A single gene encodes membrane-bound and free forms of GP-2, the major glycoprotein in pancreatic secretory (zymogen) granule membranes

(phosphatidylinositol-glycan-linked protein/polarized epithelial cell/exocrine gland compartmentation/protein sorting/apical secretion)

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ABSTRACT GP-2, a 75-kDa glycoprotein, was isolated from dog pancreatic zymogen granule membranes (ZGMs). In a carbohydrate-shift strategy, N-terminal and internal peptide sequences were obtained on glycosylated and deglycosylated forms of GP-2, respectively, by gas-phase sequencing. Sets of mixed oligonucleotides and the polymerase chain reaction were used to obtain a double-stranded cDNA probe, which was used to isolate overlapping cDNA clones from a dog pancreatic cDNA library. The sequence of these clones revealed an open reading frame that encodes a protein of 509 amino acids, eight N-linked oligosaccharide attachment sites, and an N-terminal signal sequence absent from the mature form of GP-2 associated with ZGMs. The C terminus shows a 20-residue hydrophobic transmembrane domain preceded by a decapeptide containing potential phosphatidylinositol-glycan attachment sites. GP-2 completely released from ZGMs by exogenous phospholipase C showed similar immunochemical properties and electrophoretic mobilities compared to the form associated with ZGMs. A similar form of GP-2 was released from zymogen granules permeabilized with saponin and incubated in the absence of added phospholipase C. Kinetic analysis of GP-2 release at 0°C and 37°C suggested the presence of a granule enzyme responsible for endogenous release of GP-2 to granule contents and into the apical medium. The data indicate that GP-2 is a phosphatidylinositol-glycan-linked membrane protein released from the membrane of mature zymogen granules by an enzymatic mechanism. The cDNA structure presented here thus encodes both membrane-bound and free forms of GP-2.

The pancreas synthesizes and secretes digestive enzymes and proenzymes into the upper intestine, where postgastric hydrolysis of dietary substrates takes place. Prior to release, enzyme and zymogen forms are stored in secretory zymogen granules (ZGs) within the apical region of the cell (1). Since peptide hormones and cholinergic agents enhance release of ZG contents into the apical medium (ductular lumen), ZGs constitute an important storage compartment in the regulated secretory pathway (2–4). The process of zymogen storage, in fact, captures large quantities of cellular membranes destined for targeting to the apical plasma membrane. Isolation of ZG membranes (ZGMs) after pH-induced lysis (5) revealed that membrane proteins account for only 1–2% of total granule protein (6). Characterization of ZGMs by SDS/PAGE indicated a relatively simple protein structure (≈ 10 components), whose composition appeared to be distinct from other cytological compartments (7), and a relatively high phospholipid/protein ratio, 2.49 (8). Analysis of ZGM proteins from dog, rat, rabbit, pig, and beef indicated, in each case, that a single

glycoprotein (75–92 kDa, depending upon the species) represented the major protein (>25 –30% of protein) (7, 9–11). Topographical studies labeling intact ZGs and isolated ZGMs with neuraminidase, galactose oxidase, and NaB³H₄ (12) or lactoperoxidase and ¹²⁵I (11) have shown that this glycoprotein, GP-2, is confined to the inner leaflet of the ZGM and one study has suggested that GP-2 is linked to pig pancreatic ZGM via a glycolipid anchor (13). GP-2 remained associated with ZGMs despite vigorous extraction with divalent-cation chelators, salts, or sodium carbonate at pH 11.2 (14). Solubilization and partition of ZGM proteins with Triton X-114 showed that the majority of proteins, including GP-2, remain associated with the detergent-rich phase. Thus GP-2 has been identified as an integral membrane protein.

Pulse-chase studies with dissociated rat pancreatic cells by Havinga *et al.* (15, 16) have indicated that GP-2 is a 73-kDa species in the rough endoplasmic reticulum (0–40 min of chase) and a 78- to 80-kDa species in the Golgi compartment and ZGs (60 min of chase), findings consistent with the N-linked-glycoprotein nature of GP-2 and the addition of terminal sugars within the Golgi apparatus. With longer chase periods (4–6 hr), GP-2 assumed a slightly smaller size (75 kDa), and this form was observed in a plasma membrane fraction and in the medium bathing acinar cells. Triton X-114 analysis of pulse-chase studies indicated that GP-2 is a membrane protein at early chase intervals (0–60 min) and a soluble protein at later chase periods (1–6 hr). Immunocytochemistry studies (17) showed GP-2 labeling of Golgi, ZG, and apical plasma membranes, but GP-2 was also observed in ZG contents and in the luminal space, the latter associated with linear aggregates. The significance and the mechanism of GP-2 release from granule and apical plasma membranes have remained perplexing problems.

Here we present the complete amino acid sequence of GP-2 and its potential carbohydrate attachment sites.[†] Structural analysis of GP-2 and functional analysis of permeabilized ZGs provide important information regarding the mechanism for release of GP-2 from granule membranes.

MATERIALS AND METHODS

Purification of ZGs and ZGMs. Dog pancreatic glands were removed surgically under pentobarbital anesthesia after exsanguination into the chest cavity. The gland was placed in ice-cold 0.25 M sucrose and taken immediately to the cold room. All subsequent steps were carried out at 0–4°C. The gland was minced on a clean plastic surface with a razor blade

Abbreviations: ZG, zymogen granule; ZGM, zymogen granule membrane; PL-C, phospholipase C; cmc, critical micellar concentration; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

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[†]The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. M37032).

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and passed through a tissue press to remove connective tissue. Homogenization (1:5, wt/vol) was carried out in a glass/Teflon homogenizer with ice-cold buffer A [0.25 M sucrose/5 mM Mops, pH 6.8/0.1 mM MgSO_4 /1 mM dithiothreitol (DTT)/0.1 mM phenylmethanesulfonyl fluoride (PMSF)]. Nuclei and debris were removed by centrifugation at $500 \times g$ for 5 min. Supernatant fractions were pooled and incubated on ice until pellet fractions were extracted with buffer A (one homogenizing stroke) and sedimented as before. Supernatant fractions (containing ZGs, lysosomes, mitochondria, rough and smooth microsomes) were combined and centrifuged in a Beckman TJ6 centrifuge at $2000 \times g$ for 10 min. The supernatant was discarded and the crude ZG fraction was suspended with one homogenizing stroke in 40% Percoll (Sigma)/0.25 M sucrose/50 mM Mes, pH 5.3/0.1 mM MgSO_4 /5 mM EGTA/1 mM DTT/0.1 mM PMSF to 48 ml. This mixture was centrifuged in 12-ml polycarbonate tubes in a Beckman L8-80M ultracentrifuge using a Ti-75 rotor at $100,000 \times g$ for 20 min. The dense white band near the bottom of the tube (ZG fraction) was carefully removed and diluted to 30 ml with buffer A. ZGs were sedimented at $2000 \times g$ for 20 min to remove Percoll. The ZG pellet was suspended in 2 ml of 0.17 M NH_4Cl (pH 5.0) and incubated on ice for 10 min to lyse contaminating erythrocytes. Buffer A was added to ≈ 30 ml and ZGs were again pelleted at $2000 \times g$ for 20 min.

Purified ZGs were suspended and lysed in 8 ml of 0.1 M Na_2CO_3 (pH 11.2) containing aprotinin (20 kallikrein inhibitor units/ml), soybean trypsin inhibitor (0.1 mg/ml), PMSF (1 mM), and DTT (1 mM). The lysate was layered on a discontinuous sucrose gradient (2 ml of 0.25 M sucrose over 2 ml of 1.3 M sucrose in a 13-ml polyallomer tube). Gradients were centrifuged in a Beckman SW-40 rotor at $200,000 \times g$ for 70 min. The band at the sucrose interface (ZGM fraction) was collected and diluted into 24 ml of buffer B [0.1 M Na_2CO_3 , pH 11.2/0.3 M Na_2SO_4 /100 mM KCl/5 mM EDTA/0.1 mM PMSF/1 mM DTT containing aprotinin (20 units/ml)] and pelleted in Ti-60 tubes at $225,000 \times g$ for 60 min. The final pellet of ZGMs was suspended in $\approx 80 \mu\text{l}$ of 50 mM Mops, pH 6.8/1 mM DTT containing FOY-305 (20 $\mu\text{g}/\text{ml}$; Sanol Schwarz, F.R.G.), aliquoted into plastic vials, quick frozen in liquid nitrogen, and stored at -80°C until use. Protein was determined according to Bradford (18). Amylase (Phadebas reagent, Pharmacia) activity was measured on tissue homogenate, postnuclear supernatant, ZGs purified on Percoll, and final ZGM fractions.

Analysis of Membrane Proteins by Glycanase Digestion and SDS/PAGE. Purified ZGMs were incubated with N-Glycanase (10 units/ml) and/or O-Glycanase (5 milliunits/ml) in the presence of 0.17% SDS as described by the manufacturer (Genzyme). Intact or deglycosylated ZGMs were solubilized in 1.6% SDS/0.11 M Tris-HCl, pH 7.5/5% (vol/vol) 2-mercaptoethanol/6.6% sucrose and heated at 100°C for 3 min. Proteins were separated in one dimension by SDS/10% PAGE stained with silver nitrate (20).

Purification of GP-2 and Determination of Amino Acid Sequence. Glycosylated GP-2 [75 kDa (gp75)] and deglycosylated GP-2 [52 kDa (p52)] were purified from ZGM by SDS/PAGE and transblotted to nitrocellulose (Bio-Rad) or Immobilon-P (Millipore) in a semidry electroblot apparatus (LKB). Proteins attached to membranes were stained with Ponceau S. Individual protein bands were excised and washed in pure water. The protein sample on Immobilon-P was applied to a gas-phase sequencer (Applied Biosystems) for direct N-terminal sequencing (21). The sample on nitrocellulose was digested *in situ* with $\approx 5\%$ (wt/wt) trypsin for 16 hr at 37°C (22). Peptides were extracted with 5% acetonitrile and separated by HPLC on a Vydac C_{18} reverse-phase column using a 0–95% acetonitrile gradient in 0.1% trifluoroacetic acid. Individual peptide peaks were dried, suspended in water, and applied for gas-phase sequencing.

Polymerase Chain Reaction (PCR). Mixed oligonucleotide primers (27-mers) were synthesized (Applied Biosystems oligonucleotide synthesizer) on the basis of N-terminal and internal peptide sequences. Primers contained all possible codon degeneracies with heterologous restriction enzyme sites protected by three additional nucleotides attached to 5' ends. Full-sized primers were purified by denaturing gel electrophoresis. PCRs were conducted on template DNA (single-stranded cDNA) with reagents supplied in kit form (Perkin-Elmer/Cetus) in a DNA thermal cycler (Perkin-Elmer/Cetus) using a thermal program previously described (23). Products were separated in 3% NuSieve/1% Seakem agarose (FMC) gels and stained with ethidium bromide.

Analysis of PCR Products and cDNA Clones. DNA fragments were purified, by gel electrophoresis, removed from gel slices with NaI and glass beads (Gene Clean kit, Bio 101, La Jolla, CA), and subcloned into pBluescript II (Stratagene) by use of appropriate restriction enzymes and standard recombinant DNA methods (24). cDNA libraries were screened using a probe labeled with [α - ^{32}P]dCTP ($1\text{--}2 \times 10^9$ cpm/ μg) by use of a random priming kit (Pharmacia). Pre-hybridization, hybridization, and wash conditions were as described (23). DNA sequences were determined in both orientations by the dideoxy chain-termination method (25) with [α - ^{35}S]thio[dATP and Sequenase (United States Biochemical). Overlapping clones were constructed by using nested sets of deletions prepared with exonucleases III and VII (26).

Enzymatic Cleavage of GP-2 from ZGMs. For studies with phospholipase C (PL-C) from *Bacillus cereus*, ZGMs were purified from dog pancreatic ZGs (Percoll-purified) by alkaline lysis at pH 11.2 (see above). Purified membranes (20 $\mu\text{g}/\text{ml}$) were incubated in 50 μl of 20 mM Mes, pH 7.0/80 mM KCl with saponin (45 $\mu\text{g}/\text{ml}$) and trypsin inhibitors (aprotinin, 20 units/ml; FOY-305, 40 $\mu\text{g}/\text{ml}$) in the presence or absence of *B. cereus* PL-C (70 $\mu\text{g}/\text{ml}$). For studies that investigated the endogenous release of GP-2, dog pancreas ZGs were permeabilized with saponin (Sigma) at 45 $\mu\text{g}/\text{ml}$ [below the critical micellar concentration (cmc)]. Permeabilization allowed the release of secretory products (monitored by amylase release) and GP-2 from granule compartments. Following incubation of samples in both types of experiments, membrane-bound and free products were separated and GP-2 contained in both fractions was quantified on Western blots by a two-step ^{125}I immunolabeling procedure (to be published elsewhere).

RESULTS

ZGs were purified on self-forming Percoll gradients by a modification of earlier procedures (8, 27). ZGs, lysed in alkaline buffer at pH 11.2, yielded ZGMs with $<0.1\%$ amylase contamination after purification by sedimentation in discontinuous sucrose gradients and aqueous buffers. Control experiments indicated that amylase was not denatured by treatment with Na_2CO_3 at pH 11.2 for 60 min at 4°C . Fig. 1 shows the protein composition of purified ZGM stained with silver nitrate. At this level of protein analyzed, four major proteins can be seen with molecular mass of 90, 75 (major component), 47, and 25 kDa. N-Glycanase treatment indicated that the 75- and 47-kDa proteins contained N-linked oligosaccharide chains. N-Glycanase reduced the 75-kDa (gp75) and 47-kDa (gp47) bands to 52 kDa and 42 kDa, respectively. O-Glycanase with or without prior neuraminidase treatment showed little or no effect on the relative mobilities of ZGM proteins. These findings suggest that gp75 and gp47 contain seven to eight and two to three N-linked oligosaccharide chains, respectively.

Although purified ZGM contained minimal amylase activity, contamination of membranes has been shown to vary significantly according to isoelectric point of soluble proteins

FIG. 1. Separation of dog pancreatic ZGM proteins by SDS/PAGE. Proteins contained in Percoll-purified granule membranes were separated in 10% polyacrylamide gels and visualized by silver staining. Lane 1, molecular mass markers (kDa at left); lane 2, intact ZGMs (1.8 μ g of protein); lanes 3–6, prior to electrophoresis ZGMs were treated for 16 hr with N-Glycanase (lane 3), O-glycanase (lane 4), neuraminidase followed by O-glycanase (lane 5), and combined N- and O-Glycanase (lane 6).

(28). To avoid the influence of minor contaminants, we devised an approach that employed a band-shift strategy in our molecular cloning studies. N-terminal peptide sequence was obtained from intact GP-2 (gp75) purified by SDS/PAGE and transblotted to Immobilon-P. Internal peptide sequences were obtained from deglycosylated GP-2 (p52) purified by SDS/PAGE, transblotted to nitrocellulose, and digested overnight with trypsin. Fig. 2 shows the peptide elution profile obtained from C_{18} reverse-phase HPLC and identifies three peptides chosen for sequence analysis. Since the proteins that might contaminate gp75 could be expected to differ from those that might contaminate p52, peptide sequences obtained from contaminating proteins in either band were not expected to collaborate as partners during the PCR. Direct N-terminal sequencing from gp75 showed the sequence EQQGNRLINTXSYPXQ. Internal tryptic peptides showed NWIDVTDPQAR (peak 34), VLDLGPITR (peak 43), and SEIVAINPAR (peak 27). By using sense oligonucleotides representing the N-terminal sequence and antisense oligonucleotides representing peaks 34, 43, and 27, a 700-base-pair (bp) PCR product was obtained with the antisense oligonucleotide mixture representing peak 34 (Fig. 3).

The 700-bp fragment amplified by PCR was subcloned into the pBluescript II vector by using the unique *EcoRI* and *BamHI* sites at the 5' and 3' termini of the coding sequence,

FIG. 2. HPLC separation of peptides generated by *in situ* trypsin digestion of purified GP-2. Deglycosylated gp75 (i.e., p52) was purified by SDS/PAGE, transblotted to nitrocellulose, and digested with trypsin at 37°C for 16 hr. Liberated peptides were separated by HPLC on a C_{18} reverse-phase column. Peptide elution was monitored at 210 and 277 nm. The three peptides indicated by the arrows (27, 34, and 43) were sequenced in a gas-phase sequencer. mAU, milliabsorbance units.

respectively. One of the resulting clones, pBS700E/B, was colony-purified, verified by restriction enzyme analysis, and sequenced. The 700-bp fragment, excised by *EcoRI*/*BamHI* digestion, was labeled with [α - 32 P]dCTP and used to screen a canine cDNA library (2×10^5 clones) constructed in λ ZAP II. To our surprise, the 700-bp product representing GP-2 identified 0.5% of the clones in the library, indicating that GP-2 mRNA approximates the abundance observed in mRNAs encoding secretory proteins (0.3–20%). Eighteen positive clones from the dog cDNA library were plaque-purified. R-408 helper phage was used to rescue inserts into Bluescript plasmids. Two overlapping clones (160 bp of identical overlapping sequence) from the dog cDNA library, D-41 (1.4-kbp insert) and D-44 (1.4-kbp insert), were analyzed by restriction enzyme analysis and sequenced in both directions. The 700-bp PCR product contained 632 bp of sequence between the two primers that was identical to that observed in the cDNA structure.

The nucleotide sequence that encodes GP-2 found in the dog pancreatic ZGM (Fig. 4) has a 1527-bp open reading frame that encodes a protein of 509 amino acids. The N-terminal peptide sequence as well as each of the three internal sequences, determined by gas-phase sequencing of tryptic peptides (peaks 27, 34, and 43), were observed in the amino acid sequence deduced from the cDNA. The N terminus shows a typical signal sequence containing a hydrophobic region of 19 amino acids (Met¹ to Ala¹⁹). N-terminal sequence from the mature gp75 begins at Glu²², which indicates that the signal sequence is removed before insertion of GP-2 into the ZGM. Signal cleavage probably occurs cotranslationally in the rough endoplasmic reticulum, as previously described for pancreatic secretory proteins (4, 29). At the C terminus of the GP-2 sequence is a hydrophobic region of 20 amino acid residues suggestive of a transmembrane domain. The sequence reveals eight potential N-linked oligosaccharide attachment sites, which is in good agreement with the numbers of attachment sites predicted by mobility shifts in SDS/polyacrylamide gels after N-Glycanase treatment. The presence of 26 cysteine residues suggests that the molecule may be rich in disulfide bonds.

GP-2 was completely released from ZGMs, prepared by lysis of granules at pH 11.2, after incubation of these membranes with PL-C from *B. cereus* (Fig. 5A). In order to look for endogenous release of GP-2 from ZGMs under physiological conditions, ZGs were permeabilized with saponin at concentrations below the cmc. In the presence of 80 mM KCl, >98% of amylase was released from permeabilized granules in 10 min. Over the same period we observed a rapid release of GP-2 ($\approx$ 40%) at 0° or 37°C. Beyond 10 min we observed a time-dependent release of GP-2 at 37°C (Fig. 5B) but not at 0°C (data not shown). At 60 min, 90% of GP-2 was released at 37°C.

DISCUSSION

The complete peptide structure of dog pancreatic GP-2, the major glycoprotein in ZGMs, is presented. The presence of eight N-linked oligosaccharide attachment sites is consistent with the observed effect of N-Glycanase, which reduced the

FIG. 3. PCR yields a 700-bp probe. Mixed oligonucleotide primers were synthesized according to N-terminal and internal peptide (peak 34) sequences described in *Materials and Methods*. PCR products were electrophoresed in 3% NuSieve/1% SeaKem agarose gels and visualized with ethidium bromide. Lane 1, molecular size markers (*Hae* III digest of ϕ X174 phage DNA); lane 2, PCR products.

The structural and functional properties of GP-2 demonstrated here are similar to those of a group of phosphatidylinositol-glycan-linked ("PI-G-tailed") glycoproteins (30) observed in a variety of biological systems in prokaryotes (31), yeast (32), parasites (33, 34), and mammalian cells and tissues (32). In each case, these glycoproteins are attached to cell membranes via a phosphatidylinositol-glycan linkage and released to the external medium of the cell. However, the mechanism and significance of release of these proteins have remained obscure. Studies in polarized MDCK cells indicate that these proteins are exclusively sorted to the apical plasma

FIG. 5. Enzymatic cleavage of GP-2 from ZGMs. (A) ZGMs purified from dog pancreas ZGMs by alkaline lysis at pH 11.2 and passed through three freeze-thaw cycles (to inactivate endogenous enzymes) were incubated for 60 min at 37°C in 20 mM Mes, pH 7.0/80 mM KCl with saponin (45 μ g/ml) and trypsin inhibitors (aprotinin, 20 units/ml; FOY-305, 40 μ g/ml) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of *B. cereus* PL-C. Reaction mixtures were then placed at 0°C, and membrane (lanes 1 and 3) and supernatant (lanes 2 and 4) fractions were separated by ultracentrifugation at 200,000 $\times$ g for 40 min at 4°C. GP-2 was quantified on Western blots by an 125 I immunoblotting procedure. (B) Dog pancreatic ZGMs, permeabilized with saponin at 45 μ g/ml (below the cmc), were incubated at 37°C in 20 mM Mes (pH 7.0) with 80 mM KCl plus trypsin inhibitors as described above for various times in the absence of added PL-C. Reactions were terminated at 0 min (lanes 1 and 4), 10 min (lanes 2 and 5), and 60 min (lanes 3 and 6). Pellet (lanes 1–3) and supernatant (lanes 4–6) fractions were separated and GP-2 was quantified as described above.

membrane (35), possibly following the apical sorting of glycolipids (36). The high lateral mobility of phosphatidylinositol-glycan-linked membrane proteins, approaching those for membrane lipids, can be expected to confer specialized properties on the apical plasma membrane (19). One of these properties may be the capability to support enhanced exocytosis and endocytosis at the apical plasma membrane.

Our findings indicate that a single gene encodes the membrane-bound and free forms of GP-2. They further suggest that the mechanism for this release is a granule-associated enzyme, most likely a phospholipase, responsible for cleavage of the phosphatidylinositol-glycan anchor. The structural and functional properties of dog pancreatic GP-2, taken together with the known abundance of GP-2 in pancreatic ZGMs from a variety of species, raise the question whether GP-2 is involved in trans-Golgi assembly of ZGM domains capable of compartmentalizing secretory products targeted to the apical plasma membrane.

Note Added in Proof. Since the submission of this manuscript for review (i) we have published the complete nucleotide and amino acid sequences of rat pancreatic GP2 (37) (EMBL, accession no. X53935) and (ii) biochemical evidence has been presented that rat GP2 contains a phosphatidylinositol-glycan-linked anchor (38).

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