Absence of the Major Zymogen Granule Membrane Protein, GP2, Does Not Affect Pancreatic Morphology or Secretion*

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The majority of digestive enzymes in humans are produced in the pancreas where they are stored in zymogen granules before secretion into the intestine. GP2 is the major membrane protein present in zymogen granules of the exocrine pancreas. Numerous studies have shown that GP2 binds digestive enzymes such as amylase, thereby supporting a role in protein sorting to the zymogen granule. Other studies have suggested that GP2 is important in the formation of zymogen granules. A knock-out mouse was generated for GP2 to study the impact of the protein on pancreatic function. GP2-deficient mice displayed no gross signs of nutrient malabsorption such as weight loss, growth retardation, or diarrhea. Zymogen granules in the GP2 knock-out mice appeared normal on electron microscopy and contained the normal complement of proteins excluding GP2. Primary cultures of pancreatic acini appropriately responded to secretagogue stimulation with the secretion of digestive enzymes. The course of experimentally induced pancreatitis was also examined in the knock-out mice because proteins known to associate with GP2 have been found to possess a protective role. When GP2 knock-out mice were subjected to two different models of pancreatitis, no major differences were detected. In conclusion, GP2 is not essential for pancreatic exocrine secretion or zymogen granule formation. It is unlikely that GP2 serves a major intracellular role within the pancreatic acinar cell and may be functionally active after it is secreted from the pancreas.

EXPERIMENTAL PROCEDURES

Generation of GP2-deficient Mice—Murine pancreatic total RNA was purified as described previously (18). Two primers derived from the rat GP2 nucleotide sequence (nucleotides 44–65 and nucleotides 1732–1753, GenBank accession number M58716) that bracket the open

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‡ The abbreviations used are: GPI, glycosylphosphatidylinositol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; CDE, choline-deficient ethionine-enriched.
reading frame were used to generate murine GP2 cDNA by reverse transcription followed by the PCR. The nucleotide sequence was determined for both stands of the PCR product. GP2-containing genomic DNA was obtained by screening a 129/SVj II BAC library (IncyteGenomics, Palo Alto, CA). Using the isolated murine GP2 cDNA as a probe, two BAC clones were isolated, one of which encompassed most of the GP2 open reading frame. Genomic sequencing and mapping of GP2 gene was performed and used for construction of the targeting vector (Fig. 1).

A commercial targeting vector, pKO Scrambler NTKV-1903 (Stratagene Corp., La Jolla, CA), containing elements for positive (neomycin) and negative (thymidine kinase) selection was used to construct the targeting vector. The neomycin selection marker was flanked by a 3.6-kb EcoRI fragment that contains exon 1 but is proximal to exon 2, which contains the start codon, and a 4.0-kb ClaI/SacII fragment that contains exons 6–8. The SacII site was derived from the BAC cloning vector.

The targeting vector was introduced by electroporation into R1 embryonal stem cells derived from a 129/SvJ × 129/Sv background followed by selection with G418 and ganciclovir. Drug-resistant clones were screened using PCR and Southern blots (Fig. 1). Three individual clones were selected and injected into pseudopregnant mice. The resultant chimeric mice were bred with C57BL/6J mice. Germ line transmission of the mutated GP2 gene was confirmed with PCR and Southern blots. GP2 heterozygotes were intercrossed to produce homozygous, heterozygous, and wild-type mice for the GP2 gene. Genotype was determined by PCR using primer pairs specific for wild-type (5′-GAGATGTAGAAGCAATGGCAGGTGTCGTATGC-3′ and 5′-ATTGAGCCGGTCTTCATCAGGATGATCTGGACG-3′, 8.0-kb product) and mutant (5′-GAGATGTAGAAGCAATGGCAGGTGTCGTATGC-3′ and 5′-AAGCCGGTCTTTGCTAACCGACGGGTTCAGGAGCTGAG-3′, 5.0-kb product) mice using LA TaqDNA polymerase (Takara Biomedical, Shuzo Co., Ltd., Shiga, Japan). The positive candidates were further confirmed by Southern hybridization using a 0.6-kb PstI fragment 1.8-kb 5′ to the EcoRI fragment used in the targeting vector. After digestion of genomic DNA with XbaI, mutant GP2 DNA produces an 8.0-kb fragment and wild-type GP2 results in a 10.0-kb fragment (Fig. 1B).

The presence of GP2 mRNA was also determined by reverse transcription-PCR. PCR primers were derived from nucleotides 308–328 and the reverse complement of 753–774 (5′-TCCCTGCCAGAACATGGCAGGCTTCCTAGGCACTTCG-3′ and 5′-GGAGGTCCCTGCTACGAC-3′, GenBank accession number NM 025989). Murine β-actin served as a positive control and result in a 663-bp product. Mouse β-actin primer pairs was used as a control and result in a 663-bp product. MW, molecular weight.

**FIG. 1. Production of GP2-deficient mice.** A, shown are schematics of the wild-type GP2 gene (top), targeting vector (middle), and the resultant change in the targeted gene (bottom). Black boxes denote exons; Neo, neomycin resistance; TK, thymidine kinase gene. B, Southern blot analysis of tail DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. C, immunofluorescence studies of the pancreas with GP2 antibody (red). Nuclei are stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (blue). D, reverse transcription-PCR analysis for the presence of GP2 mRNA from total pancreatic RNA. Primer pairs derived from GP2 cDNA produce a 469-bp PCR product. Mouse β-actin primer pairs was used as a control and result in a 663-bp product. MW, molecular weight.

**FIG. 2. Light and electron microscopy.** Pancreata from wild-type (A and C) and GP2-deficient mice (B and D) were examined after staining with hematoxylin and eosin (A and B, magnification = ×400) or electron microscopy (C and D, magnification = ×17,000).
control using primers 5'-AGACGGGGTCACCCACACTGTGCCCAT-3' and 5'-CTAGAAGCACTTGCGGTGCACGATGGAGGGG-3' (GenBank™ accession number NM 007393).

Immunohistology—The pancreas was excised and embedded in Optimum Cutting Temperature compound (Sakura Finetechnical Co., Tokyo, Japan) for cryosection. Six-micrometer cryostat sections were processed as described previously (19). Rabbit anti-human GP2 antisera that cross-reacts with murine GP2 (1:200 dilution) was used as the primary antibody (20). Texas Red-conjugated donkey anti-rabbit IgG (1:100 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody. 4',6-Diamidino-2-phenylindole dihydrochloride (1:10,000 dilution, Pierce) was used to stain the nuclei.

Purification of Pancreatic Zymogen Granules—Zymogen granules were isolated from mice that were age-matched and with identical genetic backgrounds as previously described (19). Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad) with bovine serum albumin as a standard.

Isolation and Stimulated Secretion of Pancreatic Acini—Pancreatic acini from wild-type and knock-out mice were isolated using collagenase (Worthington, Lakewood, NJ) digestion as described previously (21). The viability and stimulated secretion response were determined by measuring the extent of amylase release from isolated acini in the presence or absence of 1 nM cholecystokinin octapeptide (CCK8, Sigma) or 10 μM carbachol (Sigma). Amylase released into the culture media or in detergent cell lysates was measured using the Phadebas amylase tablets (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). The results were expressed as the percentage of total amylase in the acini.

Immunoblotting—SDS-polyacrylamide gel electrophoresis (4–20% gel) was performed as described previously (22). For immunoblotting, proteins were transferred to nitrocellulose membranes (0.45 μm pore size) and probed with antibody as previously described (19, 23). Primary antibodies used included: rabbit anti-synclion antibody (1:1,000 dilution, kindly provided by J. M. Edwardson, University of Cambridge, Cambridge), rabbit anti-rat muclin antisera (1:5,000 dilution, kindly provided by Robert C. De Lisle, University of Kansas), and rabbit anti-human amylase antibody (1:1,000 dilution, Sigma). The rabbit anti-rat ZG16p antisera was produced using a cDNA encoding a glutathione S-transferase fusion protein that incorporated amino acids 37–167 of rat ZG16p subcloned into vector pGEX-1T (Amersham Biosciences). The fusion protein was expressed in Escherichia coli, JM101, and affinity-purified on a reduced glutathione agarose column as described previously (24). The resultant purified glutathione S-transferase/ZG16p fusion protein was injected into New Zealand White rabbits for the production of antisera, which was used at 1:3,000 dilution for immunoblotting.

Detection and quantitation of proteins labeled by the primary antibodies was achieved using second antibodies labeled with near infrared dyes that were measured using infrared fluorescence (Odyssey System, LI-COR Biosciences, Lincoln, NE).

Two-dimensional Gel Electrophoresis of Zymogen Granule Proteins—Zymogen granules (100 μg) isolated from wild-type and GP2-deficient mice were homogenized in a buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, and 0.5% IPG buffer (Amersham Biosciences). Supernatants were subjected to isoelectric focusing in the first dimension with Immobilon DryStrip pl 3–10 gel strips (Amersham Biosciences). SDS-gel electrophoresis was performed in the second dimension using 11% acrylamide gels. Silver-stained proteins were quantified using PDQuest 2D gel analysis software (Bio-Rad).

Experimentally Induced Pancreatitis—All mice were sex and age matched and the product of breeding heterozygotes. For the caerulein model, 6-month-old mice were fasted overnight followed by intraperito-
neal injection with 50 μg/kg caerulein (Sigma) at hourly intervals for seven total injections. The mice were killed at 1, 6, 12, 36, 48, and 72 h and 6 days after the first injection. Five mice, two males and three females, were used for each time point.

Older, 5-month-old female mice were used for the choline-deficient diet model to decrease mortality and potentially reveal less severe differences between the mice (25). The mice were fasted overnight and then fed a powdered choline-deficient diet (Teklad, Madison, WI) supplemented with 0.75% DL-ethionine (Tokyo Kasei Kogyo Co., Tokyo, Japan). Five mice from each genotype were killed 72 h after initiating the diet.

At each specified time point, the mice were sacrificed and the pancreas were removed for pathology. Serum was collected for amylase and lipase assays, which was performed by the clinical laboratories at the Department of Comparative Medicine at Stanford University. Pancreatic tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (Histotec Laboratory, Hayward, CA). Pancreatitis severity was assessed in a blinded manner by a single pathologist (S. A. Michie), who scored for the presence of vacuoles, cell death, edema, and inflammation as described previously (26).

**Ultrastructural Analysis**—For ultrastructural analysis, pancreata were minced into 1-mm³ pieces, immersion-fixed in 2% (v/v) glutaraldehyde and 0.1 M sodium cacodylate buffer at 4 °C overnight, and postfixed in 1% osmium tetroxide for 1 h at room temperature. Samples were thin-sectioned and poststained with 1% aqueous uranyl acetate for 1 h in the dark followed by visualization on an electron microscope (Phillips CM 12).

**RESULTS**

Based on the murine GP2 nucleotide sequence obtained, analysis of the open reading frame revealed a nucleotide sequence that is 90% identical to that of rat GP2 and a predicted amino acid sequence that is 80% identical. The resultant murine GP2 sequence was used to generate a probe that enabled the isolation and characterization of murine genomic clones for GP2. A targeting vector was constructed that resulted in the replacement of exons 2–5 of the murine GP2 gene with cDNA encoding neomycin resistance for positive selection of embryonal stem cell clones. Exon 2 contains the predicted start codon. The total deletion encompasses 6,934 nucleotides of the GP2 gene, which represents amino acids 1–282 of the murine GP2 protein.
GP2 Knock-out and Pancreatic Function

Pancreatitis was induced in homozygous GP2-deficient and wild-type mice with caerulein as described under “Experimental Procedures.” Scoring was performed on hematoxylin and eosin-stained pancreatic sections. ND, not detected. Values represent the mean of five mice ± 1 S.D.

| Criteria                     | Genotype | 1 h   | 6 h   | 12 h  | 36 h  | 48 h  | 72 h  | 6 days |
|-----------------------------|----------|-------|-------|-------|-------|-------|-------|--------|
| Edema                       | +/+      | 0.5 ± 0.5 | 1.8 ± 0.5 | 2.0 ± 0.0 | 1.5 ± 0.5 | 1.8 ± 0.5 | 1.3 ± 0.5 | ND     |
|                            | +/-      | 0.5 ± 0.5 | 1.5 ± 0.5 | 1.8 ± 0.5 | 1.5 ± 0.5 | 1.0 ± 0.0 | ND     | ND     |
| Acute inflammation          | +/+      | ND | 0.2 ± 0.4 | 0.3 ± 0.5 | 1.0 ± 0.8 | 1.5 ± 0.5 | 2.0 ± 0.9 | ND     | ND     |
|                            | +/-      | 0.2 ± 0.4 | 0.3 ± 0.5 | 1.0 ± 0.8 | 1.5 ± 0.5 | 2.0 ± 0.9 | ND     | ND     |
| No. necrotic cells/ 40 × field | +/+    | 0.8 ± 0.4 | 12.2 ± 0.5 | 10.8 ± 1.6 | 5.7 ± 2.5 | 4.5 ± 1.9 | ND     | ND     |
|                            | +/-      | 0.8 ± 0.4 | 12.2 ± 0.5 | 10.8 ± 1.6 | 5.7 ± 2.5 | 4.5 ± 1.9 | ND     | ND     |

*p = 0.021 for necrotic cells detected at 6 h compared with wild type.

The targeting vector was successfully used to produce three independent embryonal stem cell clones that were injected into blastocysts derived from C57BL/6J mice. Germ line transmission of the GP2 mutation was achieved. Intercross-breeding of heterozygotes was performed to produce wild-type, heterozygous, and GP2-deficient mice. GP2-deficient mice were successfully produced as confirmed by Southern blotting, PCR, and immunohistology of pancreatic tissues (Fig. 1).

Heterozygous breeding produced mice in the expected distribution for Mendelian genetics. Over 18 months, the GP2-deficient mice show no gross differences in development, growth, weight, behavior, and life span compared with wild-type mice. The homozygous mutants were fertile and loss of exocrine pancreatic function, as characterized by weight loss or diarrhea, was not observed.

Light microscopy of GP2-deficient murine pancreata revealed no apparent differences from wild-type mice (Fig. 2). Electron microscopy was also employed to assess the subcellular morphology of the pancreatic acinar cells. Pancreata derived from GP2-deficient mice displayed no quantitative changes in zymogen granule size, number, or density.

Purified zymogen granules from mutant and non-mutant mice were subjected to two-dimensional electrophoresis to evaluate for changes in zymogen granule protein content. No significant changes in protein composition were detected (Fig. 3A). All of the major digestive enzymes were present. Amylase was quantified using immunoblotting because binding to GP2 has been described previously. When normalized to the total zymogen granule protein, there was no significant difference in pancreatic amylase content between GP2-deficient and wild-type mice.

Whether proteins known to interact with GP2 are affected in GP2-deficient mice was also determined. Cross-linking studies previously determined an association between syncollin and GP2 (16). ZG16p and GP2 are both major constituents of the submembranous matrix purified from zymogen granules, potentially serving a role in protein sorting or granule formation (15). Muclin is a membrane protein present in the zymogen granule that also contains a ZP domain and has also been proposed to play a role in protein sorting (27). When quantitative protein immunoblotting was performed to compare the relative amounts of syncollin, ZG16p, and muclin to amylase in zymogen granules, there was no significant difference between wild-type and GP2-deficient mice (Fig. 3C).

Mutant mice deficient in syncollin or ITMAP1 have been produced (28, 29). ITMAP1 is another zymogen granule membrane protein that possesses a ZP domain. A common trait among the knock-out mice that have been produced for both genes is increased severity of experimentally induced pancreatitis. Thus the GP2-deficient mice were subjected to two different models of experimental pancreatitis, the caerulein and choline-deficient diet models of pancreatitis. High levels of the secretagogue caerulein are known to induce a mild form of pancreatitis characterized by edema and inflammation (30). Caerulein, an analog of cholecystokinin, was used to induce pancreatitis. The presence of pancreatitis was documented by elevations in plasma amylase and lipase levels and by histologic analysis. Plasma pancreatic enzyme levels peaked at 12 h after caerulein injections were initiated (Fig. 4). No significant differences in serum amylase or lipase levels were found between wild-type and GP2-deficient mice. GP2-deficient mice exhibited significantly higher numbers of necrotic cells than their wild-type counterpart at 6 h (Fig. 5 and Table I). At other time points, there were no significant differences in histologic scoring between wild-type and GP2-deficient mice.

Mice fed a choline-deficient diet supplemented with ethionine develop a severe form of pancreatitis that is often characterized by hemorrhage, necrosis, and high mortality rates (25, 31). The mice were sacrificed at 72 h for analysis, by which time one out of five wild-type and none of the 5 GP2-deficient mice had died. For the remaining surviving mice, no significant

FIG. 6. Primary acini cultures stimulated with cholecystokinin (CCK) or carbachol (CCH). Amylase secreted is expressed as the percentage of the total amylase present in the cells. Each experiment was performed in duplicate. Each value represents the mean ± 1 S.E. of four independent experiments. Ctrl, control.
differences in serum amylase, serum lipase levels, or histologic scores were detected between GP2-deficient and wild-type mice (Fig. 5 and Tables II).

The ability of the pancreas to secrete digestive enzymes in response to secretagogue stimulation was examined using primary cultures. Primary isolated acini were cultured from GP2-deficient and wild-type mice and stimulated with cholecystokinin octapeptide (CCK-8) or carbachol. The amount of amylase released into the culture supernatant after stimulation for 15 or 60 min with CCK-8 or carbachol did not differ between GP2-deficient and wild-type mice (Fig. 6).

**DISCUSSION**

GP2-deficient mice were produced in this study to determine the role of GP2 in the exocrine pancreas. Numerous studies have supported a role for GP2 in zymogen granule formation and secretory protein sorting. Definitive evidence that GP2 is necessary for these secretory processes has not been possible in the experimental systems used to date. The GP2-deficient mice permitted studies that establishes that zymogen granule formation and digestive enzyme packaging is not GP2 dependent. In addition, the ability to secrete digestive enzymes in response to secretagogue also appeared unaffected.

Whether other proteins that have been established to physically interact with GP2 or are co-localized within the same domain within the granule are affected by the absence of GP2 was also examined. No changes in the zymogen granule content of syncollin, ZG16p, or mucin were detected in the homozygous mutants. Thus none of the proteins examined depend on GP2 for their sorting to the zymogen granule. Likewise, an increase in the zymogen granule content of any proteins that may compensate for the absence of GP2 was also not observed as determined by two-dimensional electrophoresis.

Previous studies had established that inhibition of the formation of the glycosylphosphatidylinositol linkage or cholesterol of-rich lipid microdomains lead to aberrant formation of zymogen granules and the sorting of secretory proteins to the organelle (17). The experiments described in this work indicate that the results obtained in the previously performed studies are unlikely due to perturbation of GP2. The results are also consistent with recent work that studied the effects of GP2 overexpression in the acinar cell line AR4-2J, which found no impact of GP2 expression on secretory granule formation or cholecystokinin stimulated secretion (32). Whether other glycosylphosphatidylinositol-linked proteins present in zymogen granule such as mucin are responsible for potential protein sorting functions or organelle formation remains to be definitively determined.

Although mice deficient in syncollin and ITMAP1 have shown increased susceptibility to experimentally induced pancreatitis, the results in the GP2-deficient mice were mixed. Only GP2-deficient mice treated with supramaximal concentrations of caerulein displayed significantly more necrotic cells. In contrast, only in wild-type mice did the CDE-induced pancreatitis result in mortality (one mouse). Thus the effects of experimental pancreatitis resulted in subtle differences, if any, between the wild-type and GP2-deficient mice.

Uromodulin is the protein most similar to GP2. It is specifically expressed in the kidney and shares with GP2 a 52% identity in amino acid sequence, the presence of a ZP domain, and a glycosphatidylinositol-linkage to the membrane (33). Similar to GP2, uromodulin is secreted following its cleavage from the membrane (34). Two independent laboratories recently generated homozygous mutants for uromodulin and found uromodulin deficiency led to greater susceptibility to infection by *E. coli* that possess Type 1 fimbriae as a virulence factor (35, 36). The data support a role for uromodulin in host cell defense against infection by binding to *E. coli* and preventing bacterial adherence to the host cell. In view of the minimal effects of the GP2 mutation on normal pancreatic morphology and physiology, it is plausible that GP2 serves a similar or alternative extracellular function in the pancreas.

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