Comparison of secretory protein and membrane composition of secretory granules isolated from normal and neoplastic pancreatic acinar cells of rats

(pancreatic acinar carcinoma/two-dimensional gel electrophoresis/granule membrane glycoprotein/phospholipid composition)

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ABSTRACT The diversity of cytodifferentiation in a transplanted rat pancreatic acinar carcinoma provides a biological model system for the study of regulatory molecular events that differ from those in normal acinar cells. Secretory (zymogen) granule proteins and granule membranes of neoplastic and normal pancreatic acinar cells were compared to determine the differences in gene expression between apparently well-differentiated secretory granule-containing neoplastic cells and normal cells. Nineteen proteins observed in two-dimensional polyacrylamide gels of normal secretory granule extracts were seen also in tumor granule extract profiles, with reduced but detectable amounts of lipase and four basic proteins. In addition, tumor granule extracts contained a new protein of Mr 24,000, designated p24, which was not detectable in normal extracts. Neoplastic granule membranes, while having phospholipid composition similar to that of normal membranes, lacked or contained a greatly reduced amount of a major glycoprotein of Mr 80,000 and three other proteins of Mr 50,000, 37,000, and 36,000. The nature of p24 protein in secretory granule extracts and the significance of the reduction or absence of Mr 80,000 membrane glycoprotein in this tumor remain to be elucidated.

The well-formed secretory (zymogen) granules present in adult pancreatic acinar cells represent the final product of translational and post-translational events of a highly differentiated program of gene expression (1–3). Evidence indicates that all of the exocrine acinar cells of adult pancreas are similar in cytodifferentiation, each containing a relatively homogeneous population of secretory granules (1–4). In contrast, a transplantable pancreatic acinar carcinoma of rat established in our laboratory (5) is characterized by a continuum of cytodifferentiation ranging from cells totally lacking secretory granules to those with abundant well-formed ones (6). This diversity of cytodifferentiation in a neoplasms provides a eukaryotic cell model for the identification and study of regulatory molecular events that differ from those in normal acinar cells. Such studies are relevant in view of the notion that neoplasia reflects disordered cell differentiation and gene expression (7). In order to search for alterations that could be correlated with cytodifferentiation, it becomes necessary to define the patterns of gene expression in the granule-rich and granule-deficient subpopulations of neoplastic acinar cells. With this goal in mind, secretory proteins derived from highly purified secretory granules of pancreatic acinar carcinoma were analyzed by two-dimensional gel electrophoresis to determine whether gene expression in the apparently well-differentiated subpopulations of neoplastic acinar cells is similar to that of normal acinar cells. Polypeptide and phospholipid composition of the membranes derived from secretory granules of normal and neoplastic pancreatic acinar cells were also compared.

MATERIALS AND METHODS

Purification of Secretory Granules. Secretory granules from pooled normal adult male F344 rat pancreata and transplanted pancreatic acinar tumors (5) were isolated by a modification of the procedure described for the isolation of chromaffin granules from adenral medulla (8). The tissues were minced in ice-cold sucrose buffer (0.27 M sucrose/8 mM sodium cacodylate/0.1 mM EDTA, pH 6.5) and homogenized (10% wt/vol). The homogenate was centrifuged at 710 × gmax first for 5 min and then for 3 min in a Beckman J-21C centrifuge to pellet nuclei and large cytoplasmic fragments. The supernatant was then centrifuged at 2,600 × gmax for 10 min. The grey-white secretory granule pellet was washed with ice-cold sucrose buffer to remove the overlying mitochondria and was resuspended in a small volume of sucrose buffer. About 0.6 ml of granule suspension (~10–15 mg of protein) was layered on a 10-M sodium cacodylate/0.05 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine hydrochloride in 15-ml Corex tubes and was centrifuged at 9,500 × gmax for 40 min. The highly purified secretory granule fraction, which forms a band near the bottom of the tube (see Fig. 1), was washed twice in ice-cold sucrose buffer.

Extraction of Secretory Proteins. Secretory granule extract was prepared by lysing the granules in ice-cold 100 mM Na2CO3 (pH 11.5) for 30 min (9). An aliquot of granule lysate was centrifuged at 225,000 × gmax for 1 hr with a Beckman Ti 50 rotor to separate soluble proteins from the secretory granule membranes. Benzenzamine hydrochloride (1 mM), phenylmethylsulfonyl fluoride (1 mM), and soybean trypsin inhibitor (0.1 mg/ml) were included in the lysate to inhibit proteolytic degradation.

Purification of Secretory Granule Membranes. Secretory granule lysate, prepared as above, was layered on a step gradient consisting of 0.5 ml each of 1.3 M sucrose and 0.3 M sucrose and was centrifuged for 45 min at 300,000 × gmax in a Beckman SW 50.1 rotor. The granule membranes, which band at the 0.3 M/1.3 M interface of the sucrose step gradient, were carefully collected, suspended in 0.25 M sodium bromide to remove any adsorbed secretory proteins (10), and pelleted by centrifuging at 300,000 × gmax for 1 hr.

Isoelectric Focusing (IEF) and Two-dimensional Gel Electrophoresis. IEF of granule extracts was performed as de-

Abbreviation: IEF, isoelectric focusing.
scribed by Scheele (11). The focused IEF gels were processed for enzyme assays or for two-dimensional analysis essentially as outlined by Scheele (11).

Enzyme activities in IEF gel slices were measured as follows: lipase by the triplaminate lipase assay; amylase as described by Bernfeld (12); carboxypeptidases A and B activities as described by Greene et al. (13) with N-benzyloxymethylcarbonyl-glycyl-L-phenylalanine and hippuryl-L-arginine, respectively; ribonuclease as described by Kalnitsky (14); and trypsinogen and chymotrypsinogen by the procedure of Scheele and Palade (15).

Analysis of Phospholipids. Lipids from mitochondria, secretory granules, and secretory granule membrane pellets were extracted into chloroform/methanol (16), and the phospholipids were separated by thin-layer chromatography on 250-μm-thick silica gel plates with chloroform/methanol/water, 65:25:4 (vol/vol), as solvent (17). The plates were air-dried at room temperature for 20 min, and the spots were visualized with iodine vapor.

Other Techniques. NaN3SO4/polyacrylamide gel electrophoresis in slab gels was performed by the method of Laemmli (18). Glycoproteins were detected after NaDodSO4/polyacrylamide gel electrophoresis by the procedure of Glossmann and Neville (19). Proteins were measured by the procedure of Lowry et al. (20). Subcellular fractions were processed for electron microscopy as described (21).

RESULTS

Morphology and Purity of Secretory Granule Fractions. A majority of normal and neoplastic acinar cell secretory granules sedimented in a Percoll gradient tube (density = 1.130–1.138 g/ml or greater) as represented in Fig. 1. Mitochondria were concentrated near the top of the gradient (density = 1.049–1.054 g/ml). The opaque region of the gradient between the two fractions contained an admixture of relatively few mitochondria, smaller secretory granules, and cytoplasmic membranous fragments. The yield of secretory granules was considerably higher in normal pancreas (5–10 mg/g of pancreas) than in pancreatic tumor (1–3 mg/g of tumor). This is attributed, in part, to heterogeneity of cytodifferentiation of the neoplastic cells (6, 22).

Numerous thin sections of plastic-embedded secretory granule fractions were examined by electron microscopy to assess their purity (Fig. 2). Fractions obtained from normal pancreas and pancreatic acinar carcinoma were free of mitochondrial and endoplasmic reticulum profiles (Fig. 2). In these fractions, the activity of cytochrome oxidase, a mitochondrial marker enzyme, was not detected; the microsomal enzyme esterase activity was barely detectable (data not presented). Morphological and biochemical findings indicate that the purity of Percoll gradient-separated secretory granule fractions is considerably higher than that obtained by differential centrifugation in a preliminary study (21).

Comparison of Secretory Proteins of Normal and Neoplastic Acinar Cell Granules. The Coomassie blue-staining pattern of the two-dimensional gels of normal pancreatic secretory granule content (Fig. 3A) appeared essentially similar to that of zymogen granule proteins discharged by rat pancreatic lobules (23). The two-dimensional gel electrophoretic pattern of secretory proteins obtained from secretory granules of rat pan-
cretic acinar carcinoma (Fig. 3B) appeared qualitatively similar to that of normal rat pancreas (Fig. 3A). All 19 Coomassie blue-stained spots seen in normal secretory granule proteins were also present in pancreatic carcinoma except that spots 12 and 13 appeared as two doublets. The spots 7 and 14 in tumor granule extracts were visible but appeared faintly. In addition, a spot (pI, 5.8; apparent Mr, 24,000) was consistently seen in the two-dimensional gels of pancreatic acinar carcinoma granule proteins but not in gels of normal granule proteins. This spot is designated p24 (p for polypeptide or protein and 24 to indicate Mr, 24,000) (Fig. 3B; Table 1).

Enzymatic activity of normal and neoplastic acinar cell secretory granule proteins was assayed on fractions eluted from IEF gels (Fig. 4). Enzyme activities and the available molecular weight information about rat and guinea pig pancreatic exocrine secretory proteins (1, 11, 23, 24) allowed specific identification of various spots in the two-dimensional gels (Table 1). The nature of spot p24 remains to be determined.

**Protein Composition of Secretory Granule Membranes.**
Electron microscopic examination of the secretory granule membrane band, located at the 0.3 M/1.3 M interface of the sucrose gradient, revealed unsealed or open membranes (Fig. 5). These retained a trilaminar appearance (Fig. 5 Inset). The thickness of carbonate-treated normal and tumor secretory

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**Table 1. Comparison of secretory protein content of secretory granules purified from normal and neoplastic pancreatic acinar cells**

<table>
<thead>
<tr>
<th>Peptide number</th>
<th>Enzyme orzymogen</th>
<th>pI</th>
<th>Mr, x 10^{-3}</th>
<th>Presence in</th>
<th>Normal</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>1</td>
<td>Ribonuclease</td>
<td>8.7</td>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p24</td>
<td>---</td>
<td>5.8</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>2</td>
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<td>24</td>
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<tr>
<td>4</td>
<td>Trypsinogen</td>
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</tr>
<tr>
<td>5 and 6</td>
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<td>+</td>
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<tr>
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<td>29</td>
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<tr>
<td>9</td>
<td>---</td>
<td>4.1</td>
<td>34</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>45-48.5</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>19</td>
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**Fig. 3.** Two-dimensional gel electrophoresis (Coomassie blue-stained patterns) of secretory granules of normal rat pancreas (A) and pancreatic acinar carcinoma (B). The spots are numbered from 1 to 19; spot identification is given in Table 1. The spot designated p24 seen consistently in gels of tumor secretory granule proteins (B) is not observed in A. The arrow indicates the position of soybean trypsin inhibitor. Patterns are derived from IEF strips loaded with 180 µg of protein.

**Fig. 4.** Enzymatic activities of normal () and pancreatic acinar carcinoma (■) secretory proteins determined in IEF slab gels. Each gel was sliced into 0.5-cm segments, and the proteins were eluted by homogenization of each slice. pH increases from left to right. Enzymatic activities are expressed as follows: trypsinogen, tosylarginine methyl ester units; chymotrypsinogen, benzoyltyrosine ethyl ester units; ribonuclease, AOD per hr; amylase, µmol maltose generated per hr; and lipase, nmo1 of triolin breakdown per min. Enzymatic activities of procarboxypeptidase A and B are expressed as arbitrary units.
membrane pellets were compared by NaDodSO4/polyacrylamide gel electrophoresis (Fig. 6). Ten polypeptides were discerned in normal pancreatic secretory granule membranes (Fig. 6, lanes A–C); their Mr's ranged from 135,000 to 19,000 as estimated by comparison with known standards. Two major bands

in normal granule membranes have mean Mr's of 80,000 and 54,000 respectively; the former has been identified as a glycoprotein by periodic acid/Schiff reagent staining (Fig. 7). One other band, of Mr 120,000, also reacted positively for glycoprotein staining (Fig. 7). The polypeptide pattern of tumor secretory granule membranes in Fig. 6, lanes D and E, reveals a marked reduction or absence of glycoprotein of apparent Mr 80,000. In addition, three other polypeptides with mean Mr's of 50,000, 37,000, and 36,000 were also greatly reduced or absent (Fig. 6).

Lipid Composition of Secretory Granule Membranes. The secretory granules and their membranes contained no cardiolipin (Fig. 8, lanes C–F); this phospholipid was found only in the mitochondrial fractions (Fig. 8, lanes A and B). The membranes of secretory granules isolated from normal and neoplastic pancreatic acinar cells were similar in their composition
of phospholipids and cholesterol (Fig. 8, lanes E and F). These membranes contained considerable amounts of cholesterol, phosphatidylethanolamine, and phosphatidylcholine. Sphingomyelin was also found in the secretory granule membranes of both normal and neoplastic acinar cells, whereas phosphatidylserine and phosphatidylinositol were not detectable. Quantitative comparison of various phospholipids separated on thin-layer chromatography was not attempted because of insufficient amounts of purified secretory granule membranes.

**DISCUSSION**

Newly synthesized digestive enzymes and proenzymes, markers for acinar cell differentiation, are stored in secretory granules (1–3). The extent of expression of genes for secretory proteins can be ascertained by analyzing the composition of either the discharged secretory proteins or soluble proteins of isolated secretory granules (1–3, 11, 13, 24). Detailed analyses of secretory proteins synthesized and discharged by guinea pig and rat pancreatic lobules are available (1, 3, 11, 23, 24). Because all adult pancreatic acinar cells are apparently homogenous, the composition of discharged protein is considered identical to the protein composition of secretory granules (1, 2, 13). Recently, Iwanij and Jamieson (23) established that secretory proteins discharged in *citra* by dispersed cell clusters of the same pancreatic acinar carcinoma used in this study lack a group of basic polypeptides, which include proelastase, basic tryspinogen, basic chymotrypsinogen, and ribonuclease. In addition, two forms of procarboxypeptidase B and a major lipase species were absent or greatly reduced when compared to the secretory proteins discharged by normal pancreatic lobules. In the embryonic pancreas, amylase and chymotrypsinogen are expressed between days 15 and 18 of gestation, whereas tryspinogen, ribonuclease, proelastase, and lipase do not appear until after day 18 (25, 26). Based on the fact that their findings closely resemble those encountered in the late development of rat pancreatic rudiments, Iwanij and Jamieson (25) propose that the acinar tumor may, in part, mimic a stage of normal pancreatic development.

In contrast, the secretory proteins obtained from the granule-enriched fraction of the pancreatic acinar carcinoma contain polypeptides that were not readily identified in its discharge (23). The basic forms of chymotrypsinogen, tryspinogen, and proelastase were detectable with Coomassie blue stain, as were a major lipase species, procarboxypeptidases B, and ribonuclease. An unidentified protein (M, 24,000) also was found in the tumor granule extracts. These results suggest that, with the exception of the M, 24,000 protein, the expression of genes for secretory proteins in the zymogen granule-enriched neoplastic acinar cell subpopulation closely resembles the pattern characteristic of the adult gland. The detection in zymogen granule contents of proteins not observed in the tumor discharge in *citra* may reflect a differential expression of secretory protein genes in the cells of this neoplasm. The granule-rich tumor cells comprise less than 50% of the pancreatic acinar carcinoma population (6, 22). The remaining cells represent a spectrum of cytodifferentiation and contain a few or no well-formed zymogen granules (6, 22). The differential expression of certain secretory protein genes in different cell subpopulations may account for the relative lack or marked reduction of basic proteins in secretory proteins discharged by the tumor fragments (23).

The nature of the newly identified protein designated p24 in tumor secretory granule extracts remains unknown. This protein was detectable neither in secretory proteins isolated from granules of normal pancreas (Fig. 3A) nor in the secretory proteins discharged by normal or neoplastic pancreatic acinar cells (23). To extend our understanding of neoplastic cell differentiation and heterogeneity, it will be necessary to characterize this protein.

Despite the apparent similarity of secretory protein profiles of granules isolated from tumor and normal pancreas, striking differences are encountered in the membrane protein composition. Glycoprotein M, 80,000, a major component of normal granule membranes (10, 27, 28), was greatly reduced or absent in tumor secretory granule membranes. The role of this membrane glycoprotein is not known. It may participate in the fusion of secretory granules with the plasma membrane during exocytosis (1, 27). The marked reduction of this glycoprotein in tumor secretory granule membranes may account in part for the diminished secretory response to hormonal stimuli (23, 29). The function of the other absent or reduced polypeptides in the tumor membrane is also unclear. Further investigation is required to clarify the significance and nature of alterations involved in the expression of genes for the secretory granule membrane proteins by neoplastic pancreatic acinar cells.

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