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In resting conditions, the pancreatic granule membrane protein GP-2 is secreted by cleavage of its glycosylphosphatidylinositol anchor

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GP-2 is the major membrane protein of the exocrine pancreatic secretory granule. It is an integral protein which is anchored by a phosphatidylinositolglycan. In addition to being present in the soluble contents of the granule, GP-2 is also actively secreted by the pancreas. Although 93% of the GP-2 in the resting secretions of anaesthetized rats could be pelleted, Triton X-114 phase extraction showed that 70% of this GP-2 had lost its hydrophobic properties. Proteases have been postulated to release GP-2 from the membrane, but phospholipases also have the capacity to release the protein from the membrane by hydrolysis of its peculiar glycosylphosphatidylinositol membrane anchor. These studies show the presence of inositol 1,2-(cyclic)monophosphate on the secreted hydrophilic GP-2, confirming the involvement of an endogenous phospholipase C in the solubilization of GP-2 by the exocrine pancreas. It is therefore concluded that most of the GP-2 secreted by the pancreas of anaesthetized rats under resting conditions is released from the membrane by a phospholipase C which hydrolyses the phosphodiester bond linking GP-2 to its diradylglycerol anchor.

INTRODUCTION

The glycoprotein GP-2 is an integral protein of the pancreatic zymogen granule membrane [1]. The protein is also soluble in the zymogens stored in the granule [2] and is secreted by the pancreas [3–6], and has been reported to be present in both a soluble and a pelletable form [4,6,7] in the pancreatic secretion. It has been proposed that proteolytic degradation is responsible for the release of GP-2 from the membrane and its resulting secretion [6]. Unlike typical membrane proteins, which have part of the polypeptide embedded in the membrane, GP-2 is linked via a covalent phospholipase C (PLC)-sensitive phosphodiester bond to a glycosylphosphatidylinositol (GPI) anchor [8]. The hydrolysis of the phosphodiester bond at position 1 of the inositol ring by a PLC could therefore be another mechanism by which the protein is released into pancreatic secretions. In this report, we investigated the latter possibility in secretions produced by the resting unstimulated pancreas of anaesthetized rats. These physiological conditions yield high concentrations of secreted GP-2 [3,7]. Using Triton X-114, we separated anchored GP-2 from anchorless GP-2 in these secretions, and the respective amount of each form of the protein was estimated by competitive e.l.i.s.a.

If GP-2 is released by the action of a PLC, the soluble protein should exhibit a phosphoinositolglycan at its *C*-terminus. The presence of inositol 1,2-(cyclic)monophosphate on the secreted GP-2 molecule was checked by use of an antibody specific for this particular epitope.

MATERIALS AND METHODS

Materials

Triton X-114, soybean trypsin inhibitor and phenylmethanesulphonyl fluoride were from Sigma. Bio-Beads SM-2, Bio-Gel P-6DG desalting gel and all chemicals for PAGE were from Bio-Rad Laboratories. Zymogen granule membranes were prepared from rat pancreas according to previously published procedures [2]. Anti-CRD (trypanosome variant surface glycoprotein cross-reacting determinant) antiserum was provided by Dr. M. Ferguson, University of Oxford.

Animals

Male albino Sprague–Dawley rats weighing between 200 and 275 g were fasted overnight and subsequently anaesthetized with urethane (2 g/kg body weight). The pancreatic duct was cannulated and the bile duct clamped, and the pancreatic secretion was harvested for a period of 6 h.

Methods

Phase separation using Triton X-114 of soluble and membrane-bound proteins was performed with total pancreatic secretions and with the pellet after ultracentrifugation at 200000 *g* for 3 h. Triton X-114 separation was performed according to previously published procedures [9,10]. Briefly, Triton X-114 was added to a final concentration of 1.5% to 200 μl of protein (1 mg/ml) in 10 mM-Tris/HCl, pH 7.5. Bromophenol Blue (40 μM) and NaCl (0.5 M final concentration) were added in order to facilitate phase separation. After equilibration at 0 °C for 15 min, samples were incubated at 37 °C for 1 h in order to allow condensation of the detergent. The two phases were separated by centrifugation at 2000 *g* for 10 min. The aqueous supernatant was desalted on Bio-Gel P-6DG equilibrated with 50 mM-NH_4HCO_3, freeze-dried and run on SDS/PAGE along with the detergent phase. Due to its strong interference with migration in the gel, Triton X-114 present in the detergent phase was removed from the samples using Bio-Beads SM-2 [11] before running the gels. Treatment with *Bacillus thuringiensis* phosphatidylinositol-specific PLC (PI-PLC) (15 munits/ml) was performed for 5 h with 1 mg of protein/ml at 16 °C in 100 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl/1 mM-EDTA.

Abbreviations used: CRD, cross-reacting determinant; GPI, glycosylphosphatidylinositol; PLC, phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C.

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Immunoblotting

Electrophoretic transfer from polyacrylamide gels was carried out using standard procedures. Renaturation of the blotted proteins, saturation and incubation with 1:500 dilutions of anti-CRD antiserum were done according to Birk & Koepsell [12]. Immunoblots were developed with ^{125}I-labelled anti-rabbit Ig $(Fab')_2$ fragment from donkey (Amersham IM1340), or with the peroxidase-labelled $(Fab')_2$ (Amersham NA9340) using Amersham's enhanced chemiluminescence detection system.

GP-2 determination

The amount of GP-2 in the different fractions was determined by a competitive e.l.i.s.a. developed in our laboratory [13].

Other methods

Proteins were estimated by the Bradford assay using BSA as the standard [14]. SDS/PAGE was performed on 12% acrylamide gels using Laemmli's discontinuous buffer system [15].

RESULTS

As reported previously, the main characteristic of the proteins secreted by the pancreas of anaesthetized rats is the presence of GP-2 in a pelletable form (Fig. 1, lane C). In our study, upon ultracentrifugation of the resting secretion, more than 93% of the total GP-2 was found in the pellet when assayed with a specific e.l.i.s.a. This dramatic enrichment of GP-2 in the pellet was also confirmed by PAGE (Fig. 1, lane C). Even when probed on blots with anti-GP-2 antibodies and overexposed, the supernatant of the ultracentrifugation (Fig. 1, lane B) did not show any reactivity for GP-2 (results not shown). When Triton X-114 phase separation was performed on these secretions before or after ultracentrifugation, GP-2 was very predominantly recovered (72%) in the aqueous phase (Table 1). This was also confirmed on gels (Fig. 1, lane E). Since the concentration of Triton X-114 in the detergent phase hindered the determination of GP-2 by e.l.i.s.a., we could only rely on gels to estimate the presence of GP-2 in this fraction. As shown in Fig. 1 (lane D), GP-2 was not detected in pooled detergent phases. On the other hand, when zymogen granule membranes were similarly fractionated, most of the GP-2 was recovered in the detergent fraction, with only 3% of the GP-2 partitioning in the aqueous phase (Table 1). This control establishes the reliability of the Triton X-114 extraction and clearly shows that, in the zymogen granule membrane, all of the GP-2 is anchored, whereas in the resting secretion it has lost its anchor and is soluble. In order to distinguish whether the secreted GP-2 has been solubilized by a protease or a PLC, the reactivity of the soluble GP-2 was tested on blots for the presence of the CRD. The CRD is a cryptic epitope of most GPI-anchored proteins which is uncovered when GPI-anchored proteins are solubilized by a PI-PLC [16]. GP-2 solubilized by PI-PLC from zymogen granule membranes is positive for the CRD epitope (Fig. 2). The GP-2 present in the resting secretion also reacted positively with anti-CRD antibodies (Fig. 1, lane F, and Fig. 2). As a control, CRD reactivity was tested in membranous GP-2 before and after treatment with PI-PLC (Fig. 2). The sensitivity of the anti-CRD reactivity to mild acid treatment, which decyclizes the inositol 1,2-(cyclic)monophosphate produced by the PI-PLC cleavage [17], was also tested. A weak CRD reactivity was detected for membranous GP-2, whereas GP-2 treated with PI-PLC was highly reactive (Fig. 2). The presence of the cyclic monophosphate was confirmed

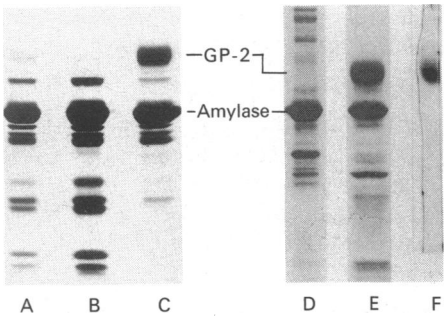

Fig. 1. Protein composition of the rat pancreatic secretions under resting conditions: determination of the hydrophilic nature of GP-2 by Triton X-114 phase separation

Lane A, total proteins secreted by the resting rat pancreas; lane B, proteins of the 200000 *g* supernatant of the resting secretion; lane C, proteins of the 200000 *g* pellet of the resting secretion; lane D, proteins of the detergent phase (membrane-associated hydrophobic proteins) from two Triton X-114 extractions of the resting pancreatic secretion, pooled in order to confirm the absence of any hydrophobic GP-2 in the fraction; lane E, proteins of the aqueous phase (soluble proteins) of one Triton X-114 extraction of the resting pancreatic secretion; lane F, immunoblot of lane E probed with antibodies specific for the *C*-terminal phosphoinositolglycan epitope CRD.

Table 1. Content of soluble GP-2 in the zymogen granule membrane and in the resting pancreatic secretion

Solubilization was determined by Triton X-114 phase partition. Centrifugation of the resting secretion was at 200000 *g* for 3 h. GP-2 content was determined by e.l.i.s.a.

Fraction	Soluble GP-2 (%)
Zymogen granule membrane	2.87±0.05
Resting secretion	70.53±2.80
Pellet from the resting secretion	70.00±0.90

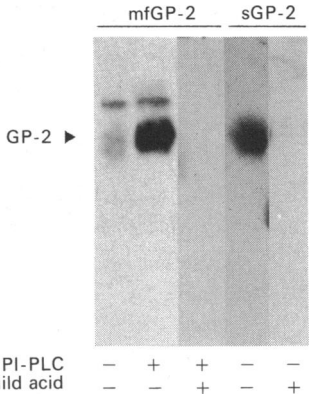

Fig. 2. Anti-CRD reactivity of GP-2 in its membrane form and in the rat pancreatic secretions obtained under resting conditions: specificity of the reaction for inositol 1,2-(cyclic)monophosphate

Identical amounts of GP-2 (100 μg) from zymogen granule membranes (mfGP-2) or pelleted rat pancreatic secretions obtained under resting conditions (sGP-2) were run on gels. After transfer to nitrocellulose membranes, blots were treated with anti-CRD antiserum as described in the Materials and methods section. In order to assess the specificity of the antibody reactivity for the inositol (cyclic)phosphate, tracks were submitted to 1 M-HCl for 30 min to decyclize the phosphate group. Zymogen granule membranes were treated with 15 munits of *B. thuringiensis* PI-PLC/ml for 5 h. Bound antibodies were detected using peroxidase-labelled anti-rabbit Ig $(Fab')_2$ using an enhanced chemiluminescence detection system (Amersham).

in the PI-PLC-solubilized membranous GP-2 and in the secreted GP-2 (Fig. 2). A higher-M_r GPI-anchored protein was also detected by this procedure. The presence of an acid-sensitive CRD on secreted GP-2 is direct evidence that the protein has been subjected to the action of an endogenous PLC.

DISCUSSION

The high level of intact GP-2 is one of the main features of the proteins secreted under resting conditions by the pancreas of the pig [3] and the rat [7]. In these secretions, most GP-2 can be pelleted ([7]; the present paper), suggesting that it is membrane-bound. The selective partition of proteins in the detergent phase of Triton X-114 is now the standard procedure for monitoring genuine hydrophobic interactions with membranes [16]. Using this technique we found that, even though GP-2 secreted by anaesthetized animals could be pelleted, it had totally lost its hydrophobic character. The possibility that addition of detergent to samples activated a PLC was rejected, as GP-2 in pelleted secretions was already reactive for CRD before any detergent addition (Fig. 2), and furthermore PI-PLC treatment could not increase this anti-CRD reactivity (results not shown). But how can GP-2 be pelleted if it is not membrane-bound? The possibility that soluble GP-2 can be trapped inside lipid vesicles and be released from these vesicles upon detergent addition is very unlikely. Indeed, according to such a proposal, all of the PI-PLC-solubilized GP-2 would have to be engulfed in a lipid vesicle by a still unknown mechanism when reaching the apex of the cell. Moreover, the very minute amount of lipids in pancreatic secretions [7] does not support this hypothesis. The possibility that we favour most is that GP-2 is associated with the zymogens, and that such a complex would readily pellet.

The most important question that this report addresses is whether GP-2 is released by a protease or a phospholipase. Here we have confirmed the presence of CRD on secreted GP-2 proving that, under resting conditions, a PLC releases GP-2 by trimming its diradylglycerol anchor. Under conditions of stimulated regulated secretion, however, zymogen granules would be responsible for secreting GP-2 as a proteolytic fragment ([2]; D. LeBel, unpublished work). But does GP-2 in the resting secretion come from granule membranes after fusion with the apical membrane? Since we have shown that the granule membrane has no phospholipase capable of releasing GP-2 [8], and that under resting conditions granule fusion is minimal, the GP-2 present in the unstimulated rat pancreatic juice is therefore less likely to directly originate from granule membrane after exocytosis. This possibility could be correct, however, if a PI-PLC is a resident enzyme of the apical membrane. After granule exocytosis, GP-2 would diffuse laterally and could then be accessible to such an apical PI-PLC for release. This PI-PLC should then be carried to the apical membrane by a vesicle different from the zymogen granule.

As zymogen granule membranes are not the most likely carrier for the secretion of GP-2 in unstimulated conditions, other possible carrier vesicles have to be considered. By definition, this vesicle should carry proteins of a composition totally different from that of the zymogen granule. The existence of such a vesicle was suggested by observations made in the pig, where the specific activity of GP-2 in secretin-stimulated secretions was exceptionally high, GP-2 even being the major component under these conditions [3]. Furthermore, studies on dispersed acinar cells showed that newly synthesized GP-2 is secreted at a much faster rate than newly synthesized amylase, the latter being primarily secreted by zymogen granules [18]. These two observations therefore argue in favour of an additional carrier vesicle which is different from the secretory granule. One potential carrier for a PI-PLC-solubilized GP-2 could be the vesicles postulated by Arvan & Castle [19] that release excess membrane from maturing secretory granules. Another possible carrier could be the vesicle that carries constitutive secretion to the apical pole of the cell. Both vesicles are known to secrete proteins that are very different from those of the secretory granule.

In conclusion, the presence of cyclic inositol phosphate on the GP-2 secreted by rat exocrine pancreas in resting conditions confirms that an endogenous PLC is directly involved in the post-translational maturation of the glycoprotein *en route* to the cell surface, which leads to the release of an anchorless GP-2 in these secretions.

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