Role of sulfated O-linked glycoproteins in zymogen granule formation

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Summary

Packaging of proteins into regulated secretory granules is mediated by the mildly acidic pH of the trans Golgi network and immature secretory granules. This need for an acidic pH indicates that ionic interactions are important. The mouse pancreatic acinar cell contains four major sulfated glycoproteins, including the zymogen granule structural component Muclin. I tested the hypothesis that sulfation and the O-linked glycosylation to which the sulfates are attached are required for normal formation of zymogen granules in the exocrine pancreas. Post-translational processing was perturbed with two chemicals: sodium chlorate was used to inhibit sulfation and benzyl-N-acetyl-α-galactosaminide was used to inhibit O-linked oligosaccharide elongation. Both chemicals resulted in the accumulation in the Golgi region of the cell of large vacuoles that appear to be immature secretory granules, and the effect was much more extensive with benzyl-N-acetyl-α-galactosaminide than chlorate. Both chemical treatments inhibited basal secretion at prolonged chase times, and again benzyl-N-acetyl-α-galactosaminide had a greater effect than chlorate. In addition, benzyl-N-acetyl-α-galactosaminide, but not chlorate, totally inhibited stimulated secretion of newly synthesized proteins. These data provide evidence for a role of sulfated O-linked glycoproteins in protein condensation and maturation of zymogen granules. Under maximal inhibition of O-linked oligosaccharide biosynthesis, anterograde post-Golgi traffic in the regulated pathway is almost totally shut down, demonstrating the importance of these post-translational modifications in progression of secretory proteins through the regulated pathway and normal granule formation in the pancreatic acinar cell.

Key words: Pancreas, Muclin, Secretory granule

Introduction

The mechanisms responsible for the formation of regulated secretory granules are poorly understood. One aspect that is becoming clear is that specific accessory or 'helper' molecules play crucial roles in regulated granule formation. These accessory molecules include proteoglycans, glycosaminoglycans, sulfated glycoproteins and lectin-like proteins (Forsberg et al., 1999; Kleene et al., 1999; Leblond et al., 1993; Kleene et al., 2000). These accessory macromolecules interact electrostatically and through specific protein-protein and protein-carbohydrate binding domains with the regulated proteins that are eventually stored in mature regulated granules. A principal mechanism for sorting of regulated proteins to the maturing granule is their aggregation at the mildly acidic pH of the trans Golgi network (TGN) and post-Golgi regulated compartments (Dannies, 2001; Leblond et al., 1993). Accessory molecules contribute to the continued aggregation of regulated secretory proteins as the granule matures, thus helping retain them in the regulated pathway.

Common constituents of regulated secretory granules are sulfated macromolecules, and in this study I investigated the roles of sulfated O-linked glycoproteins in formation of zymogen granules in the exocrine pancreas. In the pancreas, the acinar cell synthesizes 20-25 different digestive enzymes and proenzymes (zymogens), which are all stored in zymogen granules (Scheele, 1975). Although these proteins differ widely in their molecular weights and isoelectric points, they coaggregate at slightly acidic pH values known to occur in the TGN (Leblond et al., 1993), the major sorting station in the secretory pathway. Sorting continues in the condensing vacuole (immature secretory granule) as the granule contents further condense and non-regulated secretory proteins are excluded and trafficked to lysosomes and to the plasma membrane via the constitutive-like secretory pathway (Arvan and Castle, 1998; Arvan and Chang, 1987). Upon exocytosis of the zymogen granule, the digestive enzymes are exposed to the alkaline pH of the acinar/ductal lumen, and the proteins are solubilized for their transit to the duodenum (Freedman et al., 1998). These facts indicate that ionic interactions mediated by acidic pH in the distal secretory pathway and basic pH in the acinar lumen control protein packaging and subsequent release.

Recent findings show that specific molecules in the secretory pathway can have profound effects on formation of regulated secretory granules and that post-translational modification of these molecules may be key to their function. For example, in mast cells, the polyanion glycosaminoglycan heparin sulfate is required for normal granule formation (Humphries et al., 1999; Forsberg et al., 1999). Also, in neuroendocrine cells, the sulfated glycoprotein chromogranin A is necessary for secretory granule formation and proper storage of regulated secretory proteins (Kim et al., 2001).

In contrast to mast cells and neuroendocrine cells, there is less known about the specific molecules involved in forming zymogen granules in exocrine cells. The molecules in the pancreatic acinar cell that support this function are not firmly established, but they are likely to be sulfated glycoproteins

and/or proteoglycans. It appears that there are species differences in that mice have O-linked glycoproteins (De Lisle, 1994), whereas rats and guinea pigs have proteoglycans in their zymogen granules (Schmidt et al., 2000; Reggio and Palade, 1978). Recent work using rat pancreas has implicated the lectin-like molecule ZG16p in zymogen granule formation (Kleene et al., 1999) in concert with granule-membrane-associated proteoglycans (Schmidt et al., 2000). The mouse pancreatic acinar cell contains a limited number of sulfated O-linked glycoproteins, which are more easily studied biochemically than are proteoglycans. The major mouse sulfated glycoprotein is Muclin, a 300 kDa structural protein of the zymogen granule that may have a role in protein sorting in the regulated pathway (De Lisle and Ziemer, 2000). The other major sulfated proteins of the mouse acinar cell are the zymogens prolipase and proelastase IV, and p82/p75, a marker of the constitutive-like pathway associated with secretory granule maturation (De Lisle and Bansal, 1996).

In this report I focus on these sulfated glycoproteins and the role of their sulfates and O-linked carbohydrates in formation of mouse zymogen granules. When sulfation or O-linked glycosylation are perturbed, the secretory function is reduced, and this is accompanied by morphological alterations that provide a way of interpreting how the regulated pathway is affected by altering sulfated glycoprotein processing.

Materials and Methods

Preparation of granule contents and Muclin for the in vitro zymogen protein aggregation assay

Zymogens granules were purified from mouse pancreas on a Percoll gradient and osmotically lysed in 150 mM sodium bicarbonate, pH 8.0, plus protease inhibitors (phenylmethyl-sulfonylfluoride, leupeptin, pepstatin A), in the presence of the cation exchange ionophore nigericin as described previously (De Lisle and Bansal, 1996). The soluble zymogen granule contents (ZGC) and membranes were separated by ultracentrifugation at 200,000 ***g*** for 60 minutes. The soluble material contains ~10% of the Muclin in granules, but the majority of Muclin pellets with the membranes (De Lisle, 1994). The soluble zymogens were collected and depleted of Muclin by passing them over a 2 ml peanut agglutinin (PNA)-agarose column (Sigma, St.Louis, MO) several times (Fig. 1A). The column was washed with 150 mM sodium bicarbonate until the OD_{280} reached baseline. The final flow-through and the first wash of the column were pooled and considered to be Muclin-depleted ZGC. Bound Muclin could then be eluted with 0.2 M lactose (Fig. 1A). The Muclin-depleted ZGC were then exchanged into 50 mM potassium glutamate, 5 mM HEPES, 10 mM MES, pH 8.0 and concentrated by centrifugation in a 10 kDa cut-off Amicon spin filter (Amicon, Beverley, MA). The amount of Muclin that could be recovered from the PNA-agarose column was small, so large quantities of Muclin were purified from whole mouse pancreas using preparative SDS-PAGE as described (De Lisle et al., 1997). Aliquots of ZGC (50 μg protein) in a final volume of 250 μl were mixed with other proteins, $CaCl_2$ or EGTA as indicated, mildly acidified, and the pH change monitored with a pH microprobe. The protein aggregates that formed at room temperature after 15 minutes were collected by centrifugation in a microcentrifuge (16,000 ***g*** for 15 minutes). The pellets were analyzed by SDS-PAGE and Coomassie blue staining of the gels for total protein, and by western blot for Muclin (De Lisle and Ziemer, 2000).

Preparation of pancreatic acini and radiolabeling

Pancreata from mice (ND4, Swiss Webster strain, Harlan, Indianapolis, IN) were digested with purified collagenase, mechanically dispersed, and acini were purified by filtration followed by centrifugation on a step gradient of 4% bovine serum albumin as described previously (De Lisle and Bansal, 1996). The isolated acini were suspended in Hepes-buffered Ringer's solution (HR buffer) supplemented with 0-30 mM $NaClO_3$ (chlorate) or 0-32 mM benzyl-N-acetyl-α-galactosaminide (BzlGalNAc) from a 1.6 M stock in dimethylsulfoxide (DMSO). For experiments with chlorate-treated cells, all media were supplemented with NaCl to obtain a final 30 mEq extra sodium, matching the highest concentration of Na added as sodium chlorate. For experiments with BzlGalNAc-treated cells, the final concentration of DMSO was 2% in all cell aliquots, matching the maximum [DMSO] added with 32 mM BzlGalNAc. The cells were biosynthetically labeled in the presence of the indicated chemicals: 60 minutes with 100 μCi/ml [^{35}S]sulfate; 60 minutes with 1 μCi/ml [^{3}H]leucine; or 16 hours with 40 μCi/ml [^{3}H]glucosamine using media without glucose and supplemented with 22 mM pyruvate to enhance uptake of the labeled sugar. After labeling, cells were pelleted and aliquots were run on SDS-PAGE. Gels of [^{35}S]-labeled cell proteins were phosphorimaged (Cyclone, Packard Instruments, Meriden, CT). Gels of [^{3}H]glucosamine labeled protein were treated with Enhance (NEN, Boston, MA) followed by fluorography for 20 days on X-ray film. [^{3}H]leucine-labeled protein was precipitated with 10% trichloroacetic acid followed by liquid scintillation counting.

Pulse-chase analysis

For pulse chase analysis, met/cys-free medium (Sigma) was used during the cell isolation procedure to deplete the cells of these amino acids. The isolated acini were then pulse labeled with 0.5 mCi/ml [^{35}S]met/cys (Tran^{35}S-Label, ICN, Costa Mesa, CA) for 30 minutes in met/cys-free medium. The cells were washed and resuspended at 0.4-1 mg cell protein per ml of chase medium (HR with three times the usual amino acid concentration and 50 μg/ml soybean trypsin inhibitor) alone (control) or supplemented with 30 mM NaCl, 30 mM chlorate, 2% DMSO or 32 mM BzlGalNAc. The cells were aliquoted at 1 ml per well into 24-well plates and incubated at 37°C. As indicated, cells received a final concentration of 1 μM carbachol and 1 mM 8-Br-cAMP for the final 30 minutes of incubation to stimulate regulated secretion of [^{35}S]-labeled newly made protein. At the indicated times, cells were transferred to siliconized microfuge tubes (Sigma), pelleted and the media and cell pellets saved. The entire 1 ml of the media samples was precipitated with 10% trichloroacetic acid, and the pellets were solubilized in SDS-PAGE sample buffer plus 1-2 μl 1 M Tris-OH to keep the pH alkaline. Cell pellets (10% of the total) and the entire precipitated media samples were run on SDS-PAGE followed by phosphorimaging. In separate experiments, media from unlabeled cells were precipitated, run on 10% SDS-PAGE and Coomassie blue stained for quantification of released total amylase. Protein bands were quantified from scanned gels (Hewlett Packard ScanJet IIcx, Palo Alto, CA) using OptiQuant software (Packard Instruments). Muclin and its precursor pro-Muclin are of unique M_r in the acinar cell, which has been verified by immunoprecipitation (De Lisle and Ziemer, 2000). Therefore, it is straightforward to examine pro-Muclin and Muclin in whole tissue and cell homogenates by SDS-PAGE. p80, the C-terminal cleavage product of pro-Muclin, was immunopreciptated from cell homogenates as described using an antiserum to the 13 C-terminal amino acids (De Lisle and Ziemer, 2000). Carbohydrates on Muclin were assessed by lectin blotting with peanut agglutinin (PNA; specific for Gal β(1-3)GalNAc) and Maackia amurensis agglutinin (MAA; specific for NANA α(2-3)Gal) as described previously (De Lisle et al., 1998).

In vivo zymogen granule depletion and in vitro recovery

To deplete stored zymogen granules, mice were injected with a cholinergic agonist (10 μg pilocarpine per g body weight i.p.). After

1 hour, the pancreas contained ~50% of its initial amylase and Muclin content (not shown), and the majority of zymogen granules were depleted (c.f. Fig. 7A and B). The pancreas was then removed and lobules prepared by injecting HR buffer into the tissue and dissecting out the distended lobules. The lobules were injected with HR supplemented with 30 mM NaCl, 30 mM chlorate, 2% DMSO (vehicle) or 32 mM BzlGalNAc. This allowed the zymogen-granule-depleted tissue to be exposed to the test chemicals within 15 minutes of sacrifice of the mouse. The lobules were incubated at 37°C with agitation at 120 rpm in a shaking waterbath and oxygenation every 30 minutes. In control lobules, the amylase content was ~90% of untreated levels after 4 hours of incubation (data not shown), and the cells were refilled with zymogen granules (Fig. 7C,G).

Morphology and immunocytochemistry

For morphology studies, tissues were fixed in 4% paraformaldehyde, 1.6% glutaraldehyde in phosphate-buffered saline and processed for light and electron microscopy. Thick plastic sections (1 μm) were stained with toluidine blue, and images were obtained with differential interference contrast optics on a Nikon Diaphot microscope (Nikon, New York, NY). Thin sections were prepared and imaged on a JEOL 100CX-11 electron microscope. For indirect immunofluorescence, the tissue was fixed for 1 hour in 2% paraformaldehyde, 0.1% saponin to permeabilize cell membranes and cryoprotected as described previously (Barthel and Raymond, 1990). Cryosections were cut at 5 μm. Immunostaining was performed with rabbit anti-Muclin (De Lisle and Ziemer, 2000), sheep anti-amylase (The Binding Site, San Diego, CA), both diluted 1:500 in 2% normal donkey serum or rat monoclonal antibody to the lysosomal membrane protein LAMP-1 (clone 1DB4; Development Studies Hybridoma Bank, University of Iowa), diluted 1:5. The secondary antibodies were donkey anti-rabbit-FITC, donkey anti-sheep-Texas Red and donkey anti-rat-Texas Red (Jackson ImmunoResearch, West Grove, PA), and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was used to label the nuclei. Images were obtained with a SPOT II digital camera (Diagnostic Instruments, Sterling Heights, MI) on a Nikon Diaphot microscope with appropriate filter sets.

Cell viability was assessed using the LIVE/DEAD kit from Molecular Probes (Eugene, OR). Acini were incubated at 37°C under the indicated conditions for the indicated times. The LIVE/DEAD reagent, which consists of calcein-acetoxymethyl ester and ethidium-homodimer, was added and the incubation continued for 10 minutes at 37°C. The cells were then imaged on the Nikon inverted microscope, and green and red images obtained to show live (calcein-positive) and dead (ethidium-homodimer labeled nuclei) cells. The images were analyzed using Scion Image software (Scion Corp., www.scioncorp.com) to quantify the area occupied by red-labeled nuclei. Total cell death was achieved by adding Triton X-100 to 1% and was used to calculate the percentage of dead cells under the different incubation conditions.

Results

In vitro aggregation of isolated zymogens and Muclin

The hypothesis of the work presented here is that sulfated O-glycosylated proteins have important functions in normal formation of pancreatic zymogen granules. The major sulfated protein of the mouse acinar cell is Muclin, a 300 kDa O-glycosylated protein that is derived from a membrane precursor, pro-Muclin. Muclin is concentrated in the zymogen granule, is poorly secreted and has no predicted enzymatic activity, so it is likely that Muclin is a structural component of the zymogen granule (De Lisle and Ziemer, 2000; De Lisle et al., 2001). Using an in vitro aggregation assay, purified Muclin coaggregates with isolated zymogens when mildly acidified to mimic the environment of the TGN and secretory granules of the acinar cell. To test whether Muclin affects the ability of ZGC to aggregate in this assay, ZGC were depleted of Muclin on a PNA-agarose column. As shown in Fig. 1A, ZGC contain some Muclin, and we previously showed that about 10% of granule Muclin is soluble after granule lysis (De Lisle, 1994). The majority of Muclin is removed after a single pass over the PNA-agarose column (flow-through fraction 1: FT-1), and only a minor trace remains (<5% of the original amount) after the fourth pass over the column (FT-4). To demonstrate that Muclin was indeed bound to the column, 0.2 M lactose was used to elute Muclin, as shown in Fig. 1A (elution fractions 1-5: E-1–E-5). The Muclin–depleted ZGC were then used in the aggregation assay. ZGC largely depleted of Muclin are still efficiently aggregated upon mild acidification (Fig. 1B; control), and there is about a four-fold increase in the amount of protein pelleted in a microfuge (Fig. 1C). The extent of aggregation was assessed by comparing the pellets with the supernatants after aggregation, and about 7-8% of the ZGC were in the pelleted aggregates (data not shown). The effect of Muclin was tested by adding purified Muclin at a level of 10% of the amount of ZGC protein, which is similar to the ratio that exists in intact ZG. The presence of Muclin at pH 8.0 reproducibly decreased ZGC aggregation by about 40% ($P<0.001$; $n=4$ using two independent ZGC preparations). At pH 6.3, Muclin increased ZGC aggregation by about 20% (Fig. 1B,C) (not significant; $P=0.5$; $n=4$ using two independent ZGC preparations). Thus, overall, Muclin increases the efficiency of acid-mediated aggregation, primarily by decreasing the aggregation at basic pH. As shown in Fig. 1D, added Muclin also aggregates with the ZGC to a similar extent as amylase upon acidification. Importantly, Muclin by itself did not exhibit aggregation upon acidification (Fig. 1E).

The specificity of the aggregation was tested by including a constitutively secreted protein, bovine serum albumin. Serum albumin did not exhibit acid-mediated aggregation itself nor did it affect acid-mediated ZGC aggregation (Fig. 1B,C). Also tested was whether aggregation is affected by the presence or absence of Ca^{2+}, which is known to be at millimolar concentrations in the TGN. Chelation of calcium associated with the ZGC by adding 5 mM EGTA had little effect on acid-mediated ZGC aggregation nor did addition of 2 mM exogenous $CaCl_2$ (Fig. 1B,C). Neither EGTA nor Ca^{2+} had any effect up to 10 mM (not shown).

Inhibition of sulfation with sodium chlorate

Chemical inhibitors of post-translational processing were used with isolated mouse acinar cells and pancreatic lobules from in vivo zymogen-granule-depleted tissue to test the role of sulfated O-glycoproteins in granule formation and maturation. Sodium chlorate was used as a competitive inhibitor of ATP-sulfurylase, a key enzyme in synthesis of the high energy sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (Baeuerle and Huttner, 1986). Chlorate dose-dependently inhibited incorporation of [^{35}S]sulfate into pancreatic acinar cell proteins (Fig. 2A,B), which, in addition to Muclin, are p82/75, a marker of the constitutive-like secretory pathway (but unrelated to the ~80 kDa proteolytic fragment of pro-Muclin called p80) and the pancreatic digestive enzymes prolipase and

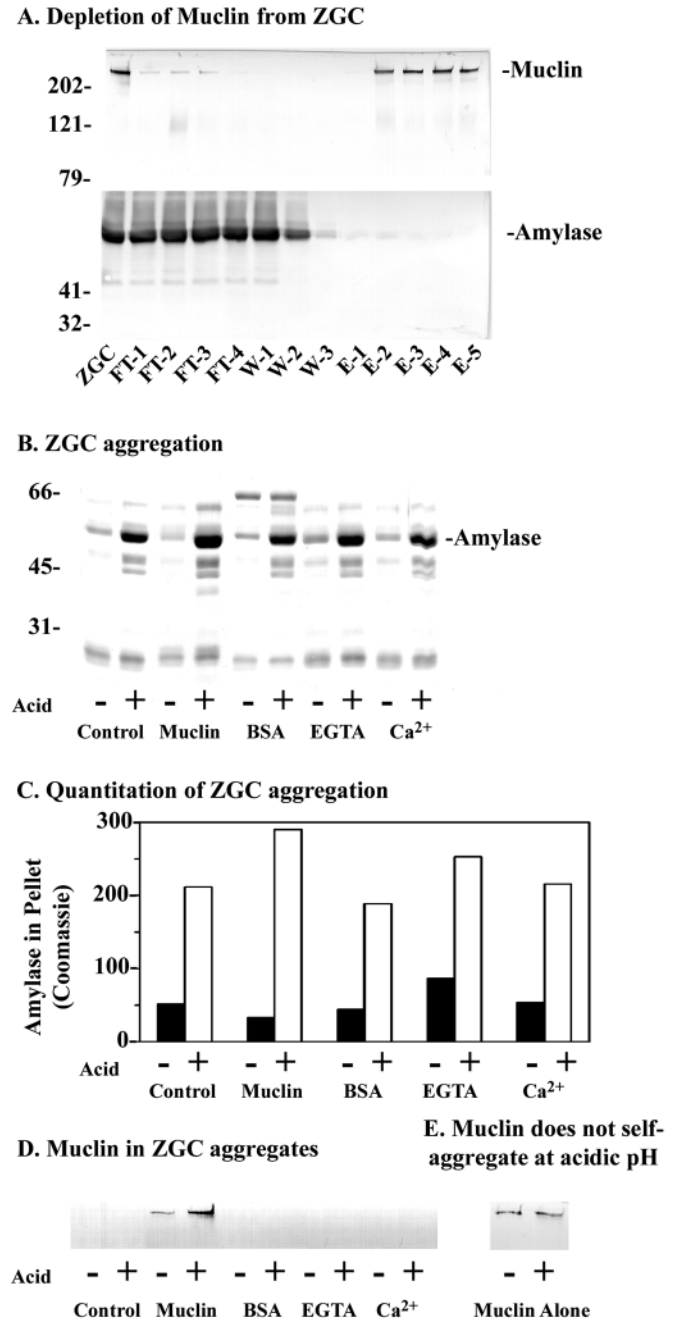

Fig. 1. Preparation of Muclin-depleted ZGC and in vitro aggregation of ZGC and purified Muclin. (A) Isolated soluble zymogens were depleted of Muclin on a PNA-agarose column. ZGC, starting material; FT, flow-through fractions; W, wash fractions; E, 0.2 M lactose eluted fractions. FT-4 and W-1 were pooled and used for the in vitro aggregation assay. (B) Muclin-depleted ZGC were incubated at pH 8.0 (Acid –) or acidified to pH 6.3 (Acid +). The aggregates were pelleted, run on 10% SDS-PAGE and stained with Coomassie blue. The control is 50 μg ZGC alone; 'Muclin' included 5 μg purified Muclin, which increased ZGC aggregation compared with the control; 'BSA' included 10 μg bovine serum albumin, which did not aggregate at acidic pH; 'EGTA' included 5 mM EGTA to chelate calcium, which did not affect aggregation; 'Ca^{2+}' included 2 mM $CaCl_2$, which did not affect acid-induced aggregation. (C) Quantification of amylase in the pellets by densitometry (arbitrary units) of Coomassie-blue-stained gel. (D) Western blot for Muclin in the pellets. No Muclin was detected in the ZGC-depleted samples, but was detected only when purified Muclin was added (Muclin). The amount of Muclin in the pellet was increased upon acidification (Acid +). (E) By western blot, Muclin in the absence of ZGC does not exhibit acid-mediated aggregation.

proelastase IV (De Lisle and Bansal, 1996). The maximal effect on sulfation was between 10-30 mM chlorate, with >95% inhibition of sulfation for all proteins (Fig. 2B). By contrast, chlorate had no effect on protein synthesis as measured by incorporation of [^{3}H]leucine into trichloroacetic acid precipitable protein (Fig. 2C).

Inhibition of sulfation and O-glycosylation with BzlGalNAc

Benzyl-N-acetyl-α-galactosaminide (BzlGalNAc), which acts as an acceptor for O-linked oligosaccharides, was used to block elongation of these sugars on glycoproteins (Delannoy et al., 1996). In the acinar cell, the majority of sulfate is on O-linked sugars (De Lisle and Bansal, 1996), so it was expected that blockage of O-linked oligosaccharide elongation would reduce sulfation as the normal sites would not be synthesized. BzlGalNAc dose-dependently inhibited sulfation of acinar cell proteins (Fig. 3A). It was noticed that a broad area of radioactivity appeared on the phosphorimages of BzlGalNAc-treated cells, that this labeled material migrated to the same region of the gels largely independently of the percentage acrylamide used and was best separated from the labeled proteins on 12.5% acrylamide (Fig. 3B). This labeled material probably represents sulfated BzlGalNAc metabolites (Delannoy et al., 1996; Huang et al., 1992). Although the incorporation of [^{35}S]sulfate into BzlGalNac derivatives was diffuse, the maximum amount of radioactivity in this material was equivalent to 169% and 49% of that incorporated into prolipase and proelastase, respectively, under control conditions. To avoid these areas, samples were run on both 7.5% and 12.5% acrylamide gels (Fig. 3A,B) for quantification of the specific protein bands. The maximum effect of BzlGalNAc on glycoprotein sulfation was at 16-32 mM BzlGalNAc, and inhibition ranged from 54% to 75%, showing some differences among the different proteins (Fig. 3C).

The effect of BzlGalNAc on total protein oligosaccharide synthesis was estimated by labeling with [^{3}H]glucosamine, which can be used by cells as a precursor for GlcNAc, GalNAc and sialic acid and thus will be incorporated into most N- and O-linked oligosaccharides (Beeley, 1985). Only Muclin was appreciably labeled with [^{3}H]glucosamine, and BzlGalNAc dose-dependently inhibited this labeling, with almost complete inhibition at 16-32 mM (Fig. 3D). BzlGalNAc had only minor effects on total protein synthesis and caused ~20% inhibition of [^{3}H]leucine incorporation into TCA-precipitable protein at 32 mM (Fig. 3E).

Effects of chlorate and BzlGalNAc on post-translational processing of pro-Muclin

I next examined the effects of chlorate and BzlGalNAc on the biosynthetic maturation of Muclin. Muclin is synthesized as pro-Muclin, a type I membrane protein that undergoes

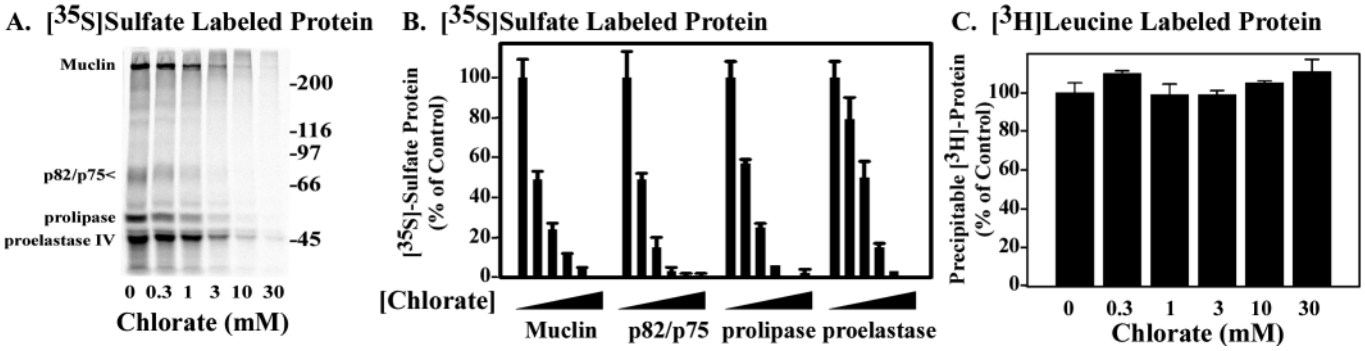

Fig. 2. Effect of chlorate on incorporation of [^{35}S]sulfate and [^{3}H]leucine into acinar cell proteins. (A) [^{35}S]sulfate labeling of isolated acini in the presence of sodium chlorate. Equal aliquots of acini (0.4 mg protein) were labeled in the presence of the indicated concentrations of chlorate. 10% of the cell pellets were run on the gels. (B) Quantification of sulfate labeling versus [chlorate]. Chlorate dose-dependently inhibits sulfation of all the major sulfated proteins and is >95% effective at 30 mM. (C) Trichloroacetic acid-precipitable [^{3}H]-protein synthesized in the presence of sodium chlorate shows that chlorate does not inhibit protein synthesis. Data are means±s.d. of triplicate samples from representative experiments.

extensive N- and O-linked glycosylation and sulfation of its O-linked sugars in the TGN. In a post-Golgi compartment pro-Muclin is proteolytically cleaved to mature sulfated Muclin, which is luminal and remains in the zymogen granule, and an 80 kDa non-sulfated glycoprotein (p80), which is trafficked to the apical plasma membrane of the acinar cell (De Lisle and Ziemer, 2000). As shown in Fig. 4, chlorate had little effect on processing of pro-Muclin to Muclin and p80. By contrast, BzlGalNac slowed the maturation process, resulting in slower disappearance of pro-Muclin (Fig. 4B) and reduced production of Muclin (Fig. 4C) and p80 (Fig. 4D). There was a slight effect of the vehicle (DMSO) on the accumulation of mature Muclin, but it was nowhere near as great as the BzlGalNAc effect. In addition, the M_r of Muclin in the presence of BzlGalNAc was less than normal, reflecting the inhibition of O-linked oligosaccharide elongation (Fig. 4A, asterisk).

Previous work using BzlGalNAc in various cultured cell lines has reported alterations in terminal sugar composition of glycoproteins, specifically increased T antigen and decreased sialic acid (Delannoy et al., 1996). Therefore, I examined binding of PNA (T antigen) and MAA (sialic acid)

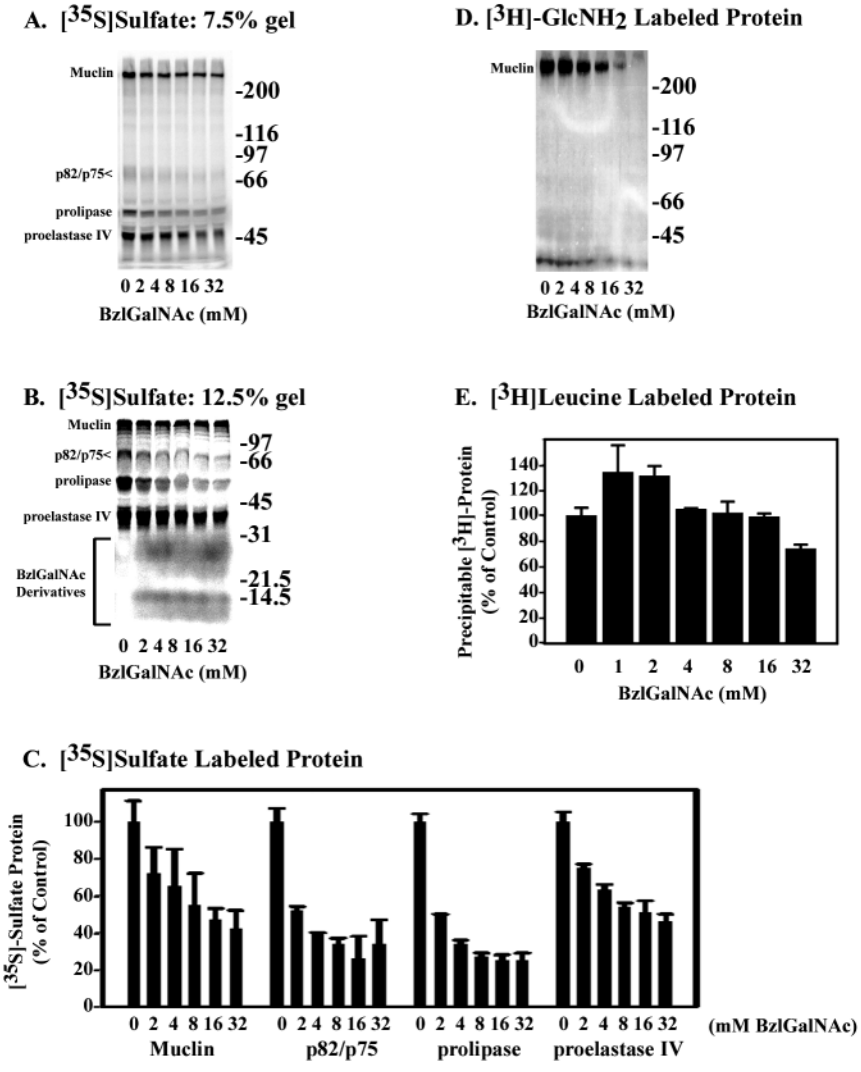

Fig. 3. Effect of BzlGalNAc on incorporation of [^{35}S]sulfate, [^{3}H]glucosamine and [^{3}H]leucine into acinar cell proteins. (A) Equal aliquots of acini (0.4 mg protein) were incubated with the indicated concentrations of BzlGalNAc. 10% of the cell pellets were run on 7.5% gels and imaged on a phosphor screen for [^{35}S]sulfate. (B) 12.5% gel using 10% of the same cell pellets shown in A. Note the labeled diffuse area representing sulfated BzlGalNAc derivatives. (C) Quantitation of sulfate labeling versus [BzlGalNAc]. Muclin and p82/p75 were quantified from 7.5% gels, and prolipase and proelastase IV were quantified from 12.5% gels. (D) [^{3}H]glucosamine labeling of equal aliquots (1 mg protein) of isolated acini in the presence of BzlGalNAc; 10% of the cell pellets were separated by SDS-PAGE followed by autoradiography. (E) [^{3}H]leucine labeling of isolated acini in the presence of BzlGalNAc. Data are means±s.d. of triplicate samples from representative experiments.

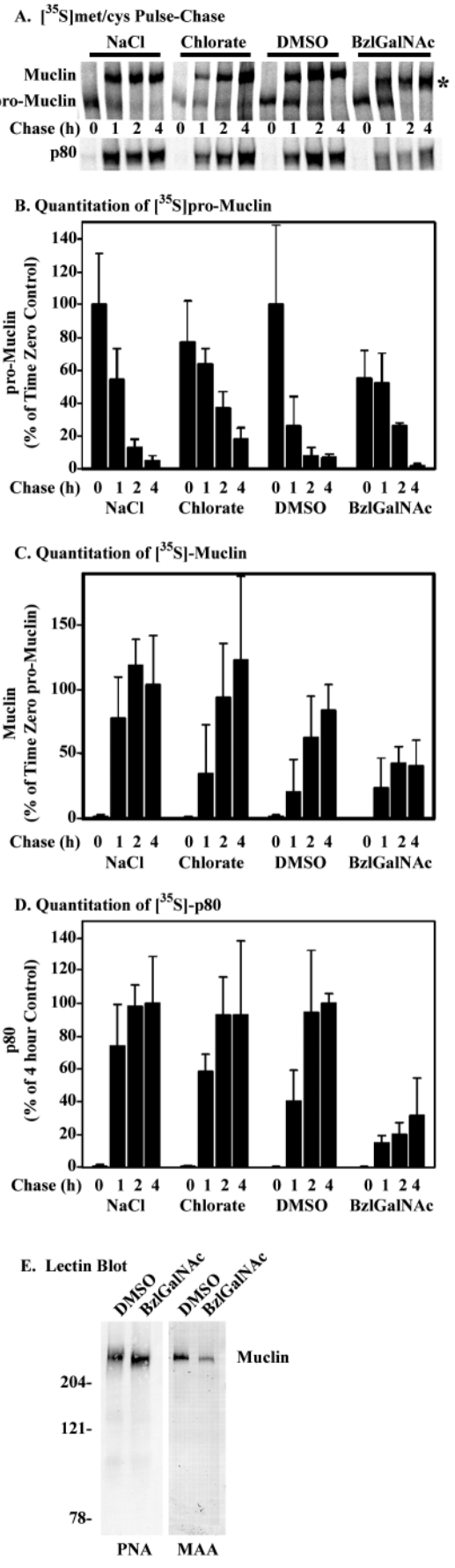

Fig. 4. Effects of chlorate and BzlGalNAc on post-translational maturation of pro-Muclin. (A) Pancreatic lobules were prepared from zymogen-granule-depleted mouse pancreas, pulse-labeled for 30 minutes in the presence of 30 mM NaCl, 30 mM chlorate, 2% DMSO or 32 mM BzlGalNAc. The samples were washed and chased in the continued presence of the chemicals for the indicated times. Equal amounts of tissue were run on SDS-PAGE (DNA equivalent to 3×10^5 cells) and phosphorimaged. p80 was immunoprecipitated from equal aliquots (DNA equivalent to 2.7×10^6 cells). The asterisk indicates the lower M_r form of Muclin produced in BzlGalNAc-treated cells. (B) Quantification of labeled pro-Muclin by phosphorimaging as a function of chase time expressed relative to the time zero incorporation of the appropriate control (NaCl and DMSO, respectively). (C) Quantification of labeled Muclin by phosphorimaging as a function of chase time expressed relative to the time zero incorporation into pro-Muclin of the appropriate control. (D) Quantification of p80 by phosphorimaging as a function of chase time expressed relative to the level at 4 hours of chase of the appropriate control. Data in B to D are from triplicate samples of a representative experiment and are means±s.d. (E) Lectin binding to pancreatic proteins. Pancreatic lobules were prepared from zymogen-granule-depleted mouse pancreas and incubated with 2% DMSO (vehicle) or 32 mM BzlGalNAc for 4 hours. Equal amounts of tissue were separated by SDS-PAGE followed by western blotting and probing with PNA (T antigen) and MAA (sialic acid).

to acinar cell proteins synthesized in the presence of chlorate or BzlGalNAc for 4 hours. It was shown previously that Muclin is the major PNA- and MAA-binding glycoprotein in the mouse acinar cell (De Lisle, 1994), and this is apparent on the lectin blots of whole pancreatic tissue shown in Fig. 4E. BzlGalNAc caused an increase in PNA binding and a reduction in MAA binding to Muclin (Fig. 4E). In addition, the reactive band in both lectin blots was of lower M_r in BzlGalNAc-treated cells, and the shift was about the same magnitude as seen in [^{35}S]met/cys pulse-labeled and chased protein (Fig. 4A).

Effects of chlorate and BzlGalNAc on basal and stimulated protein secretion

I next examined the effects of these chemicals on basal and regulated protein secretion by [^{35}S]met/cys pulse-chase analysis using freshly isolated pancreatic acini. Cells were labeled in control medium and chased in the presence or absence of the inhibitory chemicals. As shown in Fig. 5A and quantified in Fig. 5B,C, untreated acini have a basal release of newly made protein of 1.7% per hour (control). In the presence of chlorate during the chase, there was a modest inhibitory effect on basal secretion by 6 hours of chase to 65% of control (Fig. 5A,B). When stimulated with a cholinergic agonist (1 μM carbamylcholine chloride) and a membrane-permeant cAMP analog (1 mM 8-Br-cAMP) for the last 30 minutes of chase, the control cells responded with an increase in the rate of protein release of 6.8-fold compared with the basal level. Treatment with chlorate had no effect on the stimulated rate of protein secretion (7.1-fold increase compared with basal), but the cumulative amount of newly synthesized protein released was reduced, largely reflecting the decrease in basal release (Fig. 5B). Also shown in Fig. 5 is the control using 30 mM NaCl to match the additional salt added as sodium chlorate in these experiments; NaCl had no effect on basal or stimulated secretion.

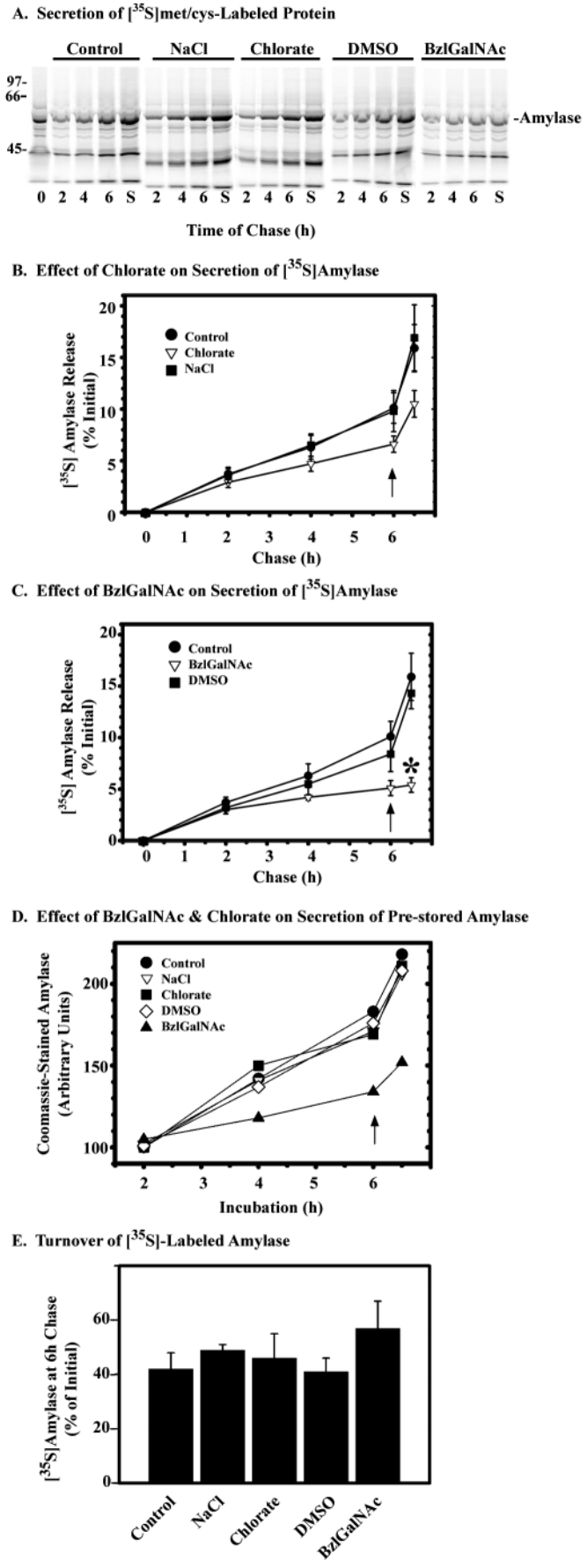

Fig. 5. Effects of chlorate and BzlGalNAc on basal and stimulated secretion of newly made proteins. (A) Phosphorimage of secreted [^{35}S]met/cys-labeled proteins. Pulse-labeled acini were washed and chased at 1 mg cell protein per ml in the indicated buffers. During the final 30 minutes of chase, 1 μM carbachol and 1 mM 8-Br-cAMP were added to stimulate regulated secretion (S). The lane marked '0' is 10% of a time zero pellet (0 hour chase) used to quantify the percentage release values. (B) The effect of 30 mM chlorate on basal and stimulated secretion (arrow indicates addition of stimulus for final 30 minutes of chase) of [^{35}S]met/cys-labeled proteins. Amylase release was quantified relative to cell content just after the pulse labeling. Data are means±s.e., *n*=4 independent experiments. (C) The effect of 32 mM BzlGalNAc on basal and stimulated secretion (arrow indicates addition of stimulus for final 30 minutes of chase) of [^{35}S]met/cys-labeled proteins. For comparison, the control data from panel B are reproduced here. Data are means±s.e.m., *n*=4 independent experiments. **P*=0.005 compared with the DMSO control after stimulation. (D) Quantification of basal and stimulated secretion (arrow indicates addition of stimulus for final 30 minutes of incubation) of prestored protein by Coomassie blue staining of secreted media from a representative independent experiment. (E) Quantitation of [^{35}S]labeled amylase 6 hours after chase under the different conditions. Data are expressed as a percentage of initial level of labeled amylase; means±range of values from two independent experiments.

Isolated acini treated with BzlGalNAc exhibited decreased basal release of newly synthesized protein to ~50% of control by 6 hours (Fig. 5A,C). More dramatically, stimulated secretion was almost totally inhibited by BzlGalNAc (Fig. 5A,C). Also shown in Fig. 5 is the effect of dimethylsulfoxide (DMSO) used as a vehicle for BzlGalNAc. DMSO had a slight effect on basal secretion (82% of control) but none on the increased rate upon stimulation of the cells.

To see if BzlGalNAc also blocked release of amylase from prestored zymogen granules, Coomassie-blue-stained gels of secreted proteins from separate experiments using unlabeled cells under the same conditions were examined. As shown in Fig. 5D, BzlGalNAc slowed basal release of prestored amylase compared with the other conditions used. However, when secretion was stimulated, there was a similar increase in the rate of amylase release from BzlGalNAc-treated cells as from the other conditions used. Thus, BzlGalNAc inhibits traffic through the secretory pathway but does not affect stimulated release of amylase that had already reached zymogen granules before addition of BzlGalNAc.

Since secretion of amylase was reduced by these treatments, it was of interest to determine whether newly made amylase was accumulating or being degraded in the treated cells. The [^{35}S]-labeled amylase remaining in the cells after 6 hours of chase was found to be similar under all conditions tested but was slightly elevated in the presence of BzlGalNAc (Fig. 5E). This result indicates that blockage of amylase release in the presence of BzlGalNAc causes some accumulation in the cell.

Morphological examination of treated cells (below) indicated that these chemical treatments were not toxic to the cells. To measure this more directly, a combination of calcein-acetoxymethyl ester and ethidium homodimer was used to label healthy and dead cells, respectively (see Materials and Methods). As shown in Fig. 6, freshly isolated acini had a small number of dead cells but most were healthy (Fig. 1A,E). Use of Triton X-100 to kill the cells shows the opposite labeling

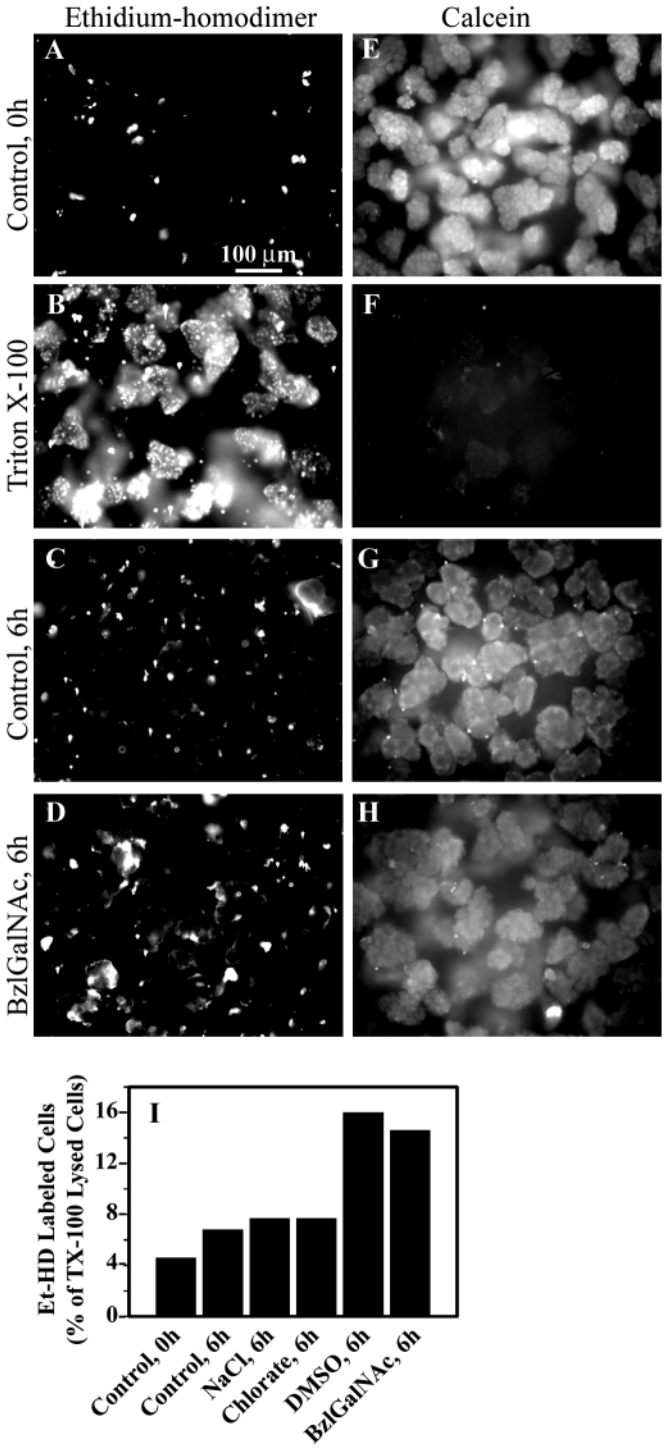

Fig. 6. Assessment of cell viability after incubation with NaCl, chlorate, DMSO or BzlGalNAc. Acini were incubated for the indicated times and then labeled for 10 minutes with calcein-acetoxymethyl ester and ethidium homodimer to label live and dead cells, respectively. (A-H) The cells were imaged by fluorescence microscopy and representative images are shown. Live cells accumulate calcein and fluoresce green (E,G,H). Dead cells are stained by ethidium homodimer and fluoresce red (A-D). Triton X-100 (1%) was used to kill the cells which cannot accumulate calcein (F) but admit ethidium homodimer, which fluorescently stains nuclei red (B). (I) Scion Image analysis software was used to quantify the amount of red staining ethidium homodimer under the different conditions. The amount of dead cells in each sample was calculated as the percentage of ethidium homodimer labeled nuclei relative to Triton X-100 killed cells.

pattern with no calcein staining and all nuclei labeled with ethidium homodimer (Fig. 6B,F). Under control conditions, after 6 hours of incubation, there was a small increase in dead cells, and there was a similar increase in the presence of NaCl or chlorate (Fig. 6I). By contrast, there was a greater amount of dead cells after 6 hours of incubation in the presence of BzlGalNAc (Fig. 6D,H,I). However, DMSO alone showed a similar increase in cell death (Fig. 6D) but, importantly, DMSO did not exhibit the blockage of stimulated release of newly synthesized amylase that BzlGalNAc did (Fig. 5C).

Morphological effects on acinar cell secretory organelles of chlorate and BzlGalNAc

I next examined the morphological effects of these chemicals on the secretory pathway of the acinar cell. To help visualize changes in newly forming secretory granules, zymogen granules were first depleted in vivo and then allowed to reform during in vitro incubations (see Materials and Methods). As shown by comparing uninjected control (Fig. 7A) with 1 hour post-pilocarpine (Fig. 7B), there was an almost total loss of zymogen granules after in vivo stimulation. The pilocarpine ZG-depleted tissue was healthy, and during a 4 hour in vitro incubation under control conditions the cells refilled with zymogen granules (Fig. 7C,E,G,I) and the amylase content recovered to about 90% of initial levels observed in unstimulated control tissue (data not shown).

When zymogen-granule-depleted lobules were incubated in vitro with chlorate, they synthesized some new zymogen granules, but the cells also had an accumulation of low density vacuoles in the perinuclear region of the cell (Fig. 7D), whereas control cells had a normal morphology (Fig. 7C). At the ultrastructural level, an accumulation of electron-lucent vacuoles was observed near the nucleus as well as normal appearing zymogen granules (Fig. 7F). The vacuoles contained varying densities of loose protein aggregates. By contrast, the control cells had normal zymogen granules, including protein aggregates in the Golgi indicating ongoing granule formation (Fig. 7E).

When zymogen-granule-depleted lobules were incubated in vitro with BzlGalNAc, they made very few normal appearing granules and had an extensive accumulation of low density vacuoles reaching from the perinuclear region toward the apical surface of the cells (Fig. 7H). At the ultrastructural level, the BzlGalNAc-treated cells contained an accumulation of electron-lucent vacuoles with some poorly condensed protein in their lumina (Fig. 7J). The control cells, treated with DMSO, were normal in appearance and had normal electron-dense zymogen granules (Fig. 7G,I).

Localization of Muclin and amylase in chlorate and BzlGalNAc-treated pancreatic cells

To examine the subcellular localizations of the major regulated secretory protein, amylase, and the major granule structural component, Muclin, frozen sections of lobules were examined following a 4 hour in vitro recovery incubation in the presence

of chlorate or BzlGalNAc. As shown in Fig. 8, amylase and Muclin are largely colocalized in control cells (A: NaCl and C: DMSO) after a 4 hour recovery incubation, as they are in untreated pancreas (not shown). The cytoplasm from the perinuclear region up to the apical plasma membrane is replete with zymogen granules. Treatment with chlorate during the recovery period caused the appearance of some larger labeled structures for both proteins (Fig. 8B; arrows). However, amylase and Muclin were still largely colocalized after chlorate treatment. By contrast, after treatment with BzlGalNAc during the recovery incubation, there are noticeable changes in amylase and Muclin distributions (Fig. 8D). Amylase labeling is in large clusters in the perinuclear region of the cells (Fig. 8D; arrowheads), corresponding to the vacuoles in Fig. 7J. On the other hand, Muclin is somewhat more diffusely localized in the cytoplasm apical to that of amylase (Fig. 8D; arrows). After BzlGalNAc treatment, amylase and Muclin are in separate compartments of the cells, with amylase remaining in the perinuclear vacuoles and Muclin progressing further towards that apical pole of the cell.

It was of interest to see if protein sorting from the TGN in general was perturbed by BzlGalNAc treatment. Therefore, the localization of the lysosomal marker protein LAMP-1 was compared with Muclin and amylase in treated tissue. Normal acinar cells have relatively few lysosomes that are located peripherally to the ZG-rich apical cytoplasm (Fig. 9A). Treatment with chlorate had no discernable alteration in LAMP-1 distribution (not shown). By contrast, BzlGalNAc-treated cells appeared to have more LAMP-1-positive structures that were somewhat larger (Fig. 9B,C). However, LAMP-1 was not seen to overlap with either Muclin (Fig. 9B) or amylase (Fig. 9C) in BzlGalNAc-treated cells. This is consistent with continued proper sorting of lysosomal proteins from the TGN in the presence of BzlGalNAc.

Discussion

Formation of regulated secretory granules involves protein sorting at the TGN and from the immature granule (Arvan and Castle, 1998). Two processes are implicated in granule

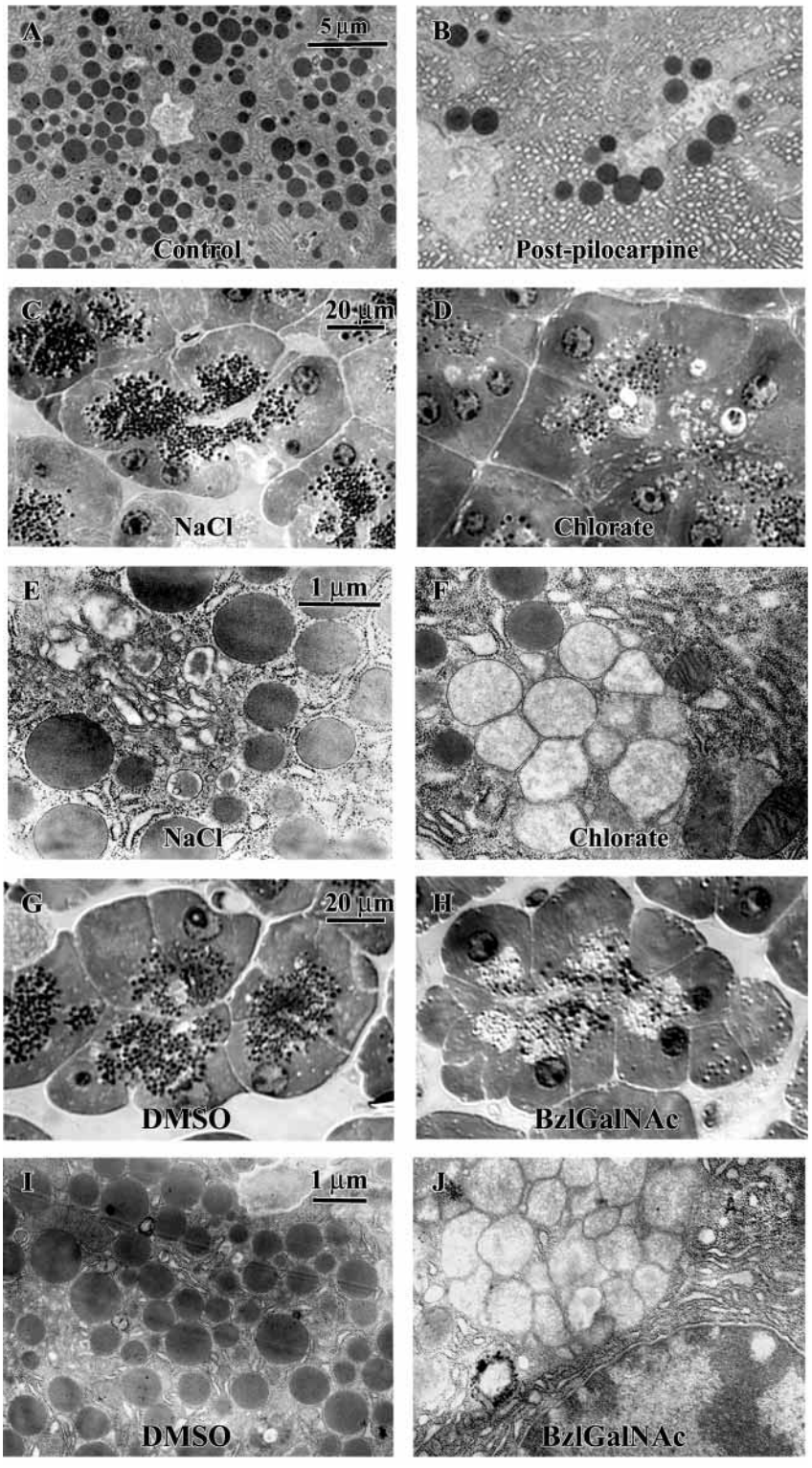

Fig. 7. In vivo zymogen granule depletion and morphological effects of chlorate and BzlGalNAc after in vitro zymogen granule repletion. Mice were injected with the cholinergic agonist pilocarpine (10 μg/g body weight). After 1 hour, the pancreas was removed, lobules were prepared and incubated in vitro for 4 hours in buffer supplemented with 30 mM NaCl, 30 mM sodium chlorate, 2% DMSO or 32 mM BzlGalNAc. The tissue was then prepared for light level and electron microscopy. (A) Untreated pancreas; (B) pancreas 1 hour after pilocarpine injection; (C,E) 30 mM NaCl; (D,F) 30 mM sodium chlorate; (G,I) 2% DMSO; (H,J) 32 mM BzlGalNAc. Note that the cells are generally healthy with intact plasma membranes and normal appearing nuclei under all conditions (C,D,G,H) and that they recover from the slight dilation of the endoplasmic reticulum that occurs after pilocarpine depletion of zymogen granules (B).

formation, content aggregation and association of the aggregates with the TGN membrane, but the relative importance of these processes is uncertain. Aggregation of regulated proteins is mediated by the mildly acidic pH of the TGN and regulated secretory granules, and this process appears to be universal among regulated secretory cells. This aggregation is likely to involve accessory proteins, notably sulfated macromolecules, which participate in the sorting events and are necessary for normal regulated granule formation. For example, knockout of the gene for N-deacetylase/N-sulphotransferase-2 (*NDST-2*), which is required for heparin sulfate synthesis, results in mast cells that are deficient in histamine and several proteases normally stored in their granules (Humphries et al., 1999; Forsberg et al., 1999). Another recent example is that downregulation of the sulfated glycoprotein chromogranin A with antisense RNA in neuroendocrine PC12 cells results in loss of dense-core regulated secretory granules accompanied by reduced storage of other granule proteins and loss of regulated secretion (Kim et al., 2001).

Understanding of the second process, association of the aggregates with TGN membranes, is less well established, but recent data show that there are cargo receptors that mediate association of the aggregating proteins with the TGN membrane. A strong case has been made that carboxypeptidase E is such a cargo receptor. In mice with defective carboxypeptidase E (Cpe^{fat}/Cpe^{fat}), redirection from the regulated pathway to the constitutive pathway occurs for several hormones and neuropeptides (Cool et al., 1997). However, whether this effect is due to loss of carboxypeptidase E itself or is secondary is not clear. These mice also have decreased expression of prohormone convertase enzymes, which are required for proteolytic cleavage of hormone precursors (Berman et al., 2001), and this processing affects hormone storage in regulated granules (Irminger et al., 1997).

The data presented in the current paper have significance for both of these processes and demonstrate that correct sulfation and O-linked oligosaccharide addition to glycoproteins in the pancreatic acinar cell are required for normal zymogen granule formation and regulated protein secretion. Inhibition of sulfation has a relatively mild effect on transit through the regulated secretory pathway in the acinar cell. The main effect is to slow delivery of newly made proteins to a regulated secretory compartment without blocking their subsequent stimulated release. Nevertheless, inhibition of sulfation causes the accumulation of vacuoles in the Golgi region of the cell, although normal zymogen granules still form. These observations indicate that when sulfation is blocked, the kinetics of granule formation are slowed but not totally blocked and that protein sorting to the regulated secretory pathway still occurs. Along with pro-Muclin, also participating in the condensation process are two sulfated digestive enzyme precursors, prolipase and proelastase.

In contrast to inhibition of sulfation by chlorate, BzlGalNAc, which blocks O-linked oligosaccharide elongation and only partially blocks sulfation, has more profound effects on the regulated secretory pathway. Not only do immature granules accumulate to a greater extent, but both unstimulated and regulated secretion of newly made protein is blocked. These effects are not caused by toxicity of BzlGalNAc for the following reasons: (i) protein synthesis is only mildly affected by BzlGalNAc; (ii) other than accumulation of immature secretory vacuoles, cellular morphology is healthy looking,

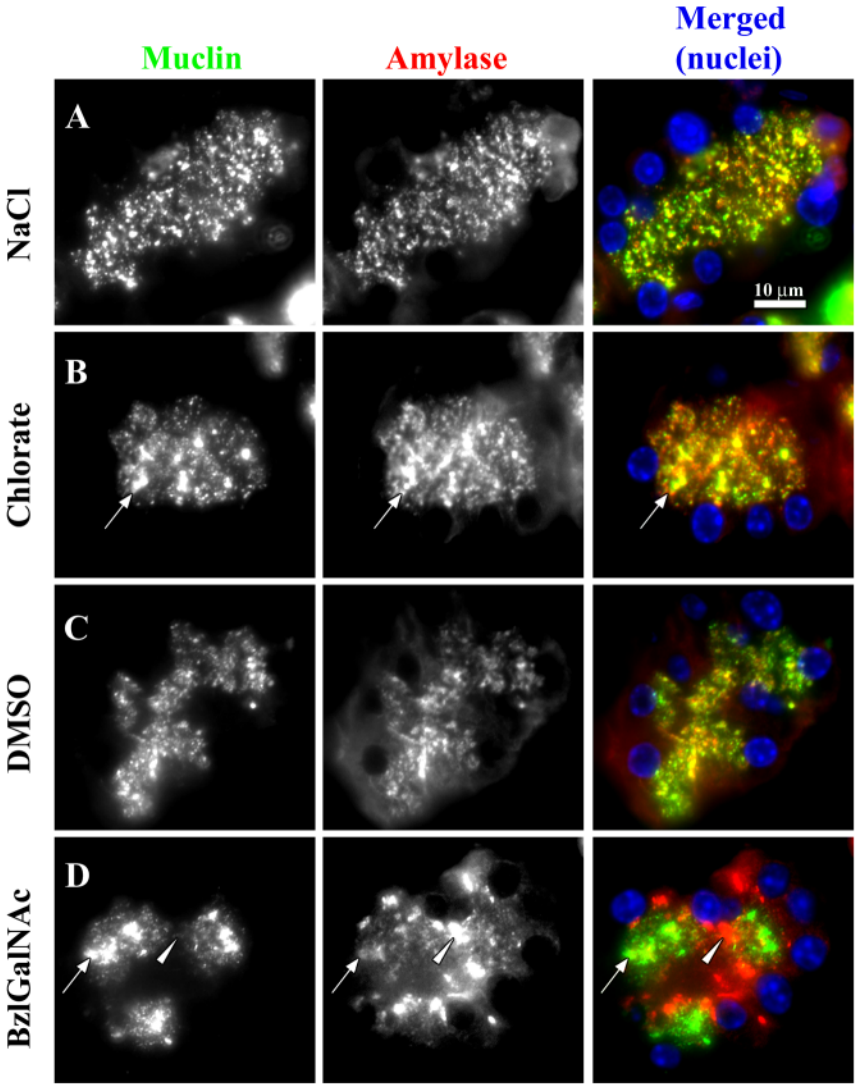

Fig. 8. Effects of chlorate and BzlGalNAc on immunolocalization of Muclin and amylase after in vitro zymogen granule repletion. Pancreatic lobules were prepared from zymogen-granule-depleted mice and incubated in vitro for 4 hours in buffer supplemented with (A) 30 mM NaCl, (B) 30 mM chlorate, (C) 2% DMSO or (D) 32 mM BzlGalNAc. Cryosections were prepared and dual immunostained for Muclin (green) and amylase (red) and for DNA with DAPI (blue). Note larger Muclin- and amylase-positive structures in chlorate-treated tissue (arrows in B) compared with controls; and dramatic dissociation of Muclin (arrows in D) and amylase (arrowheads in D) in BzlGalNAc-treated tissue.

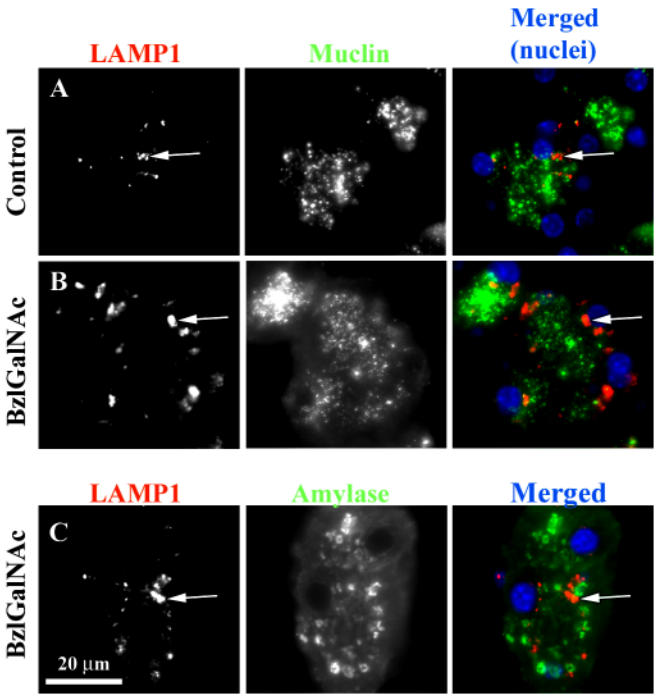

Fig. 9. Effect of BzlGalNAc on immunolocalization of LAMP-1, Muclin and amylase after in vitro zymogen granule repletion. Cryosections were prepared the same as in Fig. 8 and immunolabeled for the lysosomal membrane protein LAMP-1, Muclin and amylase. (A) In control tissue, there are sparse lysosomes (arrow) that are negative for Muclin. (B) BzlGalNAc-treated tissue has larger LAMP-1-positive structures (arrow) that are negative for Muclin. (C) BzlGalNAc-treated tissue has larger LAMP-1-positive structures (arrow) that are negative for amylase.

with intact plasma membranes and normal appearing nuclei; (iii) although there is a small increase in cell death, this is due to the vehicle (DMSO), and the small increase in cell damage is not sufficient to account for the profound effect on regulated secretion; and (iv) the stimulated secretion of prestored amylase, which is present in mature zymogen granules, from BzlGalNAc-treated cells is similar to controls. The effects of BzlGalNAc appear to be specific for protein transport in the regulated pathway, as lysosomal protein sorting is not perturbed by either chlorate or BzlGalNAc, as shown by immunolabeling for LAMP-1. In BzlGalNAc-treated tissue, there appeared to be larger organelles labeled for LAMP-1, but there was no colocalization of LAMP-1 with either Muclin or amylase.

At this time it is not possible to directly identify the key glycoprotein affected by BzlGalNAc, but we propose that these effects are caused by its perturbation of processing of pro-Muclin, which we have put forth as a putative cargo receptor in the acinar cell (De Lisle and Ziemer, 2000). Pro-Muclin is a type I membrane protein that is cleaved in a post-Golgi compartment, presumably the immature granule, to yield Muclin and the membrane protein p80 (De Lisle and Ziemer, 2000). Pro-Muclin acquires fixed negative charges via sulfation of its O-linked sugars in the TGN. Also in the TGN, the zymogens aggregate at the mildly acidic pH of this organelle and are likely to interact ionically with pro-Muclin's sulfates, as shown by the in vitro interaction of purified Muclin with isolated zymogens in a pH-dependent manner. This association is expected to facilitate packaging into immature secretory granules.

The effects of BzlGalNAc on post-translational processing of pro-Muclin are several and support the idea that pro-Muclin is a cargo receptor. First, BzlGalNAc inhibits sulfation by about 60%, and this may interfere with content aggregation and association of pro-Muclin with the content, similar to the chlorate effect. Second, cleavage of pro-Muclin is retarded by BzlGalNAc, and pro-Muclin persists longer than in control cells. Third, the eventual production of Muclin and p80 are both greatly reduced, indicating that incorrectly O-glycosylated pro-Muclin is degraded in the cell. Accompanying these biochemical changes, there is a dramatic dissociation of immunoreactive pro-Muclin/Muclin from amylase, which under normal conditions almost totally colocalize in the acinar cell. In addition, newly synthesized proteins fail to reach a stimulus-responsive compartment. Thus, incorrect post-translational processing of pro-Muclin is associated with a blockage of transport of proteins in the regulated pathway.

The mechanism of the BzlGalNAc blockage of the regulated pathway may be directly caused by loss of normally processed pro-Muclin. Others have provided evidence that lectin-like interactions of granule contents with carbohydrate-binding proteins may be important in granule formation and regulated secretion. In the mucin-secreting cell line HT29 MTX, treatment with BzlGalNAc interferes with mucin granule formation and also inhibits basal and stimulated secretion of mucins (Hennebicq-Reig et al., 1998). In addition, the cells accumulate numerous small vesicles containing brush-border-associated glycoproteins, which normally are delivered to the apical membrane (Huet et al., 1998). Because BzlGalNAc reduces glycoprotein sialylation (MAA binding) and increases terminal galactose (PNA binding), the authors of these studies suggest that sialic acid may play a key role in recognition of secretory proteins and their sorting to the apical membrane. These changes in PNA and MAA binding are also seen on Muclin in BzlGalNAc-treated acinar cells, consistent with the idea that terminal sugars on glycoproteins are important for protein sorting and granule formation in the secretory pathway. A similar proposal has been made for the lectin-like protein ZG16p, which associates with cholesterol-rich lipid rafts, the glycosylphosphatidyl-inositol-linked membrane protein GP-2 and sulfated proteoglycans in the rat acinar cell (Schmidt et al., 2001). The basic model put forth in both cases is that carbohydrates mediate association of secretory proteins with lectin-like membrane proteins of the TGN/immature granule, fostering granule formation and maturation. This model does not directly take into account the effect of the acidic pH of these organelles, but the lectin activity could easily be pH dependent, involving binding of protonated secretory proteins to the negatively charged sialic acid residues on the glycoproteins, which in turn are bound by the lectin-like membrane protein.

Additional effects of BzlGalNAc may be caused by accumulation of its metabolites in the secretory pathway. It has been shown that BzlGalNAc is modified by glycosyltransferases and complex structures such as NANA α(2-3)Gal β(1-3)BzlGalNAc are formed in BzlGalNAc-treated

cells (Delannoy et al., 1996; Huang et al., 1992). We observed [^{35}S]sulfate-labeled BzlGalNAc derivatives in treated acinar cells, which may accumulate to a significant degree in the TGN and distal secretory pathway. Such molecules are expected to have an osmotic effect, contributing to the dilute, watery state of vacuoles in the treated cells. In addition, the monovalent, sulfated BzlGalNAc derivatives may associate ionically with the aggregating mixed-charge secretory proteins. Normally, the secretory proteins are expected to interact with the polyvalent anionic sulfated glycoprotein pro-Muclin. Because the sulfated BzlGalNAc derivatives are monovalent they cannot assist in condensation. The possibility that BzlGalNAc might interfere with stimulus-secretion coupling in the acinar cell is disproved by the fact that BzlGalNAc does not affect stimulated release of prestored zymogens. Thus, its effects on secretion of newly synthesized proteins are likely to be specific to its inhibition of O-glycosylation and the possible osmotic effects on the TGN and immature granules.

In summary, there is convincing evidence that protein packaging in the regulated secretory pathway depends on electrostatic interactions driven by the charged substituents of macromolecules in the pathway and mediated by the acidic luminal pH of the TGN and post-Golgi compartments. There is also accumulating evidence that these processes involve lectin-like interactions with accessory proteins' terminal carbohydrate structures, possibly sialic acid.

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