (Jackson ImmunoResearch, West Grove, PA) and anti-Muclin as described previously (4). Labeled cells were observed on a Nikon Diaphot using appropriate fluorescence filters, and imaged using a SPOT II digital camera (Diagnostic Instruments, Sterling Heights, MI).

Statistics. Data were analyzed using one-way ANOVA with appropriate post hoc tests or by t-test when comparing basal to stimulated release values. The specific statistical analyses used are given in the figure legends.

RESULTS

AR42J cells were derived from a chemically induced rat exocrine pancreatic tumor (22). Under standard culture conditions, AR42J cells express significant levels of digestive enzymes, including amylase, but they fail to store these secretory proteins (27). AR42J cells can be induced to a more differentiated state by culturing in the presence of the synthetic glucocorticoid dexamethasone, after which they express higher levels of pancreatic digestive enzymes and they now exhibit a modest regulated secretion (27). We have shown that the sulfated mucin-type glycoprotein Muclin (Fig. 1A) is able to aggregate with pancreatic RSG proteins in vitro under conditions that mimic the TGN (4). In this work we investigated whether expression of pro-Muclin was sufficient to induce RSG in cultured AR42J cells.

AR42J cells lack endogenous Muclin expression and Ad-pro-Muclin infection results in robust expression. We evaluated whether the AR42J cell is a suitable model in which to test the role of pro-Muclin in secretory protein storage and zymogen granule formation. By Western blot analysis, AR42J cells did not express detectable Muclin, either under standard culture conditions or after 48 h in the presence of dexamethasone (Fig. 2). Immunofluorescence for Muclin was also negative in cells grown in the presence of dexamethasone, whereas the cytosol was filled with fine amylase-positive vesicular structures (Fig. 1Aa). Muclin is a major protein of the mouse pancreas, accounting for ~2% of the tissue protein (14). To express pro-Muclin to similar levels in AR42J cells, we used recombinant adenovirus with a CMV promoter-driven expression cassette. Cells that were infected with Ad-GFP-pro-Muclin express abundant Muclin, as detected by Western blot analysis (Fig. 2). By quantitative Western blot normalized to cell DNA content, GFP-pro-Muclin was expressed at 56 ± 14% of pancreatic levels in the absence of dexamethasone, and this increased to 90 ± 37% of pancreatic levels in the presence of dexamethasone (data not shown). The pro-Muclin adenovirus construct was tagged with GFP (Fig. 1A) and fluorescence was detected in virtually every cell after infection (Fig. 1B).

Expression of pro-Muclin increases amylase content of AR42J cells. As a first step in assessing whether pro-Muclin expression affects regulated secretory protein (RSP) storage, the cell content of amylase was measured in uninfected cells compared with control Ad-GFP and Ad-GFP-pro-Muclin infection. Infection with the control Ad-GFP had little effect on amylase content of the cells (Fig. 3). In contrast, infection with pro-Muclin adenovirus significantly increased the steady-state cell amylase content by ~70% over uninfected or Ad-GFP infected controls (Fig. 3).

Expression of pro-Muclin induces zymogen granules in AR42J cells. Immunofluorescent staining for amylase in Ad-GFP-pro-Muclin infected cells showed a correlation of the degree of GFP fluorescence with the strength of the amylase signal: the cells with higher GFP signal were also more highly labeled for amylase (Fig. 1C). At higher resolution, Muclin labeling was seen to be strong in the perinuclear region and in large puncta near the cell periphery (Fig. 1D,ab). Amylase labeling was strong in both regions of the pro-Muclin-infected cell and was also in the large puncta at the cell periphery (Fig. 1D,ab). By contrast, cells infected with an empty adenovirus had a fine particulate labeling for amylase (Fig. 1D,a).

To see whether expression of pro-Muclin induced recognizable zymogen granules, AR42J cells were examined by electron microscopy. Uninfected cells, either without adenovirus infection (Fig. 4A) or with control Ad-GFP infection (Fig. 4B), had occasional small electron-dense granules. Infection with Ad-GFP-pro-Muclin caused the appearance of zymogen granule-like organelles (Fig. 4C). When cells were induced with dexamethasone, either without adenovirus infection (Fig. 4D) or with control Ad-GFP infection (Fig. 4E), they had a moderate number of small granules. In contrast, Ad-GFP-pro-
Muclin infected cells in the presence of dexamethasone had more granules and those granules were of larger size (Fig. 4).

The increase in granules in pro-Muclin expressing cells was confirmed by morphometric quantitation of the area of the cell cytoplasm occupied by granules. Culturing AR42J cells with dexamethasone increased the area occupied by granules by 4.4-fold over uninduced, uninfected cells (Fig. 5). Infection with the control Ad-GFP, either without or with dexamethasone-induction, resulted in less granular area compared with the corresponding uninfected cells (Fig. 5). The negative effect of adenovirus infection on the cellular content of granules is similar to previous work (41), although they found a greater inhibition by adenovirus infection than we observed. Infection with Ad-GFP-pro-Muclin increased the area occupied by granules fourfold in the absence of dexamethasone, compared with uninduced cells infected with the control Ad-GFP (Fig. 5).

Isolated secretory granules from Ad-pro-Muclin infected, dexamethasone-induced AR42J cells are enriched in Muclin and amylase. To assess the localization of Muclin and amylase in infected cells, cells were disrupted by nitrogen cavitation and the postnuclear supernatants were fractionated on a Percoll density gradient. As shown in Fig. 6, A and B, there were two peaks of immunoreactivity in the disrupted cell fractions for both Muclin and amylase. The region of immunoreactivity at the less dense position in the gradient (peak in fractions 2 and 3) was strongly enriched for both Muclin and amylase. This position on the gradient corresponds to microsomes, including Golgi membranes and endoplasmic reticulum (15). The second peak of immunoreactivity in the heavier fractions (fractions 9 and 10) corresponds to the position of zymogen granules when rat or mouse pancreas is fractionated (15). This heavier peak was strongly enriched for amylase and less so for Muclin. The relative distributions of amylase and Muclin in the light and heavy peaks of the density gradient are consistent with the immunofluorescence patterns for these proteins (Fig. 1D, b). By electron microscopy, Percoll gradient fractions 9 and 10 contained abundant secretory granules (Fig. 6C) of the same appearance as those identified in the electron micrographs of Ad-pro-Muclin, dexamethasone-induced cells (Fig. 4F).

Expression of pro-Muclin induces regulated amylase secretion in AR42J cells. Next, it was determined whether the granules induced by GFP-pro-Muclin were functional and could release their stored protein in response to stimulation by the cholecystokinin analog caerulein. To control for nonspecific adenoviral effects, Ad-GFP infection was used for comparison. Uninduced, Ad-GFP infected cells did not respond to stimulation, but Ad-pro-Muclin infected cells exhibited a significant stimulated amylase release (Fig. 7). The efficiency of

![Graph showing Amylase Content vs. DNA Content](image)

Fig. 3. Expression of pro-Muclin in AR42J cells increases amylase content. Cells were uninfected; infected with Ad-GFP, which expresses cytosolic GFP; or infected with Ad-GFP-pro-Muclin (pro-Muclin) and were harvested 48 h later. Cell pellets were homogenized and amylase activity was measured and is expressed relative to DNA content. The data are quadruplicate samples from a representative of experiment. *P < 0.05 vs. uninfected cells (ANOVA).

![Image of cell granules](image)

Fig. 4. Expression of pro-Muclin induces zymogen granule formation in AR42J cells. Cells were uninfected; infected with the control Ad-GFP, which expresses cytosolic GFP or Ad-GFP-pro-Muclin; and cultured without (Uninduced) or with 100 nM dexamethasone (Dexamethasone) for 48 h. Uninfected (A) and Ad-GFP infected (B), uninduced cells have few identifiable secretory granules. C: Ad-pro-Muclin infected, uninduced cells have numerous small to medium secretory granules (arrowheads). Uninfected (D) and Ad-GFP infected (E), dexamethasone-induced cells have moderately numerous small granules (arrowheads). F: Ad-GFP-pro-Muclin infected, dexamethasone-induced cells have numerous large secretory granules (arrowheads).
RSP storage can be calculated from the ratio of stimulated to constitutive release, and data for the different conditions are summarized in this manner in Fig. 10. The storage efficiency of pro-Muclin expressing cells was 1.8 (Fig. 10). The increased storage efficiency in uninduced pro-Muclin expressing cells was caused by a combination of decreased constitutive amylase release and increased stimulated release, compared with control (Fig. 7). When control cells (Ad-GFP infected) were induced with dexamethasone, they exhibited an increase in stimulated amylase release and a decrease in constitutive release (Fig. 7). The storage efficiency induced by dexamethasone was 1.9, which was equivalent to that caused by expression of pro-Muclin in the absence of dexamethasone. In contrast to the modest effect in uninduced cells, pro-Muclin expressing cells treated with dexamethasone had a strong increase in the efficiency of amylase storage to a value of 5.2 (Figs. 7 and 10). The basis of the improvement was largely due to a dramatic reduction in constitutive secretion under these conditions, while the level of stimulated release was unaffected (Fig. 7). Thus the combination of pro-Muclin expression and dexamethasone-induction greatly improved the ability for amylase to be stored in the regulated pathway.

The cytosolic tail of pro-Muclin contributes to the effect on induction of regulated secretion. To test whether the C-Tail of pro-Muclin has a role in the function of the protein, a truncated pro-Muclin adenoviral construct was prepared that lacks the 16 amino acid C-Tail (Δ-TMD). When Δ-Tail was expressed in cells in the absence of dexamethasone, the amylase sorting efficiency was 1.5, slightly reduced compared with the full-length construct (Figs. 8 and 10). In dexamethasone-induced cells, expression of Δ-Tail resulted in a smaller sorting efficiency value compared with cells expressing the full-length construct (Figs. 8 and 10). The major difference comparing the Δ-Tail and the full-length constructs is that the former had a smaller effect on reducing constitutive release in dexamethasone-induced cells (Fig. 8). Thus the presence of the C-Tail is required for the maximum effect of Muclin on the regulated pathway.

For pro-Muclin to act as a cargo receptor, its transmembrane domain (TMD) may also be important. The TMD could be necessary to anchor the pro-Muclin-RSP complex to the TGN membrane for sorting into the forming RSG. The importance of the TMD was tested using a truncated adenoviral construct lacking the TMD and C-Tail domains (Δ-TMD). Expression of Δ-TMD in uninduced and in dexamethasone-induced AR42J cells resulted in similar constitutive and stimulated amylase secretion, compared with the Δ-Tail construct (Fig. 8). The storage efficiency of the Δ-TMD construct was slightly less than the Δ-Tail (Fig. 10). This indicates that the C-Tail is important for the function of pro-Muclin and that the membrane anchor by itself is relatively unimportant.
Increased cargo expression enhances regulated secretion. It has been suggested that the ability of dexamethasone to induce regulated secretion in AR42J cells is due to the increased RSP expression that occurs (20). To test this idea more directly, an Ad-amylose construct was prepared and used to induce cargo without using dexamethasone. Infection of cells with Ad-amylose resulted in an increase in amylase synthesis by ~30% as measured by $^{35}$S/mess pulse-labeling and phosphorimge quantitation (data not shown). When Ad-amylose infected cells were stimulated with caerulein there was a significant secretory response (Figs. 9 and 10), similar to Ad-pro-Muclin-infected cells (Fig. 7). However, Ad-amylose infected cells induced with dexamethasone did not exhibit a further enhancement of regulated secretion (Figs. 9 and 10), unlike that observed with Ad-pro-Muclin in the presence of dexamethasone (Figs. 7 and 10).

**DISCUSSION**

These experiments demonstrate that the type I membrane protein pro-Muclin can induce the formation of functional RSGs in the exocrine cell line AR42J. In general, regulated secretion is achieved by the retention of the protein cargo in unstimulated cells (low constitutive release) combined with a robust response to stimulation (high stimulated release). Together, these two processes result in a high ratio of stimulated to constitutive release that characterizes efficient regulated protein secretion. In uninduced AR42J cells, expression of pro-Muclin reduces constitutive release and enhances stimulated secretion. The net result is a modest regulated secretion that is similar in magnitude to that induced by dexamethasone.

![Graph showing amylase release](image-url)
Interestingly, increasing amylase synthesis in the absence of dexamethasone has a similar positive effect on regulated secretion. These results are consistent with the idea that increasing cargo levels can enhance the regulated secretory pathway (20) and that part of pro-Muclin’s effect is due to its ability to coaggregate with the other cargo proteins (4). However, in uninfected cells treated with dexamethasone, where amylase and other cargo proteins are expressed 5- to 10-fold higher than in uninduced cells (27, 33), the enhancement of the regulated pathway is modest compared with that in the presence of pro-Muclin expression.

Expression of pro-Muclin combined with dexamethasone-induction results in a strong reduction in RSP storage, resulting in much more efficient RSP storage (Fig. 10). Conceptual considerations and experimental work have provided a framework to investigate how RSGs are formed. There is evidence for two major mechanisms in RSG formation: 1) “sorting-for-entry” whereby RSPs are actively sorted into the pathway by a specific cargo receptor, or by cargo association with cholesterol-glycoplipid rich membrane domains in the TGN; and 2) “sorting-by-retention” mediated by the inherent propensity of RSG cargo proteins to aggregate with one another in the environment of the TGN and RSG, i.e., mildly acidic pH and high Ca$^{2+}$ (for review, see Refs. 2, 3, 28, 35, 38). In addition to Muclin’s role in RSP aggregation, the C-Tail of pro-Muclin further enhances the efficiency of the regulated pathway. Because Δ-TMD-pro-Muclin and Δ-Tail-pro-Muclin were still able to enhance regulated secretion, but only half as well as the full-length protein, it is likely that induction of RSGs by pro-Muclin involves both sorting for entry as well as sorting by retention.

Expression of pro-Muclin without its membrane anchor enhanced RSP storage, and, therefore, Muclin in the lumen of the secretory pathway has an important function. We recently demonstrated pH-dependent interactions of purified Muclin with several isolated zymogen granule content proteins, including amylase, that were mediated by the sulfated, O-linked oligosaccharides of Muclin (4). Furthermore, aggregation of isolated granule content proteins was modestly enhanced in the presence of Muclin (13). On the basis of these observations, Muclin in the lumen is expected to help the aggregation of the RSPs, thereby acting in the “sorting-by-retention” mechanism. Several other proteins have been suggested to have a “helper” function in RSP aggregation, and many of them are acidic sulfated macromolecules, like Muclin (35). A striking example is serglycin, a sulfated proteoglycan that is essential for mast cell RSG maturation. In a serglycin knockout mouse, mast cell granules form but they are unable to condense properly and they fail to store their RSPs (1).

The C-Tail of pro-Muclin is important for the maximal effect on inducing regulated secretion, suggesting that the C-Tail interacts with cytosolic proteins, as fits the idea of a cargo receptor. One possibility is that the C-Tail is involved in the assembly of a novel coat structure to induce RSG formation. However, there is currently no evidence for such a coat in the regulated pathway at the TGN. A second possibility is that the C-Tail recruits to the forming RSG-specific proteins that are involved in targeting the granule and regulation of its fusion with the plasma membrane. For example, in neuronal cells, members of the Rab11 family regulate the balance between constitutive and regulated exocytosis (24). In pancreatic acinar cells, it has been recently shown that Rab3D and Rab27B positively regulate RSG exocytosis (6, 7). Also, Noc2, a regulatory protein that interacts with Rab3 and Rab27, has been shown in a knockout mouse to be essential for regulated pancreatic secretion (29). Other important proteins that must be incorporated into forming RSGs are v-SNAREs, which mediate the membrane fusion event of the RSG with the plasma membrane (36). Especially relevant to the acinar cell is the v-SNARE Vamp8/endobrevin, which is on pancreatic zymogen granules and was recently demonstrated in a knockout mouse to be required for stimulated release (39). It remains to

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Fig. 9. Infection with Ad-amylose results in regulated amylase secretion in uninduced cells and has no effect on the regulated pathway in dexamethasone-induced AR42J cells. Cells were infected with Ad-amylose and were cultured for 48 h without (Uninduced) or with 100 nM dexamethasone (Dexamethasone) and then used to measure basal and caerulein-stimulated amylase release. Amylase release is expressed relative to total enzyme activity in the samples.

Fig. 10. Summary of the effects of the adenoviral constructs on the efficiency of regulated amylase storage. The data from Figs. 7–9 were used to calculate the ratio of stimulated to constitutive amylase release as a measure of the efficiency of amylase storage. A value of 1 indicates no regulated storage of amylase. *P < 0.05 vs. the respective GFP control (t-test).
be determined whether the C-Tail of pro-Muclin interacts with any of these proteins.

Induction of AR42J cells by dexamethasone combined with pro-Muclin expression achieves an efficiency of protein storage similar to that of freshly isolated pancreatic acini (40). The effects of dexamethasone on the AR42J cell are complex. Dexamethasone increases mRNA transcription for some RSPs (27), increases message stability for others (8), and can enhance protein translation (26). There is also an increase in rough endoplasmic reticulum (27) that is due to a structural reorganization of the protein translation machinery (32). In addition to increased amounts of cargo, there are changes in expression of proteins that control vesicular traffic and exocytosis. For example, the small GTPase Rab proteins Rab3A and Rab3C are downregulated, whereas Rab3B and Rab3D are upregulated, by dexamethasone (25, 31). Dynamin II, which is involved in the exit of vesicles from the TGN, is upregulated after dexamethasone induction (10). Several SNARE and SNARE-associated proteins are also upregulated in dexamethasone-induced AR42J cells, including Munc18b, syntaxins 1–4, and SNAP-23 and -25 (18).

Despite the numerous changes caused by dexamethasone, such induction results only in a modest regulated pathway. A robust regulated secretory pathway in AR42J cells was observed in the additional presence of full-length pro-Muclin. The data suggest that although dexamethasone induces expression of the protein machinery needed to assemble well-regulated and functional secretory granules, this machinery can only be fully utilized when the cells also express pro-Muclin. Because the C-Tail of pro-Muclin is needed for the maximal effect, it is consistent with the idea that the C-Tail is involved in recruiting the regulatory proteins to the forming RSG. The model system of pro-Muclin expression in dexamethasone-induced AR42J cells will be powerful for exploring how this machinery is put together and controlled to make the regulated secretory pathway in an exocrine cell. Future work will use Percoll-purified RSGs to determine whether expression of pro-Muclin enhances recruitment of such proteins to RSG and Percoll-purified RSGs to determine whether expression of secretory pathway in an exocrine cell. Future work will use

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GRANTS

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