PURIFICATION AND PARTIAL CHARACTERIZATION OF AN INTEGRAL MEMBRANE GLYCOPROTEIN FROM ZYMOGEN GRANULES OF DOG PANCREAS

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1. Introduction

Glycoproteins, long known to be functional constituents of cell surfaces, have recently been detected in intracellular membranes [1]. Membranes from the rough endoplasmic reticulum and the Golgi complex [2,3] and membranes from storage granules [4–6] of various secretory tissues, possess glycoproteins. Several functions for such intracellular, membrane-bound glycoproteins can be proposed. They may be organelle-specific and may function as determinants in membrane–membrane interactions, such as fusion. Alternatively, or additionally, membrane constituents may flow from the rough endoplasmic reticulum to the smooth endoplasmic reticulum and then to membrane-bound vesicles. Fusion of the vesicle membrane with the plasma membrane would provide a mechanism for the insertion of plasma membrane components [7,8]. According to this latter view, storage-granule membranes would contain precursors to plasma-membrane glycoproteins. The lack of detailed information regarding specific intracellular membrane glycoproteins has impeded exploration of these possibilities.

Pancreatic zymogen granules are specialized organelles for the temporary storage of secretory proteins. Although zymogen granules arise from the Golgi complex and fuse with the plasma membrane during secretion, the molecular mechanisms underlying these events are not known [9]. The characterization of subcellular membrane glycoproteins is a promising approach to this problem. A comparative analysis of zymogen-granule membrane polypeptides led us to propose that a major glycoprotein, designated as GP-2, characterizes granule membranes of the mammalian pancreas [4]. This report establishes that GP-2 from dog-pancreas zymogen granules is a glycoprotein containing 15% carbohydrate. It represents approximately 45% of the firmly-bound granule-membrane protein. As judged by surface labeling data, GP-2 is located on the zymogen granule interior. A second minor membrane glycoprotein species is located on the granule exterior.

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2. Materials and methods

2.1. *Preparation of zymogen granule membranes*

Fresh pancreases (60–200 g) from adult dog were obtained from the Veterinary Clinic, Michigan State University. Zymogen granules, prepared as previously published [4,10] were lysed at 4°C in 0.2 M $NaHCO_3$, pH 8.2, containing 50 μg soybean trypsin inhibitor (Sigma)/ml. Granule membranes were collected by centrifugation at 100 000 × *g* for 1 h. The bicarbonate extract is designated as the granule content. Contaminating mitochondria were removed from the granule membranes by discontinuous-gradient centrifugation [11]. Secretory proteins adsorbed to the granule membranes were then removed by extraction in 0.25 M NaBr [4]. Amylase activity in granule membranes, assayed by the Bernfeld method [12] was less than 0.5 units/mg protein and cytochrome *c* oxidase activity, assayed

by the method of Wharton and Tzagoloff [13], was less than 0.2 mU/mg protein. These data indicate a minimal contamination of the purified zymogen granules by mitochondria and by secretory proteins of the granule content.

2.2. *Preparation of GP-2*

Zymogen-granule membranes (2–6 mg protein) were solubilized in 10 mM sodium phosphate, pH 6.65, containing 1% SDS (w/v), 5 mM EDTA, 5% sucrose (w/v), 2% 2-mercaptoethanol (v/v) and 5 μg Pyronin B (Harleco)/ml by heating at 95°C for 5 min. The final protein concentration was ~ 1.2 mg/ml. Samples were applied to slab-gels (11 × 15 × 0.6 cm) containing a 5–17% acrylamide linear-gradient in 0.1% SDS and 0.1 M sodium phosphate, pH 6.65 [14]. The ratio of bisacrylamide to acrylamide was 1.1 g to 40 g. After electrophoresis at 3.3 V/cm for approximately 24 h at room temperature, the position of GP-2 was established as follows. Reference sections (3 mm wide) were removed from the gel and rapidly stained with Coomassie Blue G (K and K Lab) for 1–2 h [15]. The region of the unstained gel corresponding to GP-2 was excised. Gel sections containing GP-2 were electro-eluted for 18 h in the presence of 0.1 M sodium phosphate, pH 6.65, containing 0.1% SDS, and 50 μM phenylmethylsulfonyl fluoride (Sigma). The eluate was dialyzed overnight against 2 changes of distilled water containing 3 g AG-IX anion-exchange resin (Bio-Rad) and 2 ml toluene/liter. The preparation was then lyophilized and stored at −80°C.

2.3. *Analytical methods*

Protein samples (25–50 μg) were hydrolyzed in 6 N HCl at 110°C in vacuo for 24 h and 48 h and amino acid analyses were performed using an ultrasensitive system based upon that of Moore and Stein [16]. Values for serine and threonine were corrected for destruction. N-Terminal amino acid analyses utilized the dansylchloride procedure [17]. The dansylated product was identified by comparison of R_F values of dansylated amino acid standards by thin-layer chromatography on polyamide Lager sheets (Gallard-Schlesing Chemical Mfg. Corp.). Carbohydrate analyses were performed using gas–liquid chromatography as described by Bhatti et al. [18]. Prior to carbohydrate analyses, granule-protein fractions were extracted with chloroform/methanol/diethyl ether (2:1:0.1, v/v/v). After methanolysis and re-*N*-acetylation, trimethylsilyl ethers of methyl glycosides were prepared [19]. In some experiments galactosyl moieties of zymogen-granule constituents were incubated with galactose oxidase from Sigma (124 U/mg) and reduced with NaB^3H_4 from New England Nuclear (100 mCi/mmol).

Analytical electrophoresis using polyacrylamide gels (0.6 × 10 cm), containing 9% acrylamide and 1% SDS, were performed with minor modifications of the procedure of Fairbanks et al. [20]. Alternatively, protein was dissolved in phenol/acetic acid/urea/water/2-mercaptoethanol (2:1:1:1:0.5, w/v/w/v/v) and electrophoresed in gels containing 10% acetic acid [21]. To determine the apparent molecular weight of GP-2, both gel systems were calibrated using protein standards. Gels were stained with Coomassie Blue to detect protein [22] or with Schiff-periodic acid (PAS) to detect carbohydrate [20], then scanned at 550 nm on a Gilford Spectrophotometer with a linear-transport. Unstained gels were fractionated (Auto Gel Divider, Savant Instruments). Gel fractions were heated in 0.1 N NaOH/1% SDS for 1 h at 60°C, then neutralized and counted in a scintillation counter [23].

3. Results and discussion

The choice of dog-pancreas as a source for zymogen granules was dictated by earlier indications that zymogen granules are readily isolated in good yield from dog-pancreas [24] and that GP-2 represented an unusually large percentage of the protein of dog zymogen-granule membranes [4]. Figure 1 compares electrophoretograms of zymogen-granule membranes, granule content (secretory proteins) and GP-2 stained for polypeptides and for carbohydrate. The major polypeptide of dog zymogen-granule membranes accounted for 46% of the protein-stain intensity (fig.1A). Identical polypeptide profiles were obtained from gels stained with amido black [22]. The major membrane glycoprotein represented 52% of the Schiff-periodic acid stained polypeptides (fig.1D). Stained material migrating adjacent the tracking dye represents lipid [21]. Purified GP-2 yielded a single band that corresponded to the major membrane polypeptide (fig.1C, 1F). No GP-2 was detected in

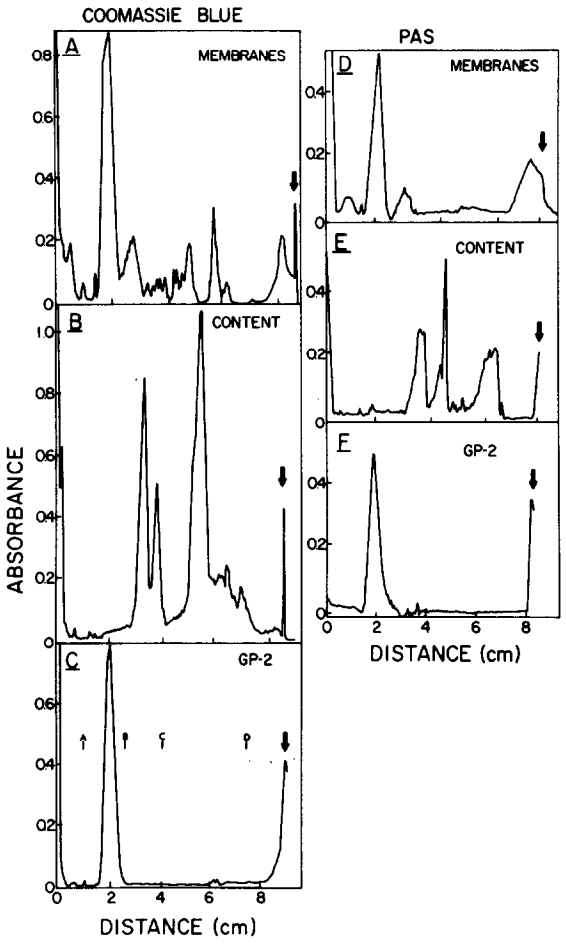

Fig.1. Electrophoretic analysis of dog zymogen-granule polypeptides. Zymogen-granule membranes, zymogen-granule content and GP-2 were electrophoresed in 9% acrylamide gels containing 1% SDS as described in Materials and methods. For gels stained for protein (Coomassie Blue) 40 μg protein was applied. For gels stained for carbohydrate (PAS), 80 μg protein was used. The following mol. wt standards were employed: (a) *E. coli* β-galactosidase (130 000). (b) Bovine-liver catalase (60 000). (c) Yeast alcohol dehydrogenase (37 000). (d) Hen lysozyme (14 300). The arrows indicate the position of the tracking-dye.

the granule content. The average recovery of GP-2 after preparative gel electrophoresis was 30% (range, 20–46% for 9 preparations). We conclude that GP-2 is the predominant integral polypeptide of zymogen granule membranes.

In the presence of SDS, GP-2 migrated as a single band with an apparent mol. wt 74 000, as judged by mobilities of protein standards (fig.1C). Varying the gel porosity from 5–12% acrylamide [12] did not alter this value. After electrophoresis in acetic acid in the absence of SDS, GP-2 yielded a single band with a mobility corresponding to mol. wt 72 000. A single *N*-terminal amino acid, valine, was detected in GP-2 preparations. As shown by table 1, granule membranes and GP-2 did not contain unusually high levels of proline, as has been reported for parotid-granule membranes [26], nor did they exhibit unusually high levels of hydrophobic amino acids. GP-2 contained approximately 31% charged amino acid and 27% apolar residues, values similar to those reported for other membrane proteins [27].

Table 2 summarizes the carbohydrate composition of zymogen-granule subfractions. Zymogen-granule membrane polypeptides contained 5–10-fold more carbohydrate, on a weight basis, than did secretory proteins of the granule content. GP-2 contained

Table 1
Amino acid composition of zymogen-granule membranes and GP-2 from dog-pancreas

Amino acid	Granule membranes[a] (mol/100 mol amino acid)	GP-2[b]
Lys	4.3	3.7
His	2.2	2.2
Arg	5.2	5.3
Asp	10.7	9.8
Glu	9.0	10.5
Thr	5.8	4.5
Ser	7.4	9.9
Pro	6.7	6.4
Gly	8.0	14.1
Ala	7.4	5.8
Cys	3.5	2.7
Val	6.7	5.0
Met	2.0	1.6
Ile	4.8	4.2
Leu	8.6	7.1
Tyr	3.6	3.7
Phe	3.7	4.3

[a] Mean values from 2 preparations, each representing 2 dogs, 40 g wet wt tissue
[b] Mean values from 2 preparations, each representing 5 dogs, 120 g wet wt tissue

Table 2
Carbohydrate composition of zymogen-granule fractions from dog-pancreas

Sugar	Fraction		
	Content[a] (mol/mg protein)	Membranes[a]	GP-2[b]
Fucose	14	86	52
Galactose	14	140	197
Mannose	13	138	93
N-Acetylgalactosamine	2.2	11	0.5
N-Acetylglucosamine	14	140	110
Sialic Acid (as NANA)[c]	4.0	56	50

[a] Mean values from 2 preparations, each representing 2 dogs (40 g wet wt pancreas)
[b] Mean values from 2 preparations, each representing 5 dogs (120 g wet wt pancreas)
[c] NANA, *N*-Acetylneuraminic acid

approximately 15% carbohydrate; the sugar moieties are those typical of mammalian glycoproteins, rather than mucopolysaccharides. The absence of *N*-acetylgalactosamine suggests that GP-2 oligosaccharides are attached to the polypeptide via asparaginyl-*N*-acetylglucosamine linkages.

The orientation of GP-2 in the zymogen granule is an important consideration in assessing the possible roles of GP-2 in exocytosis. To study the orientation of GP-2 in zymogen granules, we utilized galactose oxidase, which can oxidise exposed galactosyl moieties of glycoproteins on membrane surfaces [28]. Subsequent reduction with NaB^3H_4 leads to the incorporation of tritium into these oxidized moieties. Intact zymogen granules were treated sequentially with galactose oxidase and NaB^3H_4 (fig.2A). Only two slowly migrating glycoprotein species, not GP-2, were labeled. Several secretory proteins of the granule content were labeled by this procedure (fig.2B). These were not accessible to galactose oxidase in intact granules. When zymogen-granule membranes were treated with galactose oxidase and reduced with NaB^3H_4, four glycoprotein species, including GP-2, were labeled (fig.2C). Membranes or granule content reduced with NaB^3H_4 without prior incubation with galactose oxidase incorporated radioactivity only in glycolipid.

The present study confirms our earlier proposal [4], based upon Schiff-periodic stain profiles, that GP-2 is a glycoprotein. The following evidence supports our proposal that GP-2 is a biochemical marker for zymogen-granule membranes.

(i) This glycoprotein is present in 6 different mammalian species [4].

(ii) GP-2 is not detectable in membranes from mitochondria or rough microsomes ([4] and R. J. MacDonald and R. A. Ronzio, in preparation).

(iii) Only very low levels of GP-2 are present in Golgi-rich membranes [29]. Elsewhere we have reported that pancreatic Golgi-membranes contain glycoproteins, antigenically similar to GP-2, that are substrates for a Golgi-membrane galactosyltransferase [30]. Protein glycosylation occurring within cisternal spaces of Golgi vesicles would result in the presence of glycoproteins situated on the zymogen-granule interior.

GP-2 appears to be sequestered within granules, where its sialyl moieties could interact with basic secretory proteins or with Ca^{2+}. Studies to establish more firmly the orientation of GP-2 in granules and to detect the appearance of GP-2 in the cell-surface are in progress.

Pancreatic zymogen-granules contain a minor glycoprotein species on the granule exterior. Glycoproteins are also localized on the outer surface of chromaffin granules [6]. The origin and functions of these surface glycoproteins are unknown. An intriguing possibility is that these surface components

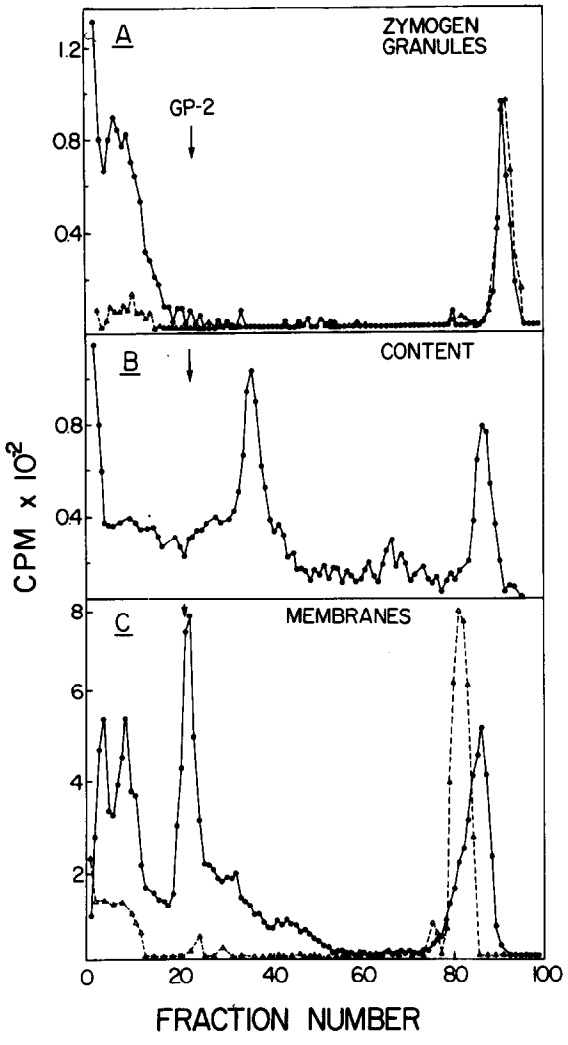

Fig.2. Electrophoretic analysis of intact zymogen-granules and granule subfractions labeled with NaB^3H_4 following galactose oxidase treatment. Zymogen-granule fractions (30–50 μg protein) were incubated in phosphate-buffered saline, pH 7.2, containing galactose oxidase (15 U/ml) and soybean trypsin inhibitor (0.25 mg/ml). For intact zymogen granules, the incubation medium contained, in addition, 0.3 M sucrose. After incubation for 60 min, NaB^3H_4 (0.1 mCi/ml) was added and incubations were continued another 15 min. Intact zymogen granules were recovered by centrifugation at 1000 × *g* and were washed several times in 0.3 M sucrose. Zymogen-granule content was precipitated with 10% trichloroacetic acid. Zymogen-granule membranes were recovered by centrifugation at 100 000 × *g* for 1 h. Samples were electrophoresed in polyacrylamide gels containing 1% SDS. Gels were fractionated and counted as described in Materials and methods. Closed circles represent radioactivity from gels of fraction treated with both galactose oxidase and NaB^3H_4. Triangles represent radioactivity in control samples treated with NaB^3H_4 alone. The arrow marks the position of migration of GP-2.

may interact with cytoplasmic elements possibly involved in secretion, for example protein kinases [31].

The storage-granule membranes characterized thus far, display relatively simple polypeptide profiles. However, it remains to be seen whether glycoproteins are common to these membranes. The isolation of the major polypeptide component of the zymogen-granule membrane holds promise for studies concerning the basic structure and function of glycoproteins in mammalian membranes.

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References

[1] Buck, C. A., Fuhrer, J. P., Soslau, G. and Warren, L. (1974) J. Biol. Chem. 249, 1541–1550.
[2] Bergman, A. and Dallner, G. (1976) Biochem. Biophys. Acta 433, 496–508.
[3] Kawasaki, T. and Yamashina, I. (1973) J. Biochem. Tokyo 74, 639–647.
[4] MacDonald, R. J. and Ronzio, R. A. (1972) Biochem. Biophys. Res. Commun. 49, 377–382.
[5] Castle, J. D., Jamieson, J. D. and Palade, G. E. (1975) J. Cell Biol. 64, 182–210.
[6] Meyer, D. I. and Burger, M. M. (1976) Biochem. Biophys. Acta 443, 428–436.
[7] Bennett, G., LeBlond, C. P. and Haddad, A. (1974) J. Cell Biol. 60, 258–284.

[8] Morré, D. J. (1975) Ann. Rev. Plant Physiol. 26, 441–481.
[9] Palade, G. E. (1975) Science 189, 347–358.
[10] Ronzio, R. A. (1973) Biochem. Biophys. Acta 313, 286–295.
[11] Meldolesi, J., Jamieson, J. D. and Palade, G. E. (1971) J. Cell Biol. 49, 109–129.
[12] Bernfeld, P. (1955) Meth. Enzymol. 1, 149–158.
[13] Wharton, D. C. and Tzagoloff, A. (1967) Meth. Enzymol. 10, 245–250.
[14] Maizel, J. V., Jr. (1971) Meth. Virol. 5, 179–246.
[15] Malik, M. and Berrie, A. (1972) Anal. Biochem. 49, 173–176.
[16] Moore, S. and Stein, W. H. (1963) Meth. Enzymol. 6, 819–831.
[17] Gros, C. and Labouesse, B. (1969) Eur. J. Biochem. 7, 463–470.
[18] Bhatti, T., Chambers, R. E. and Clamp, J. R. (1972) in: Glycoproteins (Gottschalk, A. ed) Elsevier, New York.
[19] Sweeley, C. C., Bentley, R., M Kita, M. and Wells, W. W. (1963) J. Am. Chem. Soc. 85, 2497–2507.
[20] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[21] Ray, T. K. and Marinetti, G. V. (1971) Biochem. Biophys. Acta 233, 787–791.
[22] Johnson, J. C., DeBacker, M. and Boezi, J. A. (1971) J. Biol. Chem. 246, 1222–1232.
[23] Mostafa, I. Y., Fallin, E. and Fang, S. C. (1970) Anal. Biochem. 36, 238–243.
[24] Hokin, L. (1955) Biochem. Biophys. Acta 18, 379–388.
[25] Segrest, J. P. and Jackson, R. L. (1972) Meth. Enzymol. 28, 54–63.
[26] Amsterdam, A., Schramm, M., Ohad, I., Salomon, Y. and Selinger, Z. (1971) J. Cell Biol. 50, 187–200.
[27] Ho, M. K. and Guidotti, G. (1975) J. Biol. Chem. 250, 675–683.
[28] Gahmberg, C. G. and Hakomori, S. (1973) J. Biol. Chem. 248, 4311–4317.
[29] Ronzio, R. A. (1973) Arch. Biochem. Biophys. 159, 777–784.
[30] Ronzio, R. A. and Mohrlok, S. H. (1977) Arch. Biochem. Biophys. in press.
[31] MacDonald, R. J. and Ronzio, R. A. (1974) FEBS Lett. 40, 203–206.