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Characterization of the Major Sulfated Protein of Mouse Pancreatic Acinar Cells: A High Molecular Weight Peripheral Membrane Glycoprotein of Zymogen Granules

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Abstract The major sulfated protein of the mouse pancreatic acinar cell, gp300, has been identified and characterized with monoclonal and polyclonal antibodies. gp300 is a glycoprotein of M_r = 300,000 which contains ~40% of metabolically incorporated [^{35}S]sulfate in the acinar cell. Sulfate on gp300 is resistant to hot 1N HCl, but sensitive to alkaline hydrolysis, demonstrating that the sulfate is carbohydrate-linked rather than tyrosine-linked. gp300 metabolically labeled with [^{3}H]glucosamine and [^{35}S]sulfate was chemically and enzymatically treated followed by Bio-Gel P-10 gel filtration. Both labels were resistant to treatments which degrade glycosaminoglycans. Treatment of dual-labeled gp300 with PNGase F to cleave N-linked oligosaccharides released ~17% of [^{3}H] and little [^{35}S]. Mild alkaline borohydride treatment after removal of N-linked sugar released the remainder of both labels, indicating the presence of sulfated O-linked oligosaccharides. Biosynthetic studies and PNGase F digestion indicate that the core protein is ~210 kDa, with apparent contributions of ~35 kDa N-linked sugar, and ~55 kDa O-linked sugar. Lectin blotting and glycosidase digestion demonstrated the presence of Galβ(1–3)GalNAc and sialic acid α(2–3)Gal in O-linked oligosaccharide, and Galβ(1–4)GlcNAc in N-linked oligosaccharide. Immunolocalization and subcellular fractionation showed that gp300 is a peripheral membrane protein localized to the lumenal face of the zymogen granule membrane. gp300 was not secreted in response to hormone stimulation of acini, so it is not a secretory product. Immunoblot analysis showed that gp300 is present in other gastrointestinal tissues and parotid glands. Localization of this nonsecreted sulfated glycoprotein to exocrine secretory granule membranes suggests that gp300 may have a role in granule biogenesis.

Key words: antibody, gastrointestinal tract, N-linked oligosaccharide, O-linked oligosaccharide, peripheral membrane protein, secretory granule biogenesis

Sulfated proteins are common constituents of secretory granules. Most of these sulfated proteins are proteoglycans, which have been termed "intracellular" proteoglycans [Kjellén and Lindahl, 1991]. Secretory granule proteoglycans are found in hemopoietic cells [Avraham et al., 1989; Bourdon et al., 1987; Kjellén et al., 1989], in acinar cells of the guinea pig pancreas [Reggio and Palade, 1978], and in rat salivary gland acinar cells [Blair et al., 1991]. A common feature of sulfated molecules so far described in secretory granules is that they are secreted by exocytosis from the granules. Various functions for sulfated macromolecules have been proposed. In exocrine cells, sulfated secretory granule proteins (proteoglycans) have been proposed to function in granule formation and maturation [Reggio and Dagorn, 1978; Reggio and Palade, 1978; Tartakoff et al., 1974]. In rat parotid glands, chronic isoproterenol administration results in increased expression of proline-rich basic secretory proteins, and there is a

Abbreviations used: DSA, Datura stramonium agglutinin; GAG, glycosaminoglycan; GNA, Galanthus nivaus agglutinin; Hepes, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; HR, Hepes-buffered Ringer's solution; MAA, Maackia amurneisis agglutinin; mAb, monoclonal antibody; PNA, peanut agglutinin; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; SNA, Sambucus nigra agglutinin.

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concomitant increase in chondroitin sulfate proteoglycan synthesis, suggesting the necessity for achieving a charge balance between secretory proteins and sulfated macromolecules in the granule content for proper granule formation [Blair et al., 1991].

In this report, the major sulfated protein of the mouse pancreatic acinar cell, gp300, is described and biochemically characterized using monoclonal (mAb) and polyclonal antibodies. By contrast with previously described sulfated granule components, gp300 is a high molecular weight glycoprotein rather than a proteoglycan. Additionally, gp300 is a peripheral membrane protein that is tightly associated with the granule membrane and is not released during exocytosis. The biochemical features, behavior, and subcellular localization of gp300 make it a candidate to have a role in secretory granule biogenesis in exocrine cells of the pancreas.

METHODS

Zymogen Granule Preparation and Subfractionation

Highly purified zymogen granules were prepared on a self-forming Percoll gradient as described [De Lisle and Hopfer, 1986]. Zymogen granules were osmotically lysed in 150 mM sodium acetate (pH 7.0), 0.1 mM phenylmethyl sulfonyl fluoride, with 27 μg/ml nigericin at 37°C for 15 min [De Lisle and Hopfer, 1986]. Granule membranes and content were separated by centrifugation at 100,000*g* × 60 min onto a 1 M sucrose cushion in an SW41 rotor.

Antibody Production

Monoclonal antibody (mAb) secreting hybridomas were generated from rat spleen cells and SP2/0 cells as described [De Lisle et al., 1988]. Rats were immunized i.p. with a subcellular fraction from mouse pancreas enriched for plasma membrane markers [De Lisle, 1991] solubilized in 1% Triton X-100. Hybridoma culture supernatants were screened by indirect immunofluorescence on 10 μm cryosections of 4% paraformaldehyde fixed mouse pancreas and subsequently characterized by Western blot using various subcellular fractions from pancreas. The hybridoma used in this study and the mAb produced are designated 4.15A12; the mAb is a rat IgG_{2a} antibody. The polyclonal antiserum to gp300 was generated to SDS-PAGE purified gp300. Zymogen granule membranes were prepared from the pancreata of 30 mice, separated on 7.5% SDS-PAGE, and the Coomassie stained gel slices were used to immunize rabbits using a commercial service (HRP, Inc., Denver, PA).

Gel Electrophoresis and Western Blotting

Protein samples were separated on reducing SDS-PAGE according to Laemmli [1970]. Samples were solubilized in SDS-PAGE sample buffer by sonication at room temperature with a probe type sonicator (Heat Systems, Farmingdale, NY) or by incubation at 37°C for 30 min. Molecular weight standards were from Bio-Rad (Richmond, CA) and unreduced human secretory IgA from colostrum (Sigma, St. Louis, MO) was used as a 390 kDa marker [Benacerraf and Unanue, 1979].

For immunoblotting, proteins resolved on SDS-PAGE were transferred to nitrocellulose by the method of Towbin et al. [1979] with the inclusion of 0.1% SDS in the transfer buffer for higher efficiency of transfer [Wang et al., 1989]. Immunoblotting was performed as described [De Lisle et al., 1988]. mAb 4.15A12 was diluted 1:1 in blocking solution and detected with a donkey anti-rat alkaline phosphatase secondary antibody (Jackson Immunoresearch Labs., Inc., West Grove, PA). The polyclonal antiserum to gp300 was diluted 1:5,000 in blocking solution and detected with a donkey anti-rabbit alkaline phosphatase secondary antibody (Jackson Immunoresearch Labs., Inc., West Grove, PA).

To separate integral membrane proteins from hydrophilic proteins, Triton X-114 solubilization and phase-separation were used according to Bordier [1981]. For analysis by electrophoresis, protein in the aqueous phase was trichloroacetic acid precipitated in the presence of deoxycholate as carrier.

Carbohydrate Analysis by Lectin Blot

Carbohydrate on gp300 was analyzed using Glycan Detection and Glycan Differentiation kits from Boehringer Mannheim (Indianapolis, IN), used according to the manufacturer's instructions [Haselbeck and Hosel, 1990] to probe Western blots of zymogen granule membranes. Glycosidase treatments and lectin blots of the nitrocellulose were performed with an Immunetics Miniblotter (Immunetics, Cambridge, MA) similar to that described [Horst et al., 1990]. The lectins used were Galanthus nivalus agglutinin (GNA), Sambucus nigra agglutinin (SNA), Maackia amurneisis agglutinin (MAA), peanut

agglutinin (PNA), and Datura stramonium agglutinin (DSA), and the enzymes were O-glycosidase (6 mU/ml; endo-α-N-galactosaminidase, EC 3.2.1.97; Boehringer Mannheim, Indianapolis, IN; or Oxford Glycosystems, Inc., Rosedale, NY), neuraminidase (0.2 U/ml; Clostridium perfringens, EC 3.2.1.18; Oxford Glycosystems, Inc., Rosedale, NY), and PNGase F (8 U/ml; peptide N-glycosidase F, EC 3.2.2.18; Boehringer Mannheim, Indianapolis, IN) using buffers recommended by the suppliers. All enzyme digestions were overnight at 37°C followed by extensive washing and then lectin blotting.

Metabolic Labeling and Characterization of Sulfated Proteins of Pancreatic Acini

Pancreatic acini were prepared from mouse pancreas by collagenase digestion as described [De Lisle and Logsdon, 1990]. To label sulfated molecules, acini were incubated for 1–2 h in Hepes-buffered Ringer's (HR) supplemented with 0.1–0.5 mCi/ml carrier free [^{35}S]sulfate (New England Nuclear, Boston, MA). Proteins were separated by SDS-PAGE and radioactivity was quantified in dried gels using an Ambis Mark II radioanalytic detection system (Ambis, San Diego, CA). Images for reproduction were obtained by autoradiography of the same gels with Kodak X-OMAT AR film.

To test for tyrosine-linked sulfate, [^{35}S]sulfate labeled protein was resolved on SDS-PAGE, Coomassie stained, destained, and treated with 95°C 1 N HCl [Huttner, 1984] followed by drying the gel and detection on the Ambis system. To test for carbohydrate-linked sulfate, total cellular protein samples labeled with [^{35}S]sulfate were hydrolyzed in 0.2 M $Ba(OH)_2$, neutralized with H_2SO_4, and radioactivity in the supernatant was determined by liquid scintillation counting [Huttner, 1984].

Carbohydrate Analysis

Isolated acini were incubated for 18 h in HR with glucose replaced by 22 mM pyruvate and containing 100 μCi/ml [^{3}H]glucosamine and 100 μCi/ml [^{35}S]sulfate. Total cell protein was separated on 7.5% SDS-PAGE, and the radioactive band corresponding to gp300 was excised from the gel for analysis. To test for the presence of glycosaminoglycans in gp300, gel slices were digested with 1 mg/ml pronase E (Sigma Chemical Co., St. Louis, MO), followed by chemical and enzymatic treatments known to degrade glycosaminoglycans [Hart, 1976]. The pronase was preincubated for 60 min at 37°C to destroy any contaminating enzymatic activities and the gel slices were digested for 24 h at 37°C. The digest was boiled to destroy the pronase, and the digested material was fractionated on Bio-Gel P-10, using 0.1 M ammonium bicarbonate, 0.02% NaN_3 as the elution buffer. Radioactivity eluting near the void volume (blue dextran, M_r = 2,000,000) was pooled, lyophilized, and sequentially treated according to Hart [1976] with nitrous acid, chondroitinase ABC (EC 4.2.2.4, Boehringer Mannheim, Indianapolis, IN), and endo-β-galactosidase (EC 3.2.1.103, Oxford Glycosystems, Inc., Rosedale, NY). Enzyme digestions were for 20 h at 37°C.

Dual labeled samples were also sequentially treated with PNGase F (8 U/ml for 24 h at 37°C), alkaline borohydride [50 mN NaOH, 1 M $NaBH_4$ for 24 h at 37°C; Beeley, 1985], and pronase. The gel slices were extensively rinsed between steps, and the rinses were pooled, lyophilized, and fractionated on Bio-Gel P-10.

Immunoprecipitation

Metabolically labeled acini were used for immunoprecipitation. Acini were labeled with Tran^{35}S-label ([^{35}S]methionine and [^{35}S]cysteine; ICN, Costa Mesa, CA) by incubation in HR containing 0.5 mCi/ml Trans^{35}S-label for up to 1 h. Labeled acini were chased in the same buffer with 0.2 mM unlabeled met and cys. Acinar cell proteins were also metabolically labeled for immunoprecipitation with [^{35}S]sulfate or [^{3}H]glucosamine as above. For use with the mAb, labeled acini were solubilized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.5), 10 mM EDTA, 0.1 mM phenylmethyl-sulfonyl fluoride, and 1 mM benzamidine) and immunoprecipitated as described [De Lisle et al., 1988]. For use with the polyclonal antiserum, labeled acini were sonicated in 2% SDS and diluted 10-fold in RIPA before immunoprecipitation.

Indirect Immunofluorescence

Indirect immunofluorescence was performed on 0.5–2.5 μm cryosections from perfusion-fixed (4% paraformaldehyde in phosphate buffered saline, pH 7.0) mouse tissues. Sections were prepared with a cryo-ultramicrotome (Research and Manufacturing Company, Tucson, AZ; CR-21 cryo-attachment with an MT-7 ultramicrotome). Sections were incubated with 4.15A12 (undiluted hybridoma cell culture supernatant),

washed in phosphate-buffered saline, and then incubated with a rabbit anti-rat Ig-FITC (Dako, Carpinteria, CA) diluted 1:100 in 2% normal rabbit serum. Alternatively, sections were labeled with anti-gp300 rabbit antiserum (1:1,000 diluted in 2% normal donkey serum) and donkey anti-rabbit IgG-FITC (Jackson Immunoresearch Labs, Inc., West Grove, PA) diluted 1:100 in 2% normal donkey serum. Labeled sections were observed on a Nikon Diaphot and photographed with Kodak T-Max 400 ASA black and white film.

Miscellaneous Techniques

Relative amounts of gp300 in subcellular fractions were quantified by a dot blot immunoassay using 4.15A12 as described [De Lisle, 1991]. Protein was quantified by the method of Bradford [1976] using the Bio-Rad reagent and bovine serum albumin as the standard. Amylase activity was determined by the method of Jung [1980] using Procion Yellow (Polysciences, Inc., Warrington, PA) conjugated to corn starch (Sigma Chemical Co., St. Louis, MO). For fluorography of [^{3}H] labeled samples on SDS-PAGE, gels were treated with Enlightening (NEN) before drying them, and exposure was at −70°C.

RESULTS

In an attempt to obtain antibodies to apical membrane proteins, rat monoclonal antibodies (mAb) were generated against a plasma membrane preparation from mouse pancreas. One of the mAbs, designated 4.15A12, was found to immunoblot a single band of high molecular weight with an apparent molecular mass ~300 kDa in the presence of reducing agent (Fig. 1), and 285 kDa in the absence of reducing agent (not shown). The antigen is called gp300 because it is a glycoprotein (below). The higher electrophoretic mobility of gp300 in the absence of reducing agent indicates that it has intrachain disulfide bonds but is not covalently coupled via disulfides to other proteins. Subcellular localization of this antigen was explored by immunoblotting of Western transferred protein. gp300 was detected in plasma membranes (not shown) as well as whole pancreatic homogenate but was stronger in isolated zymogen granules (Fig. 1). When isolated granules were osmotically lysed and separated into content and membrane fractions, gp300 immunoreactivity was greatly enriched in the membrane fraction (Fig. 1).

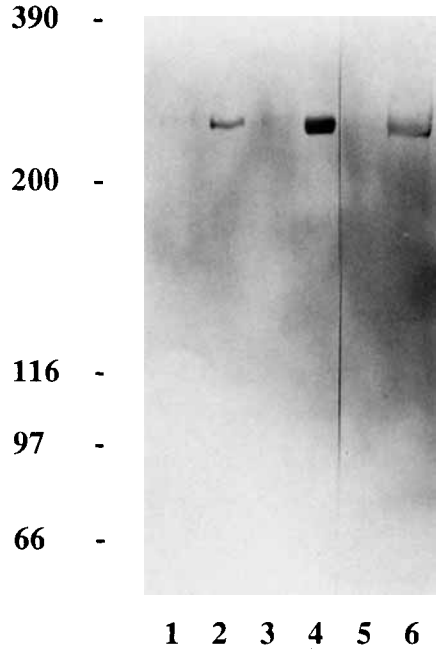

Fig. 1. Immunoblotting of gp300 in subcellular fractions and Triton X-114 phase separated granule membranes. Equal aliquots (5 μg) of (lane 1) pancreatic homogenate, (lane 2) isolated zymogen granules, (lane 3) zymogen granule content, and (lane 4) zymogen granule membranes were run on a 5% polyacrylamide gel with 2-mercaptoethanol, transferred to nitrocellulose, and blotted with mAb 4.15A12 and a rabbit anti-rat alkaline phosphatase. Another aliquot of granule membranes was subjected to Triton X-114 phase separation and the (lane 5) detergent and (lane 6) aqueous phases were run on the gel for immunoblotting. Molecular mass standards are as indicated in kDa.

To determine the nature of the association of gp300 with the granule membrane zymogen granule membranes were treated in various ways. Osmotic lysis of isolated granules released over 90% of the amylase activity (a content marker) but released less than 2% of gp300 into the soluble fraction (Fig. 2). KBr washing of zymogen granule membranes removed greater than 90% of the remaining amylase activity, while greater than 90% of the gp300 was still membrane associated (Fig. 2). Treatment of the granule membranes with the high pH carbonate buffer released more than 99% of gp300 (Fig. 2), demonstrating it is a peripheral membrane protein. Another characteristic of peripheral membrane proteins is that they are often hydrophilic proteins and will partition into the aqueous phase after Triton X-114 solubilization and temperature-induced phase separation [Bordier, 1981]. When zymogen granule membranes were solubilized in 1% Triton X-114 and were parti-

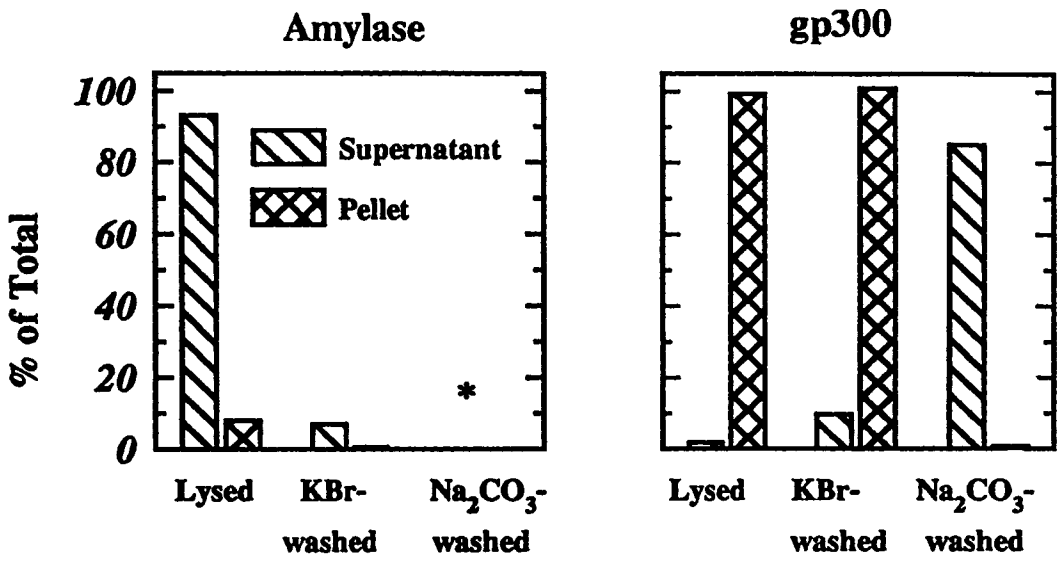

Fig. 2. Differential release of amylase and gp300 from purified zymogen granules and zymogen granule membranes. Isolated zymogen granules were osmotically lysed and the granule membranes (pellet) and content (supernatant) were separated. Washing of the membranes consisted of resuspension in the indicated solutions, 30 min incubation on ice, and separation by ultracentrifugation. Amylase (left panel) was quantified by enzymatic activity and gp300 (right panel) was quantified by dot-blot immunoassay. Equal aliquots of zymogen granules were used for the different treatments and the overall recovery of amylase activity was 97 ± 6.2% (n = 4) and recovery of gp300 immunoreactivity was 93 ± 13% (n = 5). The KBr buffer consisted of 0.25 M KBr, 10 mM MOPS (pH 6.5), and protease inhibitors. The sodium carbonate buffer consisted of 0.1 M Na_2CO_3 (pH 11.5) and protease inhibitors. *, Amylase activity was destroyed by sodium carbonate treatment.

tioned into detergent and aqueous phases, gp300 was only detected in the aqueous phase (Fig. 1).

Biochemical characterization (below) showed that gp300 has glycoconjugates characteristic of lumenal or extracellular proteins. The above experiments showed that gp300 is tightly adherent to the granule membrane, and although it is lumenal it is not expected to be secreted. To directly test whether gp300 is secreted, release of gp300 from secretagogue-stimulated and resting acini was quantified. Stimulation of isolated acini with 70 pM cholecystokinin octapeptide caused secretion of amylase as well as small amounts of gp300 (Fig. 3). However, in contrast to amylase, the amount of gp300 released relative to the total cellular content was only 1/30th that of amylase secretion.

gp300 was found to be a glycoconjugate using a glycan detection kit based on oxidation and derivatization of sugars with digoxigenin (not shown). The nature of the glycoconjugates on gp300 was explored by lectin blot analysis. gp300 was found to bind PNA, MAA, and DSA (Fig. 4) but not GNA or SNA (not shown). PNA binding indicates terminal Gal in O-linked oligosaccharide, usually Galβ(1-3)GalNAc [Goldstein and Hayes, 1978]. PNA binding to gp300 was not sensitive to O-glycosidase digestion suggesting

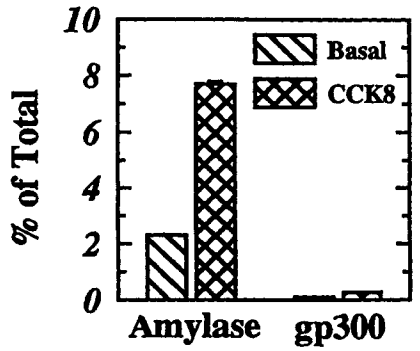

Fig. 3. Comparison of hormone-stimulated amylase release to gp300 release. Pancreatic acini were incubated for 30 min at 37°C without (Basal) or with 70 pM cholecystokinin octapeptide (CCK8) and amylase and gp300 in the media and cell pellets were quantified by enzyme activity and dot-blot immunoassay, respectively. Triplicate determinations (means ± SD) from a representative experiment (error bars not shown are too small to see).

that the disaccharide may be substituted which blocks O-glycosidase action. The interfering substituent is not sialic acid because neuraminidase digestion did not make gp300 sensitive to O-glycosidase. PNA binding to gp300 was also insensitive to PNGase F (Fig. 4). MAA binding indicates the presence of sialic acid α(2-3)Gal on

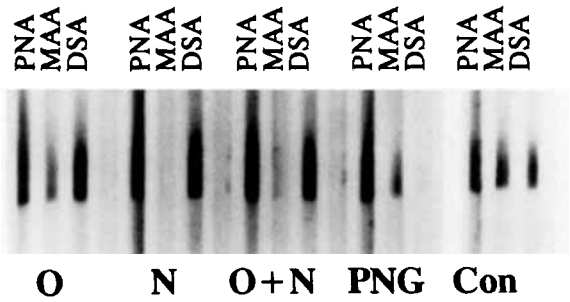

Fig. 4. Glycoconjugate analysis of gp300 on Western blots. Purified zymogen granule membranes were separated on 5% SDS-PAGE, Western blotted, and the nitrocellulose was treated with various glycosidases followed by probing with digoxigenin-coupled lectins and an anti-digoxigenin-alkaline phosphatase conjugated antibody. Glycosidase digestions were as labeled: O, O-glycosidase; N, neuraminidase; O + N, O-glycosidase and neuraminidase; PNG, PNGase F; Con, Control. Lectins used were as labeled.

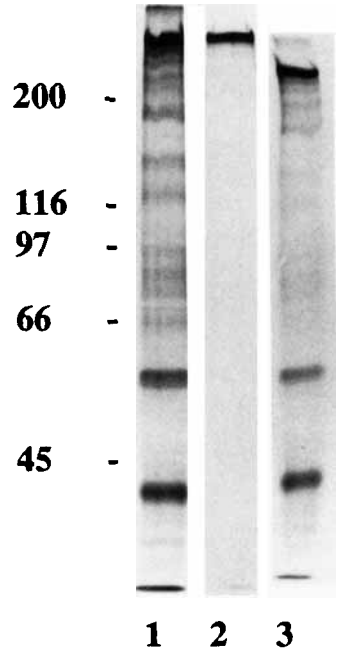

Fig. 5. Metabolic labeling of mouse acinar cells with [^{35}S]sulfate. [^{35}S]sulfate labeled acinar cell protein resolved on 7.5% SDS-PAGE: (lane 1) Total [^{35}S]sulfate labeling pattern; (lane 2) immunoprecipitated gp300 with mAb 4.15A12; (lane 3) [^{35}S]sulfate labeling pattern after treatment of the wet gel with 1 N HCl at 95°C to cleave tyrosine-linked sulfate. The acid treatment shrank the gel so the molecular mass standards do not apply to this lane.

either N- or O-linked glycoconjugates [Goldstein and Hayes, 1978]. MAA binding was lessened by O-glycosidase, was removed by neuraminidase, and was not removed by PNGase F. These results indicate sialic acid on O-linked carbohydrate. DSA binding indicates Galβ(1-4)GlcNAc [Goldstein and Hayes, 1978]. DSA binding was removed by PNGase F but was unaffected by the other enzymes, indicating that this disaccharide is N-linked in gp300.

The characteristics of gp300 were in some ways similar to high molecular weight components of the guinea pig zymogen granule [Reggio and Palade, 1978] which were found to contain sulfated GAGs. Therefore, it was examined whether gp300 is also a sulfated macromolecule. Labeling of mouse acini with [^{35}S]sulfate revealed at least 14 sulfated macromolecules in the acinar cell (Fig. 5, lane 1). The most prominently labeled band (~40% of the total label) ran at the position of gp300 and was directly identified as gp300 by immunoprecipitation with mAb 4.15A12 (Fig. 5, lane 2).

To determine whether the sulfate on acinar cell macromolecules is linked to protein via tyrosine or to carbohydrate, sensitivity to acid and alkali hydrolysis was determined. Metabolically incorporated [^{35}S]sulfate was acid resistant on SDS-PAGE (Fig. 5, lane 3). By contrast, metabolically incorporated [^{35}S]sulfate was alkali labile. After $Ba(OH)_2$ hydrolysis and neutralization, less than 5% of the starting radioactivity was soluble. Thus, at least 95% of sulfate on acinar cell macromolecules is carbohydrate linked.

To test for the presence of GAGs on gp300, [^{3}H]glucosamine and [^{35}S]sulfate metabolically dual-labeled gp300 was pronase digested, followed by various enzymatic and chemical treatments of the glycopeptides, and size fractionation on Bio-Gel P-10. On Bio-Gel P-10 chromatography of pronase digested [^{3}H]/[^{35}S] labeled gp300, two major peaks were obtained near the void volume (Fig. 6A). No significant amounts of either tracer was degraded by any of these treatments (Fig. 6B–D), indicating the absence of GAGs in gp300.

To further examine the nature of sulfated oligosaccharides, [^{3}H]/[^{35}S] labeled gp300 was digested with PNGase F followed by Bio-Gel P-10 fractionation. About 17% of [^{3}H] and a small amount of [^{35}S] (less than 3%) was released by PNGase F (Fig. 7A). When glycopeptides produced by pronase were digested with PNGase F, the peak marked in Figure 6A shifted its elution volume to the same as shown in Figure 7A (not shown), presumably due to release of N-linked oligosaccharide from pronase-resistant peptide. After PNGase digestion dual-labeled gp300 was treated with mild alkaline

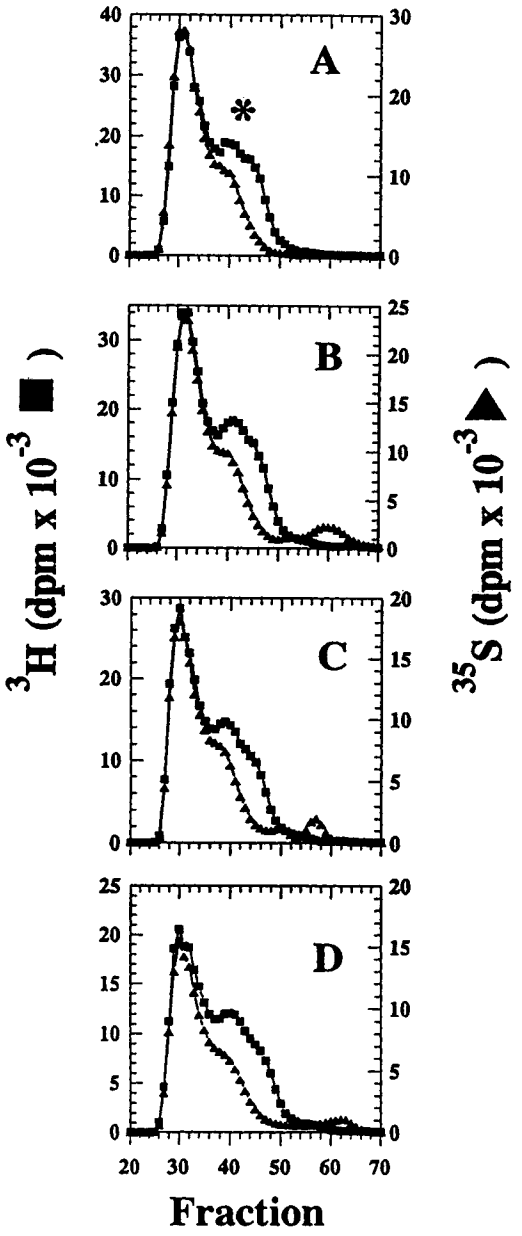

Fig. 6. Glycosaminoglycan analysis of [^{3}H]glucosamine and [^{35}S]sulfate labeled gp300. [^{3}H]glucosamine and [^{35}S]sulfate labeled gp300 was pronase digested and fractionated on Bio-Gel P-10. The labeled fractions were pooled for further analysis. After each treatment, material eluting in the original fractions was pooled and treated with the next reagent. (**A**) Pronase digestion; (**B**) Nitrous acid treatment; (**C**) chondroitinase ABC digestion; (**D**) Endo-β-galactosidase digestion. The asterisk in (A) indicates the glycopeptide containing N-linked carbohydrate (releasable by PNGase F; see Fig. 7A).

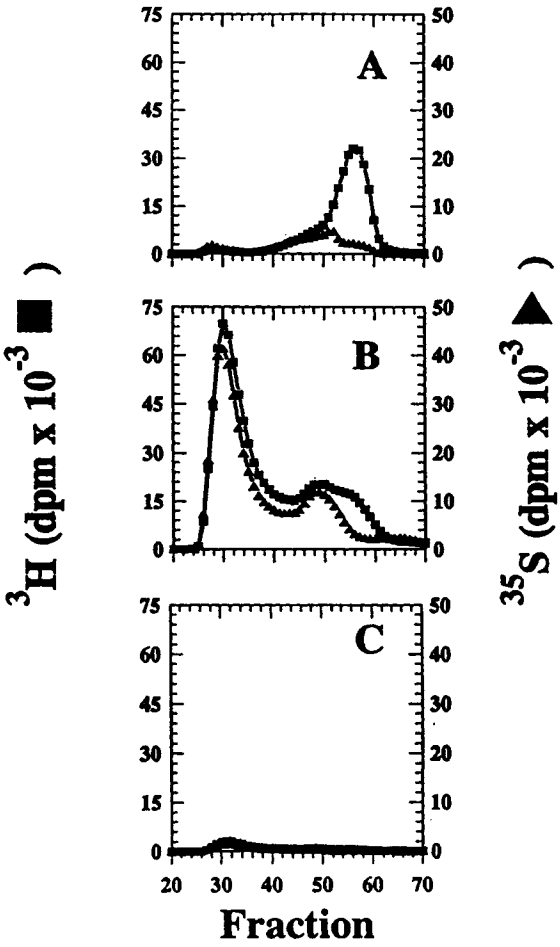

Fig. 7. N- and O-linked oligosaccharide analysis of [^{3}H]glucosamine and [^{35}S]sulfate labeled gp300. [^{3}H]glucosamine and [^{35}S]sulfate dual-labeled gp300 was sequentially treated as indicated followed by Bio-Gel P-10 fractionation. **A:** PNGase F digestion to release N-linked oligosaccharides. **B:** Alkaline borohydride treatment of PNGase F resistant material to release O-linked oligosaccharides. **C:** Pronase digestion to release residual label. The different panels are plotted with the same scales for comparison.

borohydride to remove O-linked oligosaccharides by β-elimination. This treatment released the remaining [^{3}H] and all of the [^{35}S] which fractionated as two major peaks on Bio-Gel P-10 (Fig. 7B). Thus, the majority of carbohydrate on gp300 is O-linked, and these O-linked oligosaccharides contain all the sulfate on this molecule.

To investigate the biosynthesis of gp300, the polyclonal antiserum was used to immunoprecipitate Tran^{35}S-label pulse-labeled gp300. The immunoprecipitates were untreated or PNGase F digested to remove N-linked oligosaccharides. The earliest band immunoprecipitated was ~245 kDa, and PNGase F digestion reduced its M_r by ~35,000 (Fig. 8). With increasing time of chase, the 245 kDa band decreased in intensity and the mature 300 kDa band appeared and increased in intensity. At all times, the same change in M_r after PNGase F digestion was observed. The

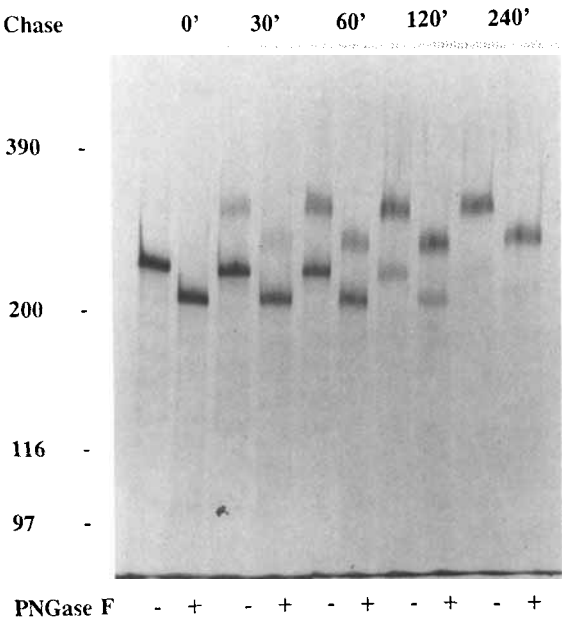

Fig. 8. Analysis of biosynthesis of gp300 by pulse chase metabolic labeling with Tran^{35}S-label. Acini were metabolically labeled with Tran^{35}S-label for 60 min followed by washing and chasing in the presence of excess unlabeled amino acids. Samples were immunoprecipitated with the polyclonal antiserum at the indicated times of chase (min), and treated without (−) or with PNGase F (+), run on 5% SDS-PAGE and autoradiographed.

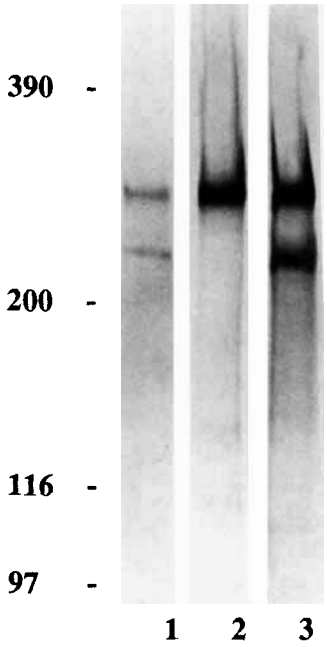

Fig. 9. Comparison of [^{3}H]glucosamine, [^{35}S]sulfate, and Tran^{35}S-label labeled gp300 by immunoprecipitation. Acini were metabolically labeled followed by immunoprecipitation of gp300 with the polyclonal antiserum and separation on 5% SDS-PAGE. (lane 1) 100 μCi/ml [^{3}H]glucosamine for 2 h in glucose-free, pyruvate supplemented HR; (lane 2) 100 μCi/ml [^{35}S]sulfate for 16 h in sulfate-free HR; and (lane 3) 0.5 mCi/ml Tran ^{35}S-label for 16 h in HR.

half-time of maturation of gp300 was ~60 min as determined by Ambis quantitation of label in the immunoprecipitates. Metabolically labeled and immunoprecipitated gp300 showed no shift in M_r after digestion with O-glycosidase, either without or with neuraminidase pretreatment (not shown). This result is consistent with the lectin blot analysis (above) and suggests that the O-linked oligosaccharides are substituted, which blocks O-glycosidase action [Beeley, 1985].

When [^{3}H]glucosamine labeled gp300 was immunoprecipitated with the polyclonal antiserum, labeled bands of 245 kDa and 300 kDa were observed (Fig. 9, lane 1). When gp300 was immunoprecipitated from Tran^{35}S-labeled acini, the polyclonal antiserum immunoprecipitated 245 kDa and 300 kDa bands (Fig. 9, lane 3), while the mAb only immunoprecipitated the 300 kDa band (not shown). When acini were labeled with [^{35}S]sulfate, both the monoclonal and the polyclonal antibodies immunoprecipitated only the 300 kDa band (Fig. 5, lane 2 and Fig. 9, lane 2, respectively). These data indicate that the mAb only recognizes the mature 300 kDa form, while the polyclonal antiserum also recognizes biosynthetic precursors. In addition, sulfate is only found in the mature form of gp300.

Immunolocalization of gp300 with 4.15A12 on semithin cryosections of pancreas revealed a halo pattern (Fig. 10A), confirming its zymogen granule membrane localization. Living cells incubated with mAb 4.15A12 became labeled on their apical membranes, consistent with a lumenal localization for gp300 and surface expression following exocytosis (not shown). With the polyclonal antiserum, secretory granules in Paneth cells of the small intestine (Fig. 10B), chief cells of the stomach, and small apical granules in crypts of the small intestinal epithelium were immunostained (not shown). Western blotting with the polyclonal antiserum of various tissues showed immunoreactive bands in pancreas, small intestine (both duodenum and jejunum), and parotid gland (Fig. 11). In pancreas, two bands were observed: one at ~300 kDa and a weaker band at 245 kDa. Besides a strong 300 kDa band, many other bands were observed in small intestine. A single weak immunoreactive band to parotid tissue was observed, and it was

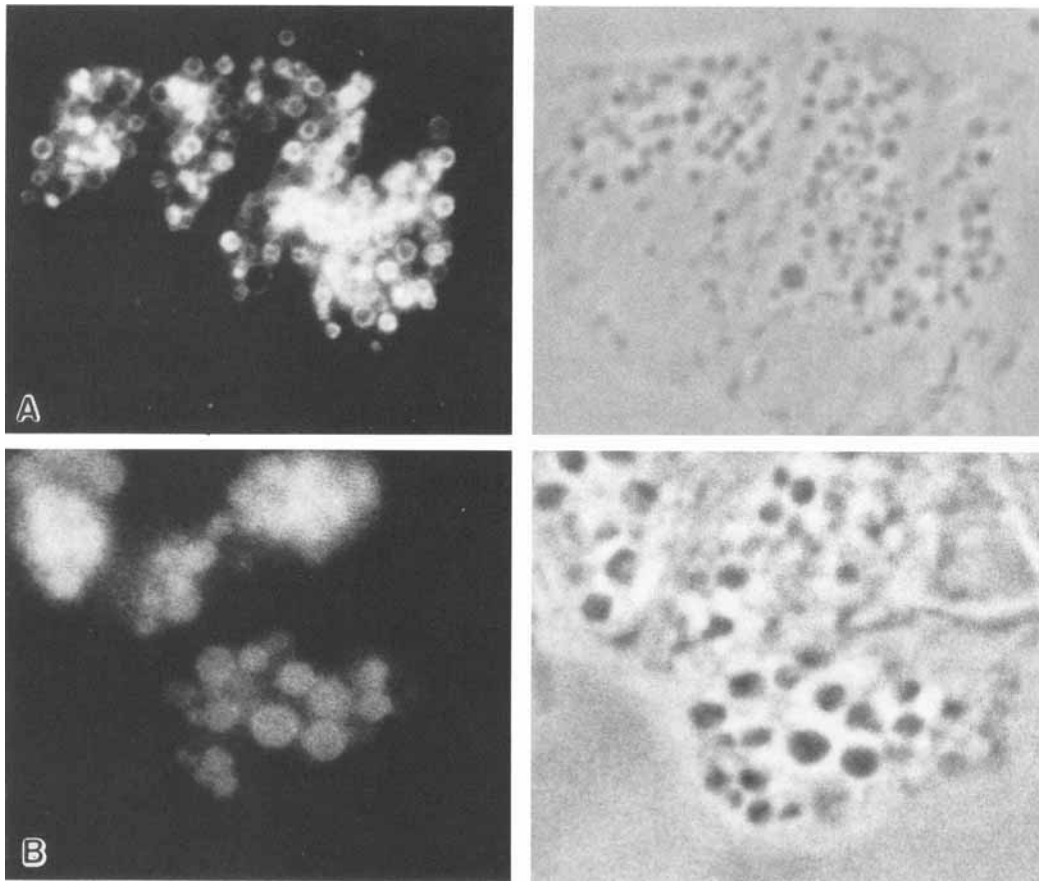

Fig. 10. Indirect immunofluorescence localization of gp300 in pancreas and small intestine. **A:** Cryosection (0.5 μm) of pancreas immunofluorescently labeled with mAb 4.15A12 and corresponding phase contrast micrograph. Zymogen granule membranes are labeled and appear as halos. **B:** Cryosection (2.5 μm) of small intestine immunofluorescently labeled with the anti-gp300 antiserum and corresponding phase contrast micrograph. Granules in Paneth cells at the base of the intestinal crypt are labeled. ×2,325.

smaller by ~10 kDa than the 300 kDa band in pancreas. By Western blotting, stomach was negative (Fig. 11). Lack of immunoreactivity in Western blots of stomach, while immunofluorescence showed labeling of chief cell granules, may be due to the small amount of chief cell granules relative to the rest of the tissue. Other tissues, including kidney, brain, heart, skeletal muscle, and liver were negative by immunoblotting with the polyclonal antiserum (not shown).

DISCUSSION

Monoclonal and polyclonal antibodies were generated and used to characterize the major sulfated protein of the mouse pancreatic acinar cell, a glycoprotein of M_r ~300,000 (gp300). Subcellular fractionation and immunolocalization studies demonstrated that gp300 is localized to the zymogen granule membrane, and that it is a peripheral membrane protein on the lumenal face of the granule membrane. Release of gp300 from hormone-stimulated acini was less than 1% of cell total indicating that gp300 is not a secretory protein. Immunolocalization and Western blotting showed that gp300 or similar molecules are also present in other gastrointestinal tissues and parotid salivary gland. The widespread occurrence of gp300 and the fact that it is not secreted suggest that gp300 is a common component of exocrine granules in gastrointestinal tissues. Furthermore, it is proposed that gp300 functions as a structural component of exocrine secretory granules.

An antigen of the mouse gastrointestinal tract, which by Western blotting and immunolocalization has similarities to gp300, recently has been described [Calvert et al., 1993]. This antigen, called MIM-1/39, is unlikely to be the same as

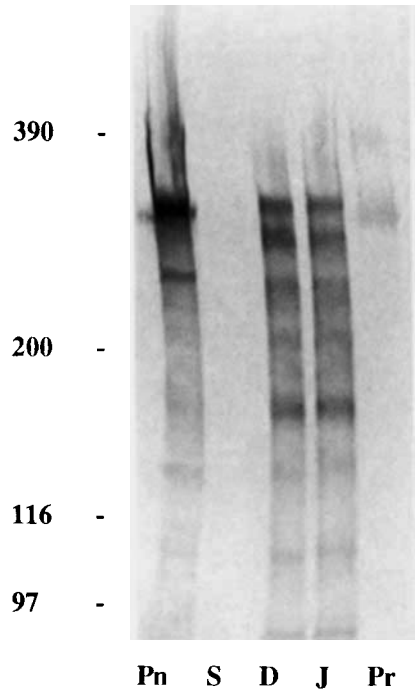

Fig. 11. Immunoblotting of gp300 in different tissues. Equal amounts of tissue protein (200 μg/lane) were separated on 5% SDS-PAGE and transferred to nitrocellulose. The blot was probed with the polyclonal antiserum to gp300. Pn, pancreas; S, stomach; D, duodenum; J, jejunum; Pr, parotid.

gp300 because MIM-1/39 is secreted and found in pancreatic juice while gp300 is not secreted to any appreciable extent.

gp300 could be metabolically labeled with both [^{3}H]glucosamine and [^{35}S]sulfate, demonstrating that it is a sulfated glycoconjugate. Chemical and enzymatic treatments of gp300 metabolically dual-labeled with [^{3}H]glucosamine and [^{35}S]sulfate demonstrated that about 17% of [^{3}H] incorporated into gp300 was in the form of N-linked carbohydrate, while the majority of the incorporated [^{3}H] and all the [^{35}S] were in O-linked oligosaccharides. The O-linked carbohydrate was insensitive to treatments which degrade GAGs, so gp300 does not appear to be a proteoglycan.

Biosynthetic studies of gp300 showed that the first detectable product was a 245 kDa protein which was reduced ~35 kDa by PNGase F digestion. Thus, the peptide core is believed to be ~210 kDa which is modified by the addition of ~35 kDa of N-linked oligosaccharide. The mature 300 kDa band appeared with a half-time of chase ~60 min and no intermediates between the 245 kDa and 300 kDa bands were observed. The 300 kDa band also showed a decrease of ~35 kDa upon PNGase F digestion, so the O-linked carbohydrate accounts for ~55 kDa of the apparent mass of the mature protein. Since no intermediates between 245 kDa and 300 kDa were observed at different times of chase the addition of O-linked sugar to the protein probably occurred rapidly. O-linked oligosaccharide addition begins in the *cis*-Golgi, or maybe even the intermediate compartment between the endoplasmic reticulum and the Golgi [Perez-Vilar et al., 1991]. O-linked carbohydrate addition to the LDL receptor has been studied in a mutant CHO cell which lacks an epimerase necessary for synthesis of UDP-GalNAc [Kozarsky et al., 1988]. In those studies, O-linked sugar addition to the LDL receptor was found to be rapid with no intermediates observed between the N-linked glycoprotein and the fully mature protein, even though the mature protein contains 18 mucin-like O-linked oligosaccharides. Thus O-linked carbohydrate addition can be a rapid process.

Sulfated macromolecules, especially proteoglycans, are widespread components of secretory granules, including endocrine [Burgess and Kelly, 1984], exocrine [Reggio and Palade, 1978; Blair et al., 1991], and blood cell granules [Kolset and Gallagher, 1990]. The best characterized secretory granule proteoglycans are the serglycins of blood cells, which have been demonstrated to be involved in regulation of substrate specificity of the proteases with which they associate [Trong et al., 1987]. It is unlikely that the core protein of gp300 is a serglycin because expression of serglycin is restricted to hemopoietic cells [Bourdon et al., 1987; Kjellén and Lindahl, 1991]. It is possible that gp300 contains a serine-glycine repeat domain, as there is evidence that a family of such proteins exists with widespread expression [Bourdon et al., 1987] and the many serines would provide sites for attachment of O-linked oligosaccharides in gp300. gp300 as a molecule is distinct from known sulfated granule components in that, although it is sulfated, it is a glycoprotein rather than a proteoglycan. Also in contrast to secretory granule proteoglycans so far described, gp300 is unique in that it is not secreted.

It has been postulated that sulfated macromolecules in the pancreas participate in the aggregation of regulated secretory proteins [Jamieson and Palade, 1971; Reggio and Dagorn, 1978; Reggio and Palade, 1978; Tartakoff et al., 1974]. Autoradiographic studies of mouse pancreas showed that sulfate is added in the Golgi com-

plex and follows the secretory pathway through condensing vacuoles (immature granules) and zymogen granules [Berg and Young, 1971]. Recent biochemical studies have shown that sulfation, both on tyrosine [Baeuerle and Huttner, 1987; Huttner, 1988] and on carbohydrate [Rosa et al., 1992] occurs in the *trans* Golgi network. Zymogen granules also form at the *trans* Golgi network [Beaudoin and Grondin, 1991], and these observations place sulfation and granule formation in the same compartment. In addition, there is evidence that sulfate is added to maturing exocrine granules as well. Electron probe X-ray elemental microanalysis of the secretory pathway in parotid gland has shown that immature granules lose Na, K, and Cl but increase their content of sulfate as they mature [Wong et al., 1991]. This finding is consistent with the idea that material is added to the immature granule, probably via vesicular transport. It has also been demonstrated that isolated immature parotid zymogen granules have the capacity to incorporate [^{35}S]sulfate into GAGs [Blair et al., 1991]. Thus, sulfation is closely correlated to both secretory granule biogenesis and granule maturation.

Morphological observations of granule formation and maturation in the exocrine pancreas show that protein is aggregated in the *trans* Golgi network where the condensing vacuole forms [Beaudoin and Grondin, 1991; Sesso et al., 1980]. Secretory protein aggregation in the *trans* Golgi network is believed to be mediated by the acidic pH and high calcium concentrations in this compartment [Orci et al., 1986, 1987]. In vitro studies of pancreatic secretory proteins demonstrated exogenous GAGs aggregate with these proteins in a pH-dependent manner [Reggio and Dagorn, 1978]. In addition, purified granule contents (presumably including soluble GAGs) demonstrate pH- and calcium-dependent aggregation [Leblond et al., 1993]. Thus, pH-dependent association of secretory products and proteoglycans may account for this initial aggregation event. As the aggregate forms it must then interact with membrane component(s) that act as a receptor(s) for the secretory material and somehow participate in budding of the immature granule off the Golgi complex. It has been postulated that such receptors, or "sortases," are responsible for this process [Chanat and Huttner, 1991; Kelly, 1991; Rindler, 1992]. Since gp300 is tightly membrane associated, this glycoprotein could be a "sortase" which could bind the forming aggregate to the nascent granule membrane via sulfate-mediated ionic interactions. Therefore, gp300 may be important in granule formation. Since gp300 is not secreted, it also is expected to recycle with the granule membrane and could be reutilized in formation of new granules.

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REFERENCES

Avraham S, Austen KF, Nicodemus CF, Gartner MG, Stevens RL (1989): Cloning and characterization of the mouse gene that encodes the peptide core of secretory granule proteoglycans and expression of this gene in transfected rat-1 fibroblasts. J Biol Chem 264:16719–16726.

Baeuerle PA, Huttner WB (1987): Tyrosine sulfation is a trans-Golgi-specific protein modification. J Cell Biol 105: 2655–64.

Beaudoin AR, Grondin G (1991): Secretory pathways in animal cells: With emphasis on pancreatic acinar cells. J Electron Microsc Tech 17:51–69.

Beeley JG (1985): "Glycoprotein and Proteoglycan Techniques." New York: Elsevier.

Benacerraf B, Unanue ER (1979): "Textbook of Immunology." Baltimore: Williams and Wilkins.

Berg NB, Young RW (1971): Sulfate metabolism in pancreatic acinar cells. J Cell Biol 50:469–483.

Blair EA, Castle AM, Castle JD (1991): Proteoglycan sulfation and storage parallels storage of basic secretory proteins in exocrine cells. Am J Physiol 261:C897–C905.

Bordier C (1981): Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 256:1604–1607.

Bourdon MA, Shiga M, Ruoslahti E (1987): Gene expression of the chondroitin sulfate proteoglycan core protein PG19. Molec Cell Biol 7:33–40.

Bradford MM (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Burgess TL, Kelly RB (1984): Sorting and secretion of adrenocorticotropin in a pituitary tumor cell line after perturbation of the level of a secretory granule-specific proteoglycan. J Cell Biol 99:2223–2230.

Calvert R, Millane G, Pothier P, Beaulieu J-F (1993): An intestinal secretory protein is found in most glands associated with the gastrointestinal tract: Von Ebner's and salivary glands, gallbladder, and pancreas. J Histochem Cytochem 41:1223–1231.

Chanat E, Huttner WB (1991): Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. J Cell Biol 115:1505–1519.

De Lisle RC (1991): A quantitative dot-blot immunoassay for integral membrane proteins: Preparation of pancreatic plasma membranes containing both basolateral and apical markers. Anal Biochem 192:1–5.

De Lisle RC, Hopfer U (1986): Electrolyte transport properties of pancreatic zymogen granules: implications for pancreatic secretion. Am J Physiol 250:G489–G496.

De Lisle RC, Logsdon CD (1990): Pancreatic acinar cells in culture: expression of acinar and ductal antigens in a growth-related manner. Eur J Cell Biol 51:64–75.

De Lisle RC, Logsdon CD, Hootman SR, Williams JA (1988): Monoclonal antibodies as probes for plasma membrane domains in the exocrine pancreas. J Histochem Cytochem 36:1043–1051.

Goldstein IJ, Hayes CE (1978): The lectins: carbohydrate-binding proteins of plants and animals. Adv Carbohydrate Chem and Biochem 35:127–350.

Hart GW (1976): Biosynthesis of glycosaminoglycans during corneal development. J Biol Chem 251:6513–6521.

Haselbeck A, Hosel W (1990): Description and application of an immunological detection system for analyzing glycoproteins on blots. Glycoconjugate J 7:63–74.

Horst CJ, Johnson LV, Besharse JC (1990): Transmembrane assemblage of the photoreceptor connecting cilium and motile cilium transition zone contain a common immunologic epitope. Cell Motility Cytoskeleton 17:329–344.

Huttner WB (1984): Determination and occurrence of tyrosine O-sulfate in proteins. Meth Enz 107:200–23.

Huttner WB (1988): Tyrosine sulfation and the secretory pathway. Annu Rev Physiol 50:363–376.

Jamieson JD, Palade GE (1971): Condensing vacuole conversion and zymogen granule discharge in pancreatic exocrine cells: Metabolic studies. J Cell Biol 48:503–522.

Jung DH (1980): Preparation and application of procion yellow starch for amylase assay. Clin Chim Acta 100:7–11.

Kelly RB (1991): Secretory granule and synaptic vesicle formation. Curr Opinion Cell Biol 3:654–660.

Kjellén L, Lindahl U (1991): Proteoglycans: Structures and interactions. Annu Rev Biochem 60:443–475.

Kjellén L, Pettersson I, Lillhager P, Steen M-L, Pettersson U, Lehtonen P, Karlsson T, Ruoslahti E, Hellman L (1989): Primary structure of a mouse mastocytoma proteoglycan core protein. Biochem J 263:105–113.

Kolset SO, Gallagher JT (1990): Proteoglycans in haemopoietic cells. Biochim Biophys Acta 1032:191–211.

Kozarsky K, Kingsley D, Krieger M (1988): Use of a mutant cell line to study the kinetics and function of O-linked glycosylation of low density lipoprotein receptors. Proc Natl Acad Sci USA 85:4335–4339.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Leblond FA, Viau G, Lainé J, Lebel D (1993): Reconstitution in vitro of the pH-dependent aggregation of pancreatic zymogens en route to the secretory granule: implication of GP-2. Biochem J 291:289–296.

Orci L, Ravazzola M, Amherdt M, Madsen O, Perrelet A, Vassalli J-D, Anderson RGW (1986): Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. J Cell Biol 103:2273–2281.

Orci L, Ravazzola M, Anderson RGW (1987): The condensing vacuole of exocrine cells is more acidic than the mature secretory granule. Nature 326:77–79.

Perez-Vilar J, Hidalgo J, Velasco A (1991): Presence of terminal N-acetylgalactosamine residues in subregions of the endoplasmic reticulum is influenced by cell differentiation. J Biol Chem 266:23967–23976.

Reggio H, Dagorn JC (1978): Ionic interactions between bovine chymotrypsinogen A and chondroitin sulfate A.B.C. J Cell Biol 78:951–957.

Reggio HA, Palade GE (1978): Sulfated compounds in the zymogen granules of the guinea pig pancreas. J Cell Biol 77:288–314.

Rindler MJ (1992): Biogenesis of storage granules and vesicles. Curr Opinion Cell Biol 4:616–622.

Rosa P, Mantovani S, Rosboch R, Huttner WB (1992): Monensin and brefeldin A differentially affect the phosphorylation and sulfation of secretory proteins. J Biol Chem 267:12227–12232.

Sesso A, Assis JEP, Kuwajima VY, Kachar B (1980): Freeze-fracture and thin-section study of condensing vacuoles in rat pancreatic acinar cells. Acta Anat 108:521–539.

Tartakoff A, Greene LJ, Palade GE (1974): Studies on the guinea pig pancreas: Fractionation and partial characterization of exocrine proteins. J Biol Chem 249:7420–7431.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci 76:4350–4354.

Trong HL, Neurath H, Woodbury RG (1987): Substrate specificity of the chymotrypsin-like protease in secretory granules isolated from rat mast cells. Proc Natl Acad Sci 84:364–367.

Wang K, Fanger BO, Guyer CA, Staros JV (1989): Electrophoretic transfer of high-molecular-weight proteins for immunostaining. Methods Enzymol 172:687–696.

Wong JG, Izutsu KT, Robinovitch MR, Iversen JM, Cantino ME, Johnson DE (1991): Microprobe analysis of maturation-related elemental changes in rat parotid secretory granules. Am J Physiol 261:C1033–C1041.