A Potential Endogenous Ligand of Annexin IV in the Exocrine Pancreas

CARBOHYDRATE STRUCTURE OF GP-2, A GLYCOSYPHOSPHATIDYLINOSITOL-ANCHORED GLYCOPROTEIN OF ZYM OGEN GRANULE MEMBRANES*

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We demonstrated previously that annexins IV, V, and VI, proteins of the calcium/phospholipid-binding annexin family, have glycosaminoglycan binding properties (Ishitsuka, R., Kojima, K., Utsumi, H., Ogawa, H., and Matsumoto, I. (1998) J. Biol. Chem. 273, 9935–9941). In this study, we investigated the endogenous ligands of annexin IV in the exocrine pancreas. Immunohistochemical study of bovine pancreas showed that annexin IV localized in the apical cytoplasmic region of pancreatic acinar cells where zymogen granules are concentrated. Because it is the major component of the zymogen granule membrane, the glycosylphosphatidylinositol-anchored glycoprotein GP-2 was suggested to play a role in apical sorting and secretion of zymogens. We isolated GP-2 from porcine pancreas extract and determined the structure of its N-linked oligosaccharides by two-dimensional mapping. The major carbohydrate structures of porcine GP-2 were trisialo-triantennary and tetrasiA-tri-antennary complex-type oligosaccharides. Dot-blot assay showed that annexin IV interacts with GP-2 in the presence of calcium and that it recognizes the terminal sialic acid residues linked through α2–3 linkages to the carbohydrate of GP-2. Lectin blot assay showed that Maackia amurensis mitogen, a plant lectin specific for the trisaccharide sequence Siaα2–3Galβ1–4GlcNAc of N-linked oligosaccharides, has strong affinity for GP-2. Thus, M. amurensis mitogen was used as a specific probe for GP-2 in the histochemical staining of the bovine pancreas. GP-2 was found to localize exclusively in the same apical cytoplasmic region of pancreatic acinar cells as annexin IV does. These results suggest that GP-2 is an endogenous ligand of annexin IV in the exocrine pancreas.

Annexins are a family of structurally related proteins that bind to phospholipids in a calcium-dependent manner. They consist of four or eight conserved repeating structures of ~70 amino acid residues and have an N-terminal domain, which is highly variable in both sequence and length, that distinguishes different family members. Their exact biological functions in vivo have not yet been elucidated completely, although a number of in vitro experiments have indicated that some annexins exhibit membrane channel activity, inhibit phospholipase A2, and blood coagulation, function in regulation of membrane traffic and exocytosis, and transmit intracellular signals as a kinase substrate (1–3).

We reported previously that a protein isolated from bovine tissues, p33/41, is a carbohydrate-binding protein specific for sialoglycoproteins and glycosaminoglycans and is identical to annexin IV (4). Further, we elucidated recently the glycosaminoglycan binding properties of annexins IV, V, and VI and showed that each of them has its own specific binding activities to glycosaminoglycans (5). Immunoblot analysis of various bovine tissues revealed high expression of annexin IV in kidney, liver, pancreas, and intestine (4). In bovine kidney, annexin IV was found to localize in the apical plasma membrane in renal proximal tubules (6). Our preliminary experiments using bovine pancreas sections showed that annexin IV localized in the apical cytoplasmic region of pancreatic acinar cells. Localization of annexin IV in these epithelial cells suggests its functional contribution to the exocytotic pathway. In the exocrine pancreas, digestive enzymes are stored in secretory zymogen granules (ZGs)1 within the apical region of acinar cells and released to the luminal ducts in response to hormone signals. It has been proposed that GP-2, the most abundant glycoprotein in ZG membranes (25–40% of membrane protein) (7), functions as a critical component in the secretory granule formation and in regulated membrane trafficking along the apical secretory pathway (8). GP-2 is attached to the granule membrane via a glycosylphosphatidylinositol (GPI) anchor (9) and is heavily glycosylated with N-linked oligosaccharides accounting for ~15–30% of its molecular mass (10, 11). Taking into account the carbohydrate binding activity of annexin IV, we postulate that annexin IV contributes to granule formation, presumably by interaction with GP-2.

In this study, we isolated zymogen granule membranes (ZGMs) from bovine pancreas and examined interaction between GP-2 and annexin IV in vitro. We determined the structure of N-linked oligosaccharides on porcine GP-2 and revealed

1 The abbreviations used are: ZG, zymogen granule; GPI, glycosylphosphatidylinositol; ZGM, ZG membrane; FL-PLC, phosphatidylinositol phospholipase C; HRP, horseradish peroxidase; GST, glutathione S-transferase; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholinethanesulfonic acid; KIU, kallikrein inhibitor unit; PVDF, polyvinylidene difluoride; CBB, Coomassie Brilliant Blue; HPLC, high performance liquid chromatography; THG, Tamm-Horsfall glycoprotein; MAM, Maackia amurensis mitogen; RCA, Ricinus communis agglutinin.
the structural elements that annexin IV recognizes. The localization of GP-2 in exocrine pancreas was investigated and compared immunohistochemically with that of annexin IV. The possible contribution of annexin IV to the exocrine pancreas is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine pancreas, porcine pancreas, and bovine urine were obtained from a local slaughterhouse. Rat pancreas was excised from freshly killed rats purchased from Japan Biological Materials Center (Tokyo, Japan). Percoll, soybean trypsin inhibitor, phosphatidylinositol phospholipase C (PI-PLC), bovine transferrin, calf fetuin, and bovine pancreatic DNase B were purchased from Sigma. The synthetic serine protease FOY-305 (camostate) was from Sanol Schwarz (Mannheim, Germany). Neuraminidase from Vibrio cholerae, N-glycosidase F from Flavobacterium meningosepticum, and Triton X-100 were from Roche Molecular Biochemicals. α2,3-Sialidase from Salmonella typhimurium LT2, standard pyridylamine-oligosaccharides, and pyridylamine-luciglucone oligomers were from Takara Shuzo (Kyoto, Japan). Biotinylated or horseradish peroxidase (HRP)-conjugated plant lectins were from Seikagaku Kogyo (Tokyo, Japan). HRP-conjugated biotin-avidin complex was purchased from Vector Labs (Burlingame, CA). Monosaccharides, 2-amino pyridine, acrylamide, SDS, and other gel electrophoresis reagents were obtained from Wako Pure Chemicals (Osaka, Japan). Bovine serum albumin (BSA) and bovine thyroglobulin (Avantisam Biosciences), and recombinant protein was produced as a GST fusion protein as described previously (12). Rabbit polyclonal antibody to annexin IV was prepared in our laboratory. HRP-conjugated goat anti-rabbit IgG antibody was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). The Gel filtration resin Sephadex G-15 was from Seikagaku Kogyo (Tokyo, Japan). A PALPK Type A column (4.6 × 150 mm) was from Takara Shuzo (Kyoto, Japan). A Shim-pack CLC-ODS column (6 × 150 mm) was from Shimazu Co. (Kyoto, Japan). A TSK-GEL Amide-80 column (4.6 × 250 mm) was from Tosoh Corp. (Tokyo, Japan).

**Isolation of ZGMs and Enzymatic Release of GP-2**—The pancreatic ZGM from V. cholerae was purified by the following procedures, based on the method of Fukuoka et al. (13). Briefly, murine, porcine, and bovine pancreatic glands were homogenized, and ZGMs were isolated by centrifugation of the homogenate on a self-forming continuous Percoll gradient (40% Percoll (v/v) at 100,000 g for 20 min). The dense white band near the bottom of the tube (ZG fraction) was carefully harvested and washed with buffer containing 5 mM MOPS, pH 6.8, 1 mM dithiothreitol, 20 mM Na2CO3, pH 11.2. Purified ZGMs were suspended in stock buffer (50 mM MOPS, pH 6.8, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). The lysate was layered on a discontinuous sucrose gradient (0.25 M/1.3 M) and centrifuged at 200,000 g for 20 min. The supernatant was lyophilized, and the reducing ends of the oligosaccharides were pyridylaminated as described by Kondo et al. (17). Pyridylamine-oligosaccharides were first separated by gel filtration on a Sephadex G-15 column. Then each fraction was separated further by reverse-phase HPLC on a Shim-pack CLC-ODS column and subsequent size-fractionation HPLC on a TSK-GEL Amide-80 column. Elution and detection were performed as described by Tomiya et al. (18). The structures of pyridylamine-oligosaccharides were determined by the two-dimensional sugar mapping method using these two kinds of HPLC in combination with α2,3-sialidase (from S. typhimurium) digestion.

**Purification of Tamm-Horsfall Glycoprotein (THG)**—THG was purified from NA urine, described by Tamm and Horsfall (19, 20) with some modification. Briefly, NaCl in limited amounts was added to the bovine urine containing 0.2% NaN3 to a final concentration of 1.0 M. After incubation at 4 °C overnight, the solution was centrifuged at 58,000 × g for 20 min. The precipitated crude THG was suspended in a small amount of water and dialyzed against water containing 0.2% NaN3. Precipitation of THG in this fraction was repeated as above. The final dialyzed solution was lyophilized and stored at −20 °C.

**Glycoprotein Binding Assay**—Various amounts of glycoprotein samples were dot-blotted onto PVDF membrane and subjected to desialylation or desulfation as described above. The membranes were allowed to react with 10 μg/ml biotinylated recombinant GST-annexin IV, followed by HRP-conjugated biotin-avidin complex as described above in the presence of 5 mM CaCl2 or 2 mM EDTA as a calcium chelating agent. Each membrane was developed with 3,3′-diaminobenzidine tetrahydrochloride and avidin complex and washing in phosphate-buffered saline, the sections were blocked with 2% skim milk and 1% normal goat serum. The sections were incubated with biotinylated MAM for 2 h and then washed in phosphate-buffered saline. After incubation with the secondary antibodies or HRP-conjugated biotin-avidin complex and washing in phosphate-buffered saline, the sections were developed with 3,3′-diaminobenzidine tetrahydrochloride and H2O2, and then stained with hematoxylin. Desialylation treatment with neuraminidase from V. cholerae at 37 °C for 2 h was carried out before blocking of sections with skim milk, and then they were allowed to react with biotinylated MAM.

**RESULTS**

**Analysis of Carbohydrate Structure of GP-2**—Prior to investigating the interaction of annexin IV with GP-2, we analyzed the carbohydrate structures of GP-2. In a previous study, GP-2 was shown to possess sialylated N-linked oligosaccharides (13), but carbohydrate structures have not been elucidated in detail. Another glycoprotein, THG, is produced mainly in renal epithelial cells. THG and GP-2 are encoded by a homologous gene family (25), and these proteins have remarkable similarity in their primary structures. The carbohydrate structures of THG have already been studied in detail, so we first characterized the carbohydrate structures of GP-2 by their lectin reactivities and sugar composition compared with those of THG.

On SDS-PAGE, ZGM fractions isolated from bovine pancreas extracts were found to contain GP-2 with a molecular mass of 4 × 10^6 m trifluoroacetic acid at 100 °C for 4 h. Hydrolyzed saccharides were extracted from the PVDF membrane with MeOH and H2O, then transferred to a glass tube. Pyridylamination of monosaccharides was carried out according to the protocol of a TaKaRa PALSTATION model 4000. The resulting monosaccharide mixtures were analyzed by high-performance liquid chromatography (HPLC) using the anion exchanger column PALPK Type A (4.6 × 150 mm). Elution was performed with 0.7 mM citric acid, pH 9.0, 10% acetonitrile at 37 °C at a flow rate of 0.3 ml/min. The fluorescence was recorded using excitation and emission wavelengths of 310 and 380 nm, respectively.

**Two-dimensional Mapping Analysis of Pyridylamine-oligosaccharides from pGP-2—pGP-2 was denatured by 2% SDS at 100 °C for 5 min. Then N-linked oligosaccharides of pGP-2 were released by exhaustive digestion with N-glycosidase F, pH 8.6, at 37 °C for 24 h. The supernatant was lyophilized, and the reducing ends of the oligosaccharides were pyridylaminated as described by Kondo et al. (17). Pyridylamine-oligosaccharides were first separated by gel filtration on a Sephadex G-15 column. Then each fraction was separated further by reverse-phase HPLC on a Shim-pack CLC-ODS column and subsequent size-fractionation HPLC on a TSK-GEL Amide-80 column. Elution and detection were performed as described by Tomiya et al. (18). The structures of pyridylamine-oligosaccharides were determined by the two-dimensional sugar mapping method using these two kinds of HPLC in combination with α2,3-sialidase (from S. typhimurium) digestion.
The ZGM fraction was solubilized with 2% SDS and 2% 2-mercaptoethanol, loaded onto a 9.5% acrylamide gel, and then electroblotted onto a PVDF membrane. Proteins were stained with 0.1% CBB and allowed to react with biotinylated RCA (lane 5). The values on the left indicate the migration positions of the molecular mass markers (lane 1).

**FIG. 2. Lectin blot analysis of ZG fractions.** ZG fractions were solubilized with 2% SDS and 2% 2-mercaptoethanol, loaded onto a 9.5% acrylamide gel, and then electroblotted onto a PVDF membrane. Proteins were stained with 0.1% CBB (lane 2) and allowed to react with biotinylated MAM (lane 3), biotinylated RCA (lane 5), and HRP-conjugated ConA (lane 7). Desialylation with neuraminidase from *V. cholerae* was carried out after electroblotting onto a PVDF membrane and then the ZG fractions were allowed to react with biotinylated MAM (lane 4) and biotinylated RCA (lane 6). The values on the left indicate the migration positions of the molecular mass markers (lane 1).

**TABLE I**

Lectin reactivities of murine, porcine, and bovine GP-2 and bovine THG

| Lectin | Specificity | Rat GP-2 | Porcine GP-2 | Bovine GP-2 | Bovine THG |
|--------|-------------|---------|-------------|--------------|-----------|
| MAM    | Terminal sequence of Siaα2–3Galβ1–4GlcNAc | + | + | + | + |
| SSA    | Terminal sequence of Siaα2–6Gal | – | – | – | ± |
| WGA    | Terminal Sia and bisecting GlcNAc | + | + | + | + |
| ConA   | Biantennary type oligosaccharides | ± | + | + | + |
| PHA-L  | Tri- and tetraantennary-type oligosaccharides | + | + | + | + |
| PHA-E  | Bi- and triantennary-type oligosaccharides with bisecting GlcNAc | + | + | + | + |
| LCA    | Bi- and triantennary oligosaccharides with Fuc | + | + | + | + |
| RCA    | Terminal Gal residues | – | – | – | – |
| PNA    | Terminal Gal residues of O-linked oligosaccharides | – | – | – | + |

**TABLE II**

Neutral sugar and hexosamine compositions of murine, porcine, and bovine GP-2 and bovine THG

The relative number of each monosaccharide was calculated as a proportion of 3 mol of mannose because 1 mol of complex-type oligosaccharide contains 3 mol of mannose residues (ratio). Determinations were done in triplicate. The abbreviations used are: GalNAc, N-acetylglucosamine; GlcNAc, N-acetylgalactosamine; Man, mannose; Fuc, fucose; Gal, galactose.

| Sugar | Ratio GP-2 | Ratio Porcine GP-2 | Ratio Bovine GP-2 | Ratio Bovine THG |
|-------|-----------|--------------------|-------------------|-----------------|
| GalNAc| 0.0       | 0.0                | 0.9               | 2.5             |
| GlcNAc| 44.2      | 5.4                | 42.0              | 43.7            |
| Man   | 24.5      | 3.0                | 21.7              | 23.6            |
| Fuc   | 8.3       | 1.0                | 7.9               | 7.9             |
| Gal   | 23.0      | 2.8                | 24.9              | 22.3            |

98 kDa as the major component (Fig. 1). Identification of GP-2 was carried out by N-terminal amino acid sequencing (data not shown). The ZG fractions isolated from bovine pancreas extracts were electroblotted onto a PVDF membrane and allowed to react with various lectins (Fig. 2). Because the zymogen granules contain a large amount of enzyme precursors, bovine GP-2 is a minor component in the fraction (Fig. 2, lane 2). Many enzyme precursors were suggested to have biantennary-type N-linked oligosaccharides that were recognized by concanavalin A (21) (Fig. 2, lane 7). MAM, specific for the terminal sequence Siaα2–3Galβ1–4GlcNAc of N-linked oligosaccharides (22, 23), reacted strongly with only bovine GP-2 (Fig. 2, lane 3). After desialylation with neuraminidase, MAM lost its affinity for bovine GP-2 (Fig. 2, lane 4). In addition, bovine GP-2 interacted with neither *Sambucus sieboldiana* agglutinin, specific for the terminal sequence Siaα2–6Gal (24) (Table I), nor with *Ricinus communis* agglutinin (RCA), specific for terminal Gal residues of N-linked oligosaccharides (26, 27) (Fig. 2, lane 5). After treatment with neuraminidase, RCA recognized bovine GP-2 (Fig. 2, lane 6). These results suggested that almost all terminals of the N-linked oligosaccharides of bovine GP-2 contain sialic acid residues linked through α2–3 linkage. This may be a unique feature of the GP-2 oligosaccharides among a number of glycoproteins in the zymogen granule fraction. The lectin reactivity and sugar composition of the murine, porcine, and bovine GP-2 and bovine THG were investigated and are summarized in Table I and Table II, respectively. The detailed carbohydrate structures of human THG have already been revealed by means of one- and two-dimensional 1H NMR spectroscopy by Hard et al. (28). They showed that the major oligosaccharides of human THG were of the sialylated, tetra-antennary-type N-linked oligosaccharide with smaller amounts of the tri- and bi-antennary types. Most of the terminal sialic acid residues were linked through α2–3 linkages, although Siaα2–6Gal, Gal3SO4 has occurred as minor terminal structural elements. These carbohydrate structures of human THG were consistent with the results of lectin blot analysis (Table I) and sugar composition analysis (Table II) of bovine THG. As shown in Table I, the carbohydrate structure of GP-2 from three
different species and bovine THG had similar characteristics in their oligosaccharides, such as the antennary type, the linkages of sialic acid residues, and the locations of fucose residues. Further, they had similar sugar compositions. These results suggested that they have similar features in their carbohydrate structures. GP-2 oligosaccharides were suggested to contain only small amounts of O-linked oligosaccharides, because little GalNAc, which is essential for the O-linked oligosaccharides, was detected in sugar composition analysis (Table II). An interesting finding was observed in the lectin reactivity experiments with bovine THG; after desialylation, bovine THG still reacts strongly to MAM, even though MAM is known as a sialic acid-specific lectin. As shown in Fig. 3, MAM interacted strongly with bovine THG corresponding to 106 kDa (Fig. 3, lane 6), indicating that sialic acids had been removed successfully, and galactose residues were exposed. MAM still had strong affinity for bovine THG after desialylation treatment (Fig. 3, lane 5), which differs from the reactivity of bovine GP-2, suggesting that MAM has the ability to bind to sulfate groups on sugar chains of THG. Methanolysis is an effective desulfation method that is expected to release both the sialic acids and sulfate groups of glycoproteins. As shown in Fig. 3, lanes 7 and 8, methanolysis completely abolished the reactivity of THG with MAM. Strong binding of RCA after methanolysis showed that this chemical treatment specifically eliminates sialic acids and sulfate groups of oligosaccharides without disruption of the structural framework of the oligosaccharides. It is noteworthy that MAM is capable of binding to sulfate groups on galactose residues instead of sialic acids as negative charged determinants.

Next, we performed two-dimensional mapping analysis of pyridylamino-oligosaccharides to determine structures of N-linked oligosaccharides from porcine GP-2. GP-2 prepared from porcine pancreas has a higher yield than bovine pancreas, so porcine GP-2 was used for the analysis. The pyridylamino-oligosaccharides were separated into three fractions by gel filtration, and each fraction was further separated into seven peaks. The elution positions (expressed as glucose oligomers) were plotted on a two-dimensional sugar map and compared with those of the respective standard oligosaccharides (18). The fractions obtained on digestion with a2–3-sialidase were analyzed again, their structures also being identified. The results showed that all sialic acid residues on the oligosaccharides were linked through α2–3 linkages. The structures of oligosaccharides derived from the seven peaks are shown in Table III. The mol % of each oligosaccharide was calculated on the basis of its peak area.

After N-glycanase treatment of porcine GP-2, its 91-kDa molecular mass was reduced to 56 kDa. The major oligosaccharides of porcine GP-2 were of the trisialo-triantennary-type (48.4 mol %) and tetrasialo-tetra-antennary-type (32.4 mol %) oligosaccharides. Other minor components were sialylated, tri-, or tetra-antennary type oligosaccharides. These carbohydrate structures of porcine GP-2 were consistent with the results of the lectin blot analysis. The mol ratios of GlcNAc:Man:Fuc:Gal of the oligosaccharides A and B in Table III were 5:1:3:3 and 6:3:1:4, respectively, which is approximately consistent with the results of the sugar composition analysis (Table II).

Interaction of Annexin IV with GP-2 and THG—Binding activities of annexin IV toward bovine GP-2 and bovine THG were examined by dot-blot assay, and the results were shown in Fig. 4. Annexin IV was found to interact with both bovine GP-2 and bovine THG in the presence of 5 mM CaCl₂. After desialylation of bovine GP-2, annexin IV lost affinity for it, indicating that annexin IV recognized sialic acid residues of bovine GP-2 oligosaccharides. On the other hand, asialo bovine THG was still recognized by annexin IV. To eliminate not only sialic acid
residues but also sulfate groups, methanolysis was used as described above. After this treatment, annexin IV lost affinity for bovine THG, suggesting that annexin IV strongly recognized the few sulfate residues of bovine THG oligosaccharides. In the same way, binding activities of annexin IV and MAM toward various glycoproteins were examined, and the results obtained are summarized in Table IV. These results indicate that negatively charged modifications with C-3 of galactose residues in N-linked oligosaccharides are important for carbohydrate recognition by annexin IV. Furthermore, annexin IV was shown to preferentially bind to bulky branched oligosaccharides, such as tri- or tetra-antennary-type carbohydrate structures.

**Localization of Annexin IV and GP-2 in Exocrine Pancreas**—Formalin-fixed and paraffin-embedded sections of normal bovine pancreas were stained immunohistochemically using polyclonal antibodies specific to bovine annexin IV. The carbohydrate structures of calf fetuin (54), bovine transferrin (55), and bovine pancreatic ribonuclease B (56) have already been revealed by the concentration of each glycoprotein when the value of the reflection at 370 nm was 1500.

To investigate the localization of GP-2, MAM was used for histochemical staining instead of a specific antibody against GP-2. Although monoclonal antibodies against rat GP-2 and dog GP-2 stained nothing in the bovine pancreas sections, MAM strongly stained the apical region of acinar cells. Because GP-2 is a secretory granule membrane protein, it should be concentrated in the zymogen granules. As shown in Fig. 5b, the characteristic MAM staining was consistent with the localization of zymogen granules. Because MAM recognizes only GP-2 in the zymogen granule fractions (Fig. 2), the staining by MAM in Fig. 5b was suggested to represent the localization of GP-2. After desialylation treatment, MAM stained nothing in the exocrine pancreas (Fig. 5c). Thus, it was confirmed that MAM specifically recognized sialic acid residues of GP-2 oligosaccharides. The restriction of GP-2 to the apical region of acinar cells seems to be similar to that of annexin IV.

**DISCUSSION**

GP-2 has been found in the pancreas of all species studied so far, including rat, rabbit, dog, sheep, cow, and pig, as a major component of zymogen granule membranes (7). Further, cDNA sequences of dog, rat, and human GP-2 have been determined (13, 29, 30). The number of potential N-glycosylation sites in these cDNA sequences were eight in dog GP-2, eight in rat GP-2, and eleven in human GP-2. GP-2 is highly glycosylated with N-linked oligosaccharides, accounting for ~15–30% of its molecular mass (10, 11). For example, N-glycanase treatment of dog GP-2 reduced its molecular mass by 23 kDa, which suggests that all eight N-glycosylation site are glycosylated (13).

**Table IV**

**Glycoprotein binding properties of annexin IV and MAM**

| Glycoprotein | Acidic residues linked to Gal | Major antennary-type | Annexin IV | MAM |
|--------------|------------------------------|----------------------|------------|-----|
| GP-2         |                              |                      | +          |     |
| Asialo GP-2  |                              |                      | ++         | ++  |
| THG          |                              |                      | ++         | ++  |
| Asialo THG   |                              |                      | ++         | +   |
| Methanolysed THG |                      |                      | ++         | +   |
| Calf fetuin (54) |                              |                      | ++         | +   |
| Bovine transferrin (55) |                              |                      | ++         | +   |
| Bovine ribonuclease B** |                              |                      | ++         | +   |

[Fig. 4. Binding assay of annexin IV toward GP-2/THG family of GPI-anchored protein. Varying amounts of bGP-2 (a) and bTHG (b) were dot-blotted onto PVDF membrane and then incubated with biotinylated GST-annexin IV followed by HRP-conjugated avidin-biotin complex in the presence of 5 mM CaCl\(_2\) (●) or 2 mM EDTA (○). Binding toward desialylated GP-2/THG (●) and desialylated/desulfated GP-2/THG (○) was examined in the presence of 5 mM CaCl\(_2\) as above. The intensity of the staining was measured with a densitometer as described in Fig. 4 and represented by pluses and minuses determined by means of mass spectrometry and \(^1\)H-NMR spectroscopy. *, minor elements; **, high mannose-type oligosaccharide (56).]
In this study, the carbohydrate structure of porcine GP-2 was elucidated for the first time. N-Glycanase treatment of porcine GP-2 reduced its molecular mass by 35 kDa, which corresponds to the mass of about ten N-linked oligosaccharides. The major oligosaccharides of porcine GP-2 are sialylated tri-/tetra-antennary-type N-linked oligosaccharides with core fucose residues. Their terminal sialic acid residues are all linked through α2–3 linkages. These carbohydrate structures are very similar to those of human THG elucidated by Hard et al. (28). It is interesting that GP-2 and THG have similarity not only in amino acid sequences but also in carbohydrate structure. Their highly branched and sialylated oligosaccharides may contribute to their function in vivo.

THG is produced in the TALH (thick ascending limb of Henle) cells and distal collecting duct cells (31). These renal epithelial cells contain the Na⁺, K⁺, and Cl⁻ cotransporters responsible for reabsorption of anions and cations from the renal tubular fluid (32). Because THG is the most abundant protein in human urine with daily excretion of 50–100 mg (33), its function has been investigated over the past four decades. For example, it has been suggested to play a role in regulation of the circulating activity of cytokines (34), in inhibition of certain types of bacterial infection in the bladder and urinary tract (35), or in regulation of water permeability in the nephron (36), but the detailed function of THG remained unknown. In this study GP-2, as well as THG, was shown to be a good ligand for annexin IV, and both THG and annexin IV are produced abundantly in the kidney. The possibility that annexin IV contributes to physiological events regulated by THG in the kidney is interesting, but individual expression sites of annexin IV and THG so far investigated are not quite identical; annexin IV is localized in epithelial cells of renal proximal tubules and collecting ducts (6). Interaction between annexin IV and THG in the kidney is feasible, but further studies are necessary.

Both GP-2 and THG are GPI-anchored proteins associated with the membrane of secretory granules of polarized epithelial cells and secreted at the apical region of plasma membranes (37–39). Although GPI-anchored proteins are normally found attached to the outer surface of the plasma membrane, GP-2 and THG are released endogenously into luminal ducts of pancreas and kidney, respectively. It is interesting that GP-2 and THG exhibit similar pH- and ion-dependent self-aggregation properties in vitro (25) and that they were shown to be prominent components in protein precipitations in pancreatic fluid (40) and renal casts in urine (41), respectively. Thus, they were suggested to play a critical role in regulated secretory pathways. Their acidic carbohydrate moiety may be responsible for the self-aggregation property. A recent study of polarized GP-2 secretion in Madin-Darby canine kidney cells showed that GP-2 that has defects in glycosylation in the Golgi apparatus was not incorporated into zymogen granules, and it was transported to the basolateral membranes (42). These results suggested that the carbohydrate of GP-2 is important for its apical targeting. The carbohydrate structure of GP-2 revealed in this study will help to elucidate the role of GP-2.

In this study, dot-blot assay showed that annexin IV interacts with GP-2 in the presence of calcium. Further, immunohistochemical study on the exocrine pancreas showed that annexin IV, as well as GP-2, localized exclusively in the apical cytoplasmic region of acinar cells. Zymogen granules were reported to have a 10–13 mM internal Ca²⁺ concentration (9). These results suggested the possibility that GP-2 is the endogenous ligand of annexin IV. Recently Scheele et al. (8) showed that PI-PLC, which cleaves the GPI anchor and removes GP-2 from the membrane, also releases proteoglycans in the zymogen granules. Using electron microscopy, they showed that GP-2 and proteoglycans constitute a fibrillar matrix on the surface of zymogen granules. They presented the hypothesis that the GP-2/proteoglycan submembranous matrix may play a role in membrane sorting during granule assembly in the
trans-Golgi networks and in trafficking of ZG membranes from the apical plasma membrane after exocytosis. Recently we found that proteoglycans with molecular masses of >200 kDa are present in the peripheral components of zymogen granule membranes. Because annexin IV has the ability to interact with various glycosaminoglycan chains in the presence of calcium, annexin IV may play a role in cross-linking between GF-2 and proteoglycans. Further investigations are required to prove this hypothesis.

Because annexins lack hydrophobic N-terminal signal sequences, they have been considered to be primarily intracellular proteins and to function only in the cytoplasmic region. However, recent studies have shown that annexins are actually exported from cells and expressed on the cell surface. Yeatman and co-workers (43, 44) demonstrated the presence of annexins I-VI on the external surface of higher metastatic cell lines (45). The specific extracellular transport and expression mechanisms of annexins have not yet be defined.

Further study of the localization of annexin IV in acinar cells will elucidate its ligands and the transport mechanisms to the apical membrane.

Immunohistological study showed that annexin IV localized not only in pancreatic acinar cells but also in centroacinar cells, which are classified as ducetal elements. It is interesting that annexin IV localized mainly in the nucleus of centroacinar cells, a different localization from that observed in acinar cells. Ductal elements have various ion channels and secretory electrolytes such as Cl⁻, HCO₃⁻, Na⁺, and K⁺ to alkalize the acinar lumen in response to combined cholesterol and secretin stimulation (46). This alkalization solubilizes and releases aggregated enzymes of zymogen granules into the acinar lumen. Thus, ductal elements were thought to essentially regulate the secretion of pancreatic enzymes (47). The N-terminal domains of annexins contain phosphorylation sites for different protein kinases such as protein kinase C and tyrosine kinases, as well as protein-protein interaction domains. Annexins are reported to play a role in vitamin tyrosine kinase signaling in a variety of cell types (48–50). The annexin IV observed in nuclei of ductal elements in this study may play a role in signaling transduction in the regulated secretion pathway. A recent study of tyrosine phosphorylation of annexin V by the vascular epithelial growth factor receptor suggested that annexin V functions as a signaling protein that regulates vascular endothelial cell proliferation (51). Although annexin V is known to locate in the cytosol of various cells, the nuclear location of annexin V has also been reported (52). The nuclear location of annexin V appears to be controlled by signaling pathways involving serum factors and tyrosine kinases (53). The function of annexin IV in the ductal elements is now under investigation.

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