Binding of the Golgi Sorting Receptor Mucln to Pancreatic Zymogens through Sulfated O-linked Oligosaccharides

Igor Boulatnikov and Robert C. De Lisle‡

From the Departments of Anatomy and Cell Biology, University of Kansas School of Medicine, Kansas City, Kansas 66160

Sorting and packaging of regulated secretory proteins involves protein aggregation in the trans-Golgi network and secretory granules. In this work, we characterized the pH-dependent interactions of pancreatic acinar cell-regulated secretory proteins (zymogens) with Mucln, a putative Golgi cargo receptor. In solution, purified Mucln co-aggregated with isolated zymogens at mildly acidic pH. In an overlay assay, \[^{[35S]}\text{sulfate biosynthetically labeled Mucln bound directly at mildly acidic pH to the zymogen granule content proteins amylase, pro-

lipase, pro-carboxypeptidase A1, pro-elastase II, chymotrypsinogen B, and Reg1. Denaturation of Mucln with reducing agents to break the numerous intrachain disulfide bonds in Mucln’s scavenger receptor cysteine-rich and CUB domains did not interfere with binding. Non-sulfated \[^{[35S]}\text{Methyl-

cysteine-labeled Mucln showed decreased binding in the overlay assay. Extensive Pronase E digestion of unlabeled Mucln was used to produce glycopeptides, which competed for binding of \[^{[35S]}\text{sulfate-labeled Mucln to zymogens. The results demonstrate that the sulfated, O-glycosylated groups are responsible for the pH-dependent interactions of Mucln with the zymogens. The behavior of Mucln fulfills the requirement of a Golgi cargo receptor to bind to regulated secretory proteins under the mildly acidic pH conditions that exist in the trans-Golgi network.

All eukaryotic cells synthesize and transport both membrane and soluble proteins through the endoplasmic reticulum and the Golgi complex to the cell surface, where they are delivered by unregulated exocytosis, a process called constitutive secretion (1). Some cells also have the capacity to store proteins in secretory granules, which are exocytosed upon neural or hormonal stimulation of the cell, and this is called the regulated secretory pathway (1). Sorting and packaging of proteins in the regulated pathway involves protein selection at the trans-Golgi network (TGN)\(^1\) (sorting-for-entry) as well as removal of residual lysosomal enzymes and constitutively secreted proteins during post-Golgi maturation of secretory granules (sorting-by-retention) (for review, see Ref. 2). The underlying process operating in the regulated secretory pathway is the aggregation of regulated proteins, which excludes constitutively secreted proteins.

Protein aggregation in the secretory pathway relies on a variety of mechanisms for interaction of regulated proteins. The most widespread mechanism is the pH-dependent aggregation of regulated proteins in the TGN, which has a pH of about 6.0 (3). To complete the process of granule formation and keep the content proteins aggregated, secretory granules are either mildly acidified (pancreatic zymogen granules, pH \(-6.0\) to \(-6.5\) (4)) or moderately acidified (neuroendocrine granules, pH \(-5.5\) to \(-5.0\) (5)). Regulated protein storage in some cells also relies on calcium, which is at millimolar concentrations in the secretory pathway compared with submicromolar levels in the cytosol (6).

Despite understanding these processes in a general way, the details of packaging of regulated secretory proteins are not well understood. The major protein of the pancreatic zymogen granule is amylase, and although this enzyme co-aggregates with other zymogens, it does not self-aggregate at mildly acidic pH (7, 8). Amylase was shown to associate with an SH3 binding domain of soluble rat zymogen granule protein ZG29p (9). There is also evidence that amylase can interact with N-glycosylated proteins (10). Whether either of these components exists in sufficient amounts in the secretory pathway to account for amylase sorting is unknown.

Sulfated proteoglycans and glycoproteins are also likely to be involved in protein packaging in zymogen granules (11–13), but the nature of these interactions is not known. We previously showed that zymogen granule formation in mouse acinar cells requires O-glycosylation as well as sulfation (11). When sulfation was inhibited, regulated protein secretion was not inhibited but newly formed granules were large and poorly condensed. When O-glycosylation was blocked, both regulated and constitutive secretion were strongly inhibited. These results suggest important roles for sulfated O-linked oligosaccharides in the secretory pathway. A candidate for mediating these interactions is pro-Mucln, a type I membrane protein that is the precursor to mature Mucln, the major O-glycosylated protein as well as the major sulfated macromolecule of the acinar cell (14). Because the inhibitors used in the previous studies target all sulfated and O-glycosylated proteins, they cannot show whether pro-Mucln is central to these processes. In the current work, we studied the interactions of purified Mucln with isolated zymogens and show that the sulfated, O-linked oligosaccharides of Mucln interact with purified zymogens in a pH-dependent manner.

EXPERIMENTAL PROCEDURES

Preparation of Pancreatic Acini and Radiolabeling of Mucln—Pancreatic acini were prepared from mouse pancreas (20–25 g male ND4 mice, Swiss Webster strain; Harlan, Indianapolis, IN) by digestion with purified collagenase (Worthington, Freehold, NJ) as described previously (11). To prepare \[^{[35S]}\text{sulfate-labeled Mucln, acini were suspended in HEPES-buffered Ringer’s solution and biosynthetically labeled for 2.5 h in the presence of 0.5 mCi/ml \[^{[35S]}\text{sulfate (ICN, Costa Mesa, CA). This paper is available online at http://www.jbc.org

Received for publication, June 6, 2004, and in revised form, July 22, 2004
Published, JBC Papers in Press, July 29, 2004, DOI 10.1074/jbc.M406213200

\(^1\) The abbreviations used are: TGN, trans-Golgi network; MES, 2-(N-morpholino)ethanesulfonic acid; ZGC, zymogen granule contents; PVDF, polyvinylidene difluoride; PNA, peanut agglutinin; CRP, cyclic AMP receptor protein; CUB, complement component C1r/C1s, Urchin EGF, and bone morphogenic protein-1.

\(^2\) This work was supported by National Institutes of Health Grant DK55998. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^3\) To whom correspondence should be addressed. Tel.: 913-588-2742; Fax: 913-588-2710; E-mail: rdelisle@kumc.edu.

\(^4\) The abbreviations used are: TGN, trans-Golgi network; MES, 2-(N-morpholino)ethanesulfonic acid; ZGC, zymogen granule contents; PVDF, polyvinylidene difluoride; PNA, peanut agglutinin; CRP, cyclic AMP receptor protein; CUB, complement component C1r/C1s, Urchin EGF, and bone morphogenic protein-1.
For other experiments, control and non-sulfated $^{35}$S/Met/Cys-labeled Muclin fractions were prepared. Acini were preincubated for 1 h in buffer supplemented with 30 mM sodium chloride (control) or 30 mM sodium chloride to deplete cellular pools of the high-energy sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (11, 15). In the continued presence of NaCl or chloride, the cells were incubated for 30 min in Met/Cys-free medium (Sigma) to deplete cellular pools of these amino acids. The excess unlabeled amino acids for 2 h to allow biosynthetic maturation of Muclin. 

**Purification of Muclin by Preparative SDS-PAGE**—Acini were solubilized with a probe-type sonicator in SDS-PAGE sample buffer (without reducing agent) and run on a PrepCell following the manufacturer’s instructions (model 491; Bio-Rad). Initial experiments showed that Muclin migrated to an $R_f$ of 0.5–0.6 on a 3% acrylamide/0.5% agarose separating gel, so this gel composition was used in the PrepCell to optimize resolution of Muclin from other cell proteins. Elution was performed by separating gel buffer, which was used in the outer chamber of the PrepCell, and allowed concentration of the collected fractions by lyophilization. After the bromphenol blue dye front was eluted, the gel run was continued, and 95 × 2.5 ml fractions were collected. Unlabeled Muclin in PrepCell fractions was detected by immuno-dot blot (16) with a Muclin-specific antiserum (14). $^{35}$S-labeled Muclin was detected in the fractions by liquid scintillation counting. The specific activity of $^{35}$S/Sulfate-labeled Muclin was approx. $10^8$ cpm/μg of fraction. Muclin-poor fractions were pooled, lyophilized, and dissolved in 150 mM NaCl, 10 mM MOPS, and 5 mM HEPES, pH 8.0, and then dialyzed against the same buffer for 48 h at 4 °C. Control and non-sulfated $^{35}$S/Met/Cys-labeled Muclin fractions were purified in the same manner. To prepare Muclin for aggregation assays, the buffer was exchanged to 0.1 M NH$_4$HCO$_3$, pH 8.0, and 0.02% NaN$_3$, and protein was concentrated by centrifugation in a 10-KDa cutoff Amicon Ultra-15 filter device (Fisher Scientific).

**Preparation of Zymogenic Granule Content and Muclin Interaction Assays**—Zymogen granules were isolated from mouse pancreas on a self-forming density gradient of 40% Percoll (Sigma) as described previously (17). Granules were osmotically lysed to release soluble content proteins using the cation exchange ionophore nigericin (27) followed by 30-min pulse-labeling with 1 μCi of $^{35}$S/Met/Cys (TransLabel; ICN, Costa Mesa, CA) and chasing in medium containing excess unlabeled amino acids for 2 h to allow biosynthetic maturation of Muclin. 

**RESULTS**

This work explores the ability of the putative Golgi cargo receptor pro-Muclin to bind to pancreaticzymogens in a pH-dependent manner. pro-Muclin is a type I membrane protein that has a signal peptide that directs the nascent protein to the rough endoplasmic reticulum lumen (20) (Fig. 1A). As the protein traffics through the secretory pathway, it acquires the Pro-rich domain (Fig. 1, B–D). Because pro-Muclin is a membrane protein and exists at relatively low levels in the acinar cell, we purified the more abundant mature Muclin for these studies. Muclin was shown experimentally to be heavily sulfated on O-linked oligosaccharides (14). Using the NetOglyc algorithm (Ref. 22; www.cbs.dtu.dk/services/NetOGlyc/) there are many predicted sites for O-glycosylation in mature Muclin (Fig. 1A). These sites include six so-called CRP domains (23), a Thr-rich domain, and a Thr-Pro-rich domain (Fig. 1, B–D).

Muclin was purified from isolated pancreatic acini on a preparative SDS-PAGE system (Bio-Rad PrepCell). Immunoreactive Muclin eluted off the preparative gel between fractions 54–74, as shown by dot-blot and Western blot (Fig. 2, A and B, respectively). Because of its unique size (~300 kDa) among acinar cell proteins, Muclin elutes as an essentially pure protein as shown by Coomassie blue staining of the PrepCell fractions (Fig. 2D). To prepare a sensitive probe for these studies, pancreatic acini were incubated with carrier-free $^{35}$S-sul-
As shown in Fig. 2C, [35S]sulfate-labeled Muclin eluted at the same position as unlabeled Muclin (Fig. 2A). Muclin comprises approximately one half of the incorporated label and is completely separated from other sulfated cell proteins that elute with the tracking dye front; the front was allowed to run off the gel before the fraction collector was started.

Purified [35S]sulfate-labeled Muclin was used in the pH-dependent in vitro aggregation assay previously used with unlabeled Muclin (11). This entails mixing purified ZGC at pH 8.0 or 6.0, followed by pelleting the large aggregates in a microcentrifuge. Labeled Muclin efficiently co-aggregates with purified ZGC at pH 6.0 but not at pH 8.0 (Fig. 3). As a negative control, [35S]sulfate-labeled Muclin in the absence of ZGC does not self-aggregate at mildly acidic pH (Fig. 3). By mass spectrometry of trypsin-digested proteins and N-terminal sequence analysis, the six binding partners for Muclin were identified: the digestive enzymes amylase, pro-lipase, pro-carboxypeptidase A1, pro-elastase II, and chymotrypsinogen B and a pancreatic stress protein called Reg1 (24) (Fig. 4B). The pH-dependent binding was highly reproducible, and the increased binding at pH 6.0 compared with pH 8.0 ranged from about 2.5-fold for...
Muclin was also overlaid at pH 6.0 and 8.0 on PVDF membranes containing whole pancreatic or liver homogenates, or just bovine serum albumin. Radioactivity in the pellets is shown as a percentage of the total input of radioactive 

The data are means of two experiments and the bars represent one half of the range; data without error bars are from a single representative experiment. Between 1 and 5 × 10^4 cpm were used per sample. Labeled Muclin co-aggregates in the presence of ZGC at pH 6.0 but not by itself or with ovalbumin or serum albumin.

As an independent assay of pH-dependent interactions of Muclin with ZGC, Muclin was immobilized to agarose beads to use as a solid phase that could be separated from unbound zymogens at low centrifugal force. We took advantage of the fact that Muclin can be quantitatively bound to the lectin PNA (11), and we cross-linked Muclin to PNA agarose beads. When incubated with ZGC, a similar pattern of zymogens bound to agarose bead-immobilized Muclin was observed compared with the aggregation assay (Fig. 7). Binding to bead-immobilized Muclin was also pH-dependent with increased binding at pH 6.0 over pH 8.0 of amylase (1.5-fold), pro-carboxypeptidase A1 (3-fold), pro-elastase II (2-fold), and Reg1 (2-fold). Unlike the aggregation assay, there was not appreciably more binding of pro-lipase or chymotrypsinogen B to bead-immobilized Muclin (Fig. 7). The patterns of ZGC that aggregate at acidic pH and pro-lipase or chymotrypsinogen B to bead-immobilized Muclin were also observed in the overlay assay, except that one band of about 74 kDa bound in the overlay assay (Fig. 4A) and to bead-immobilized Muclin (Fig. 7). Binding to bead-immobilized Muclin but does not show pH-dependent aggregation (Fig. 7). Binding to unconjugated agarose beads was minimal and also shows that insignificant amounts of ZGC were pelleted at the low centrifugal force used to wash the beads (Fig. 7).

To test whether the oligosaccharides on Muclin mediate its

Muclin is composed of scavenger receptor cysteine-rich domains interspersed with CRP domains, and CUB domains at its carboxyl terminus (Fig. 1A). The scavenger receptor cysteine-rich and CUB domains are globular structures with multiple intrachain disulfide bonds in each domain (25, 26). The presence of intrachain disulfide bonds in Muclin was previously demonstrated by its decreased mobility on SDS-PAGE after treatment with a reducing agent (14). To test whether the acidic pH-dependent binding of Muclin to zymogens is sensitive to the protein conformation, we performed the overlay assay in the presence of a reducing agent (Tris(2-carboxyethyl)phosphine) to disrupt the numerous disulfide bonds. Reduction of Muclin had no effect on binding to ZGC proteins (Fig. 6), indicating that the structure of the peptide is unimportant in binding of Muclin to ZGC proteins. This result is consistent with the proposal that the carbohydrate portion of Muclin is responsible for its association with ZGC proteins (11, 20).

As controls for the overlay assay, [35S]sulfate-labeled Muclin was also overlaid at pH 8.0 and 6.0 on PVDF membranes containing whole pancreatic or liver homogenates, or just bovine serum albumin. When overlaid on whole pancreatic homogenates, the predominant bands at pH 6.0 were the zymogens identified above (Fig. 5A). Additional bands were observed at about 100, 42, 26–27, and ≤20 kDa (Fig. 5A). There was no binding at either pH 8.0 or 6.0 when a large amount (5 μg) of bovine serum albumin was separated and transferred to PVDF (Fig. 5B). On total liver homogenates, there were only two weakly binding bands at about 116 and 30 kDa (Fig. 5B). These results show that Muclin binding in the overlay assay is fairly specific for pancreatic proteins, especially those stored in zymogen granules.
binding to ZGC proteins, purified [35S]sulfate-labeled Muclin was reduced and extensively digested with the broad-specificity protease mixture Pronase E to prepare labeled glycopeptides (18). As shown in Fig. 8A, after digestion of labeled Muclin, no bands were visible by Coomassie blue staining on either 7.5 or 15% acrylamide gels, demonstrating the effective degradation of the protein. Nevertheless, there was a peak of 35S radioactivity and a band of about 21 kDa on the gel by filmless autoradiographic analysis (Fig. 8A). Lack of Coomassie blue staining of the glycopeptides may be a result of their high carbohydrate content. The 35S-labeled glycopeptides were separated from small digestion products by gel filtration (Fig. 8B). Amino acid analysis of the isolated glycopeptides was performed and compared with the predicted O-glycosylated domains of Muclin. Because it is not known exactly where the Pronase mixture would cleave the peptide backbone and how many amino acids near O-glycosylated sites would be protected from the proteases, we evaluated several possibilities: the entire domains shown in Fig. 1A would be Pronase-resistant compared with only 1, 2, or 3 amino acids flanking each predicted O-glycosylated Ser and Thr being protected. The best fit to the measured amino acid composition occurred when it was modeled that the three amino acids flanking these sites would be protected from protease digestion (Fig. 8C).

The ability of the [35S]sulfate-labeled, Muclin-derived glycopeptides to co-aggregate with ZGC was tested and there was significant co-aggregation at pH 6.0 compared with pH 8.0 (Fig. 8D). The [35S]sulfate-labeled glycopeptides also bound to ZGC in the overlay assay at pH 6.0 (Fig. 8E), but the amount bound was reduced compared with intact labeled Muclin (Fig. 4A). A possible explanation for weaker pH-dependent binding of glycopeptides may be that the several sulfated, O-glycosylated domains in an intact Mucin molecule exhibit cooperativity that increases the affinity of binding. To test this in another way, glycopeptides were prepared from unlabeled Mucin and used as a competitor for [35S]sulfate-labeled intact Mucin in the overlay assay. When 100-fold excess unlabeled glycopeptides were added to [35S]sulfate-labeled Mucin in the overlay assay, binding at pH 6.0 was abolished (Fig. 9B) compared with control (Fig. 9A).

As a more direct test of whether the sulfate groups are important for association of Mucin with zymogens, [35S]Met-Cys-labeled control and non-sulfated Mucin were prepared. Non-sulfated Mucin was prepared by radiolabeling acini in the presence of sodium chlorate, which is a competitive inhibitor of ATP-sulfurylase, a key enzyme in biosynthesis of the high-energy sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (15). Using the overlay assay, binding of non-sulfated Mucin at pH 6.0 was reduced 40–60% for the different zymogens compared with control sulfated Mucin (Fig. 10). Thus, the sulfated, O-glycosylated domains of Mucin are at least partly responsible for its pH-dependent association with ZGC proteins.

**DISCUSSION**

In this work, we explored the interactions of Mucin with isolated pancreatic zymogens to test the proposal that pro-Mucin serves as a Golgi cargo receptor in the regulated secretary pathway of the pancreatic acinar cell. Three different assays were used to investigate the pH-dependent interactions of Mucin with pancreatic zymogen granule contents: a solution phase assay, in which both both Mucin and ZGC were used as soluble proteins; an overlay of Mucin on ZGC immobilized to PVDF membranes; and binding of soluble ZGC to Mucin immobilized to PNA-agarose beads. There were some minor differences in the ZGC proteins that associated with Mucin in the different assays; overall, however, the patterns of bound proteins were similar. In addition, Mucin bound only weakly to two proteins in total liver homogenates, showing Mucin's specificity for binding to pancreatic proteins. When non-sulfated Mucin was compared with control sulfated Mucin in the overlay assay of ZGC, there was a reduction of 40–60% compared with sulfated Mucin, demonstrating that the majority of pH-dependent Mucin binding to zymogens involves its sulfate groups. The remaining binding may be mediated by sialic acid residues, which are also present on Mucin (14). The results demonstrate that the sulfated O-glycosylated domains of Mucin are responsible for Mucin's ability to bind to several of the protein species in the zymogen granule content in a pH-dependent manner. These data support the idea that pro-Mucin is a Golgi cargo receptor.

In the pancreatic acinar cell, regulated secretory protein concentration begins in the rough endoplasmic reticulum (27),
and small aggregates of regulated secretory proteins exist at pH 7.5 (28). Protein concentration continues in the TGN and secretory granule. The TGN process can be mimicked in vitro by mildly acidifying isolated zymogens to match the pH of the TGN lumen (3), and ZGC aggregates become large enough to pellet in a microcentrifuge at pH 6 or lower (29). The major regulated protein in the pancreatic acinar cell is amylase, which comprises 20 to 30% of the total zymogen granule protein (30). Several zymogens, including amylase, do not undergo individual homotypic aggregation at mildly acidic pH, and they aggregate only when mixed together (7). The minimal protein composition required for zymogen aggregation at acidic pH is unknown. Also unknown is how the aggregated proteins are targeted to a patch of TGN membrane to form the secretory granule and whether this process requires a membrane protein that serves as a cargo receptor for the regulated secretory pathway at the TGN.

A possible mechanism for inclusion of amylase with other sorted proteins is that amylase can bind to N-linked glycoproteins in a pH- and Ca2+/H+ dependent manner (10). Although this association could explain amylase's ability to be sorted, identification of specific N-glycoprotein binding partners in the granule has not been made. It has also been shown that amylase interacts with the soluble rat zymogen granule protein ZG29p (9). This interaction occurs through an SH3 binding domain on ZG29p, and it was suggested that ZG29p is an accessory protein in aggregation of proteins in the acinar cell-regulated pathway. In neither of these examples has it been shown how the aggregates are linked to the TGN membrane.

It has been convincingly demonstrated in endoplasmic reticulum to Golgi traffic that the p24 family of membrane proteins acts as cargo receptors (for review, see Ref. 31]. The p24 proteins bind content proteins in the endoplasmic reticulum lumen and recruit coat-forming proteins to the cytosolic face of the membrane, which results in vesicle formation. For regulated secretory granule formation in neuroendocrine cells, the prohormone processing enzyme carboxypeptidase E (CPE) has been shown to act as a cargo receptor (32). CPE is a membrane protein that associates with the cargo pro-opiomelanocortin (POMC) through a pair of acidic residues and a pair of hydrophobic residues (33). In the Cpe(fat)/Cpe(fat) mouse, which has a mutated CPE gene, granule formation is impaired and regulated proteins are constitutively released (34, 35).

It is unknown whether a membrane protein cargo receptor exists in the exocrine pancreas. Several models for ZGC aggregation and sorting have been advanced. It was proposed that a submembrane proteoglycan matrix in the rat granule mediates association of zymogens with the membrane (13), but it remains to be shown how the proteoglycans associate with the membrane. There is also recent evidence for association of granule membrane components with lipid rafts that may serve as a sorting mechanism at the TGN (36), but the protein composition of such lipid rafts is not well defined at this point, and a specific membrane protein that serves as a cargo receptor has not been identified. An alternative proposal is that a membrane protein cargo receptor is unnecessary because zymogens may...
**Fig. 8.** Preparation of [35S]sulfate-glycopeptides from Muclin and use in the overlay assay. [35S]sulfate-labeled Muclin was reduced and extensively digested with Pronase E. A, intact and Pronase-digested Muclin were run on 7.5 and 15% acrylamide gels and were stained with Coomassie blue and subjected to filmless autoradiographic analysis (Phosphorimage). Pronase digestion almost completely degrades Muclin and leaves a 21-kDa glycopeptide fraction that does not stain well with Coomassie blue. B, the digest was separated on gel filtration and radioactivity in the fractions determined. C, unlabeled Muclin was Pronase E-digested, and the glycopeptides were isolated by gel filtration and submitted for amino acid analysis. The graph shows the experimentally determined amino acid composition compared with that expected if the predicted O-glycosylated Ser and Thr sites and their flanking 3 amino acids were protected from Pronase digestion (Fig. 1). D, [35S]sulfate-labeled Muclin-derived glycopeptides (input, 5 × 10⁶ cpm/sample) co-aggregate with ZGC at mildly acidic pH. E, [35S]sulfate-labeled glycopeptides (input, 7.5 × 10⁶ cpm/strip) bind to ZGC at acidic pH in the overlay assay.
Muclin Binding to Zymogens

A. Control
B. Cold Glycopeptides

Fig. 9. Unlabeled glycopeptides compete for intact [35S]sulfate-labeled Muclin binding to ZGC in the overlay assay. A, control overlay assay. B, a ~100-fold molar excess of unlabeled Muclin glycopeptides was added to the [35S]sulfate-labeled intact Muclin (3 × 10^6 cpm/strip) in the overlay assay. Unlabeled Muclin-derived glycopeptides completely block 35S-labeled Muclin binding to the ZGC.

Fig. 10. Comparison of binding of sulfated and non-sulfated [35S]Met/Cys-labeled Muclin to ZGC in the overlay assay. Muclin was [35S]Met/Cys labeled biosynthetically in the absence (control) and presence of 30 mM sodium chlorate to inhibit sulfation, and purified on a preparative gel filtration column. Equal amounts of radioactive control and non-sulfated Muclin were used in the overlay assay to assess pH-dependent binding to ZGC. The data are from a representative experiment and are presented as the ratio of binding of non-sulfated Muclin compared with sulfated Muclin at pH 6.0.

bind directly to lipid membrane (37). A drawback to this idea is that it does not explain how needed membrane proteins, such as soluble N-ethylmaleimide-sensitive factor attachment protein receptors and rab GTPases, would be selected for inclusion in the granule.

Much of the work done on the problem of ZGC aggregation and sorting has focused on the mechanism of aggregation and identification of soluble proteins that may assist the aggregation process. A role for sulfated macromolecules in zymogen granule formation is attractive because their fixed negative charge makes them good candidates for the pH-dependent sorting/aggregation process. There seem to be species differences in that guinea pigs and rats have sulfated glycosaminoglycans as components of their zymogen granules (13, 38), whereas mice have mostly sulfated O-glycans (14). Inhibition of glycosaminoglycan synthesis in rat acinar cells with p-nitrophenyl-β-D-xylopyranoside inhibits sorting of the proteoglycan serglycin as well as amylase and procarboxypeptidase A to granules (12). We have shown in the mouse pancreas that inhibition of sulfation with sodium chlorate or O-glycosylation with benzyl-N-acetyl-α-galactosaminide inhibits normal zymogen granule formation (11).

The best candidate sulfated macromolecule in the mouse pancreas is Muclin, the major sulfated, O-glycosylated protein of the mouse pancreas. Muclin is a major acinar cell protein, comprising approximately 2% of the total pancreatic protein and 6–8% of the zymogen granule protein (39). Muclin is derived from the type I membrane protein pro-Muclin by proteolytic cleavage in a post-Golgi compartment (immature secretary granule) (20). Our model is that pro-Muclin becomes heavily sulfated when it reaches the TGN, where it will associate with the aggregating granule content proteins in the acidic environment of the TGN lumen. Because it is a membrane protein at the TGN, pro-Muclin binding to zymogens will also link them to the TGN membrane. This is important because it will allow the regulated protein aggregate to be associated with a patch of membrane that contains the needed proteins to form a functional regulated secretory granule (e.g. soluble N-ethylmaleimide-sensitive factor attachment protein receptors, rabs, etc.).

The presence of sulfated O-linked oligosaccharides along the length of Muclin provides numerous sites for pH-dependent electrostatic interactions with aggregating zymogens. Muclin’s sulfates will be negatively charged at any physiological pH, whereas the zymogens will be protonated at the acidic pH of the TGN/secretory granule and will lose their charge upon exocytosis into the alkaline environment of the acinar lumen allowing solubilization and release of the zymogens (40).

It remains to be shown whether the 16-amino acid cytosolic carboxyl tail of pro-Muclin can recruit coat proteins or somehow induce formation of an immature zymogen granule. Some previous data indicate that the cytosolic tail is important for trafficking of pro-Muclin to zymogen-like granules in the pancreatic cell line AR42J (20). Other recent data using fusion peptides of the cytosolic tail with a TAT-protein transduction domain to attempt to interfere with the function of the endogenous cytosolic domain had no effect on granule formation or regulated protein trafficking (21).

The work presented here shows that Muclin satisfies one requirement of a Golgi cargo receptor, which is to interact with cargo (zymogens) under conditions that exist in the TGN. It remains to be determined whether pro-Muclin satisfies the second requirement of a cargo receptor, which is the ability to recruit coat-forming proteins to induce granule formation.

REFERENCES
1. Kelly, R. B. (1985) Science 230, 25–32
2. Arvan, P., Zhang, B. Y., Feng, L. J., Liu, M., and Kuliawat, R. (2002) Curr. Opin. Cell Biol. 14, 448–453
3. Orci, L., Ravazzola, M., and Anderson, R. G. W. (1987) Nature 326, 77–79
4. Gasser, K. W., DiDomenico, J., and Hopfer, U. (1988) Am. J. Physiol. 255, C795–C811
5. Anderson, R. G. W., and Orci, L. (1988) J. Cell Biol. 106, 539–543
6. Colomer, V., Kieska, G. A., and Rindler, M. J. (1996) J. Biol. Chem. 271, 48–55
7. Gerr, S. U., and Tseng, S. Y. (1985) Biochem. Biophys. Res. Commun. 215, 82–88
8. Jacob, M., Laine, J., and Lebel, D. (1992) Biochem. Cell Biol. 70, 1105–1114
9. Kleene, R., Classen, B., Zdanieblo, J., and Schrader, M. (2000) Biochemistry 39, 9863–9866
10. Matsushita, H., Takenaka, M., and Ogawa, H. (2002) J. Biol. Chem. 277, 4680–4686
11. De Lisle, R. C. (2002) J. Cell Sci. 115, 2941–2952
12. Biederbick, A., Licht, A., and Kleene, R. (2003) Eur. J. Cell Biol. 82, 19–29
13. Schmidt, K., Dartsch, H., Linder, D., Kern, H. F., and Kleene, R. (2000) J. Cell Sci. 113, 2335–2342
14. De Lisle, R. C. (1994) J. Cell Biochem. 56, 385–396
15. Baueuerle, P. A., and Huttner, W. B. (1986) Biochem. Biophys. Res. Comm. 141, 870–877
16. De Lisle, R. C. (1991) Anal. Biochem. 192, 1–5
17. De Lisle, R. C., Schulz, I., Tyarakowski, T., Haase, W., and Hopfer, U. (1984) Am. J. Physiol. 246, G411–G418
18. Cummings, R. D. (1993) in Glycobiology: A Practical Approach (Fukuda, M., and Kobata, A., eds.), IRL Press, New York
19. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
20. De Lisle, R. C., and Ziemer, D. (2000) Eur. J. Cell Biol. 83, 892–904
21. Tandon, C., and De Lisle, R. C. (2004) Eur. J. Cell Biol. 83, 79–89
22. Hansen, J. E., Lund, O., Tolstrup, N., Gooley, A. A., Williams, K. L., and Brunak, S. (1998) Glycoconjugate J. 15, 115–130
23. Cheng, H., Bierknes, M., and Chen, H. Y. (1996) Anat. Rec. 244, 327–343
24. Bimmler, D., Schiesser, M., Perren, A., Scheele, G., Angst, E., Meili, S., Ammann, R., and Graf, R. (2004) J. Surg. Res. 118, 122–135
25. Hohenester, E., Sasaki, T., and Timpl, R. (1999) Nat. Struct. Biol. 6, 228–232
26. Feinberg, H., Udinhaa, J. C. M., Davies, J. M., Wallis, R., Drickamer, K., and Wes, W. J. (2003) EMBO J. 22, 2348–2359
27. Oprins, A., Rahouille, C., Pesthuma, G., Klamperman, J., Geuze, H. J., and Slot, J. W. (2001) Traffic. 2, 831–838
28. Kleene, R., Kastner, B., Rosser, R., and Kern, H. (1999) Digestion 60, 305–313
29. Dartsch, H., Kleene, R., and Kern, H. F. (1998) Eur. J. Cell Biol. 75, 211–222
30. Scheele, G. A. (1975) J. Biol. Chem. 250, 5375–5385
31. Barlowe, C. (2003) Trends Cell Biol. 13, 295–300
32. Loh, Y. P., Kim, T., Rodriguez, Y. M., and Cawley, N. X. (2004) J. Mol. Neurosci. 22, 63–71
33. Loh, Y. P., Maldonado, A., Zhang, C., Tam, W. H., and Cawley, N. (2002) Ann. N. Y. Acad. Sci. 971, 416–425
34. Cool, D. R., Normant, E., Shen, F. S., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Cell 88, 73–83
35. Cawley, N. X., Rodriguez, Y. M., Maldonado, A., and Loh, Y. P. (2003) Endocrinology 144, 292–298
36. Kalus, I., Hodel, A., Koch, A., Kleene, R., Edwardson, J. M., and Schrader, M. (2002) Biochem. J. 362, 435–442
37. Laine, J., and LeBel, D. (1999) Biochem. J. 338 (Pt 2), 289–294
38. Reggio, H. A., and Palade, G. E. (1978) J. Cell Biol. 77, 288–314
39. De Lisle, R. C., Petitt, M., Huff, J., Isom, K. S., and Agbas, A. (1997) Gastroenterology 113, 521–532
40. Freedman, S. D., and Scheele, G. A. (1993) Biochem. Biophys. Res. Commun. 197, 992–999
Binding of the Golgi Sorting Receptor Muclin to Pancreatic Zymogens through Sulfated O-linked Oligosaccharides
Igor Boulatnikov and Robert C. De Lisle

J. Biol. Chem. 2004, 279:40918-40926.
doi: 10.1074/jbc.M406213200 originally published online July 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406213200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 10 of which can be accessed free at http://www.jbc.org/content/279/39/40918.full.html#ref-list-1