A Common Spectrum of Polypeptides Occurs in Secretion Granule Membranes of Different Exocrine Glands

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Abstract. A highly purified membrane preparation from rat parotid secretion granules has been used as a comparative probe to examine the extent of compositional overlap in granule membranes of three other exocrine secretory tissues—pancreatic, lacrimal, and submandibular—from several standpoints. First, indirect immunofluorescent studies using a polyclonal polyclonal anti-parotid granule membrane antiserum has indicated a selective staining of granule membrane profiles in all acinar cells of all tissues. Second, highly purified granule membrane subfractions have been isolated from each exocrine tissue; comparative two-dimensional (isoelectric focusing; SDS) PAGE of radioiodinated granule membranes has identified 10-15 polypeptides of identical pI and apparent molecular mass. These species are likely to be integral membrane components since they are not extracted by either saponin-sodium sulfate or sodium carbonate (pH 11.5) treatments, and they do not have counterparts in the granule content. Finally, the identity among selected parotid and pancreatic radioiodinated granule membrane polypeptides has been documented using two-dimensional peptide mapping of chymotryptic and tryptic digests.

These findings clearly indicate that exocrine secretory granules, irrespective of the nature of stored secretion, comprise a type of vesicular carrier with a common (and probably refined) membrane composition. Conceivably, the polypeptides identified carry out general functions related to exocrine secretion.

In all eukaryotic cells vesicular carriers serve as shuttles between the Golgi complex and the cell surface to bring about the export of secretory products and the delivery of plasmalemmal components. The presence of secretion granules reflects the capacity of certain cells to devote at least a part of this shuttle system to concentration and intracellular storage of secretory products for intermittent mobilization and discharge in response to external stimuli. The processes of granule formation, exocytosis, and compensatory membrane reinternalization and recycling that define this kind of shuttling pathway have been described in some detail for both exocrine and endocrine systems (14, 32). At present, however, very little information exists concerning the function of specific components of the carrier membranes. Further, it is not clear whether carrier membranes involved in exocrine and endocrine secretion, either with or without an intervening storage phase, contain identical or analogous polypeptides that might have a general role in the shuttle process.

This study seeks to evaluate the extent of compositional overlap between the secretion granule membranes of four different exocrine glands—the parotid, submandibular, lacrimal, and pancreatic. An extensively characterized and highly purified membrane fraction from rat parotid granules, for which a maximum of 5% of the total protein can be ascribed to residual secretory polypeptides or contaminating membrane sources (7), has been used as a compositional standard for comparative investigations at three levels of increasing refinement. First, polyclonal polyclonal (rabbit) antibodies were developed against the entire membrane fraction. Indirect immunofluorescence studies using these antibodies have shown prominent granule membrane labeling in all four tissues. Second, highly purified granule membrane fractions were isolated from lacrimal, pancreatic, and submandibular tissues and freed of adsorbed soluble polypeptides by saponin-sulfate treatment. Comparative examination to parotid granule membranes by subsequent radioiodination, two-dimensional (isoelectric focusing; SDS) PAGE, and autoradiography has identified an apparent compositional overlap involving 10-15 polypeptides of identical (or nearly identical) pI and molecular mass. Finally, the identity of selected parotid and pancreatic radioiodinated polypeptides has been documented using two-dimensional cellulose peptide mapping of chymotryptic and tryptic digests. As a result of these findings, our future studies relating to the basic mechanisms of exocrine secretion, and possibly even the general operation of vesicular carriers between the Golgi region and the cell surface, are now focused on a subset of granule membrane proteins.

Materials and Methods

Preparation and Immunochemical Characterization of Anti-Membrane Antiserum

New Zealand white rabbits (female, 2-2.5 kg) were bled 1 wk before immunization to obtain preimmune serum. Purified parotid secretion granule...
membranes (100–150 μg protein; isolated as described previously [7]) solubilized in 0.5% Triton X-100, were emulsified with complete (and then incomplete) Freund's adjuvant. The schedule of immunization and bleeding, as well as site of injection (intradermal, intramuscular, along mammary lines) were carried out essentially according to the protocol of Papermaster et al. (33).

To identify the antigenic determinants recognized by the antisera, immunoprecipitations were conducted using radiiodinated parotid granule membrane samples by procedures already described in detail (10). Antigen-antibody complexes were isolated with goat IgG (Cowan I) and subjected to one-dimensional SDS PAGE. Labeled antigens were identified by autoradiography.

Immunocytochemical Procedures

Rats (100–125 g) were perfused transcardially with tissue culture medium followed by ice-cold 3% formaldehyde (deproteinized from paraformaldehyde) in 0.12 M sodium phosphate, pH 7.4. Tissues of interest were excised, and fixation in the same solution was continued for 3 h at 0°C. Tissue blocks were then washed in several changes of phosphate buffer, infiltrated with a series of buffered sucrose solutions, and used for cutting frozen sections (either 4–6-μm thick [12] or 0.5–1-μm thick [17]). Indirect immunofluorescence on frozen sections was performed as described (12), using rhodamine-conjugated goat IgG directed against rabbit IgG, for detecting the binding of primary antibody.

Isolation of Secretion Granule Fractions from Rat Exocrine Tissues

From Lacrimal Gland. Exorbital lacrimal tissue (1.5–2.5 g from ten 100–125 g animals) was minced with razor blades and homogenized in 0.3 M sucrose, 0.5 mM MgCl₂ and 0.2 μg/ml N,N'-diphenyl-p-phenylene-diamine (DPPD) at 15% (wt/vol) using a TissuePrep (Tekmar Co., Cincinnati, OH) for 5 s at 1,900 rpm followed by three passes at 1,300 rpm in a Bredelfer Teflon-glass homogenizer. Centrifugation (13 min at 60,000 g) yielded supernatants, which were decanted and saved, and pellets containing microsomal and particulate fractions. The latter were resuspended in the original volume of the same medium, and homogenization (Teflon-glass only) and centrifugation were repeated. The first and second supernatant fractions were combined and designated NS₁; the remaining pellet, used only for immunoprecipitations, was designated NS₄ (see also Section on Immunoprecipitation).

Purification of lacrimal granule membranes was conducted by a modified procedure originally used for the purification of parotid secretion granules (7). Subcellular fractions from homogenates of 4–5 g tissue (from 10–12 animals starved overnight) were adjusted to a density corresponding to that of 1.42 M sucrose, given three passes in a tight-fitting sucrose step gradient (underlayers of 1.50 M and 1.80 M sucrose [15 and 8 ml, respectively]) containing the supplements above and an overlay to tube capacity of 1.8 M sucrose containing everything except Ficoll. Centrifugation (150 min at 87,000 g, in a Beckman SW28 rotor) gave a crude granule fraction (G₁₅) at the 1.50/1.80 M sucrose interface. G₁₅ was collected, adjusted to a density equivalent to 1.55 M sucrose using 0.5 M EDTA solution, dispersed by Dounce homogenization, and loaded (8 ml/tube) in a second sucrose step gradient (underlayers of 1.50 M and 1.80 M sucrose [15 and 8 ml, respectively]) containing the supernatants above and an overlay to tube capacity of 0.8 M sucrose containing everything except Ficoll. Centrifugation (150 min at 87,000 g, in a Beckman SW28 rotor) provided the purified granule fraction (G₂₅) at the 1.50/1.80 M sucrose interface.

For all three granule fractions, NS and NP together constitute the total homogenate that is used as a basis for calculating the recoveries of all enzyme activities. All biochemical data presented represent the averages of values obtained from at least three independent preparations.

Granule Lysis and Isolation of Membrane Subfractions

After sedimentation, purified secretory granule fractions were resuspended to a 30 ml vol with lysis medium containing: (a) lacrimal: 140 mM KCl, 50 mM KSCN, 5 mM EDTA, 10 μg/ml DPPD, 0.4 mM morpholinopropanesulfonic acid, pH 7.0; (b) pancreas: 150 mM NaHCO₃, 50 mM KCl, 5.5 mM EDTA, 1.0 μg/ml DPPD, 1.0 mM benzamidazole, 0.4 mM PMSE, pH 8.5; and (c) submandibular: 190 mM KCl, 5 mM EDTA, 1.0 μg/ml DPPD, 1.0 mM benzamidazole, 0.4 mM PMSE, 2 mM Hepes, pH 6.8. The diluted granule suspensions were maintained at 0°C until clearing indicated total or nearly complete lysis (1–2 h). The resulting density of granule membranes from soluble and particulate contaminants, the granule lysates were mixed with an equal volume of 1.8 M sucrose in lysis medium (KSCN was not included during further purification of lacrimal secretion granule membranes), loaded into three centrifuge tubes, and overlaid with 0.75 M sucrose in lysis medium (25 ml/tube) and finally with lysis medium alone to tube capacity. During centrifugation (180 min at 87,000 g, in a Beckman SW41 rotor) secretion granule membranes float to the lysis medium/0.75 M sucrose interface while residual mitochondria and unlysed secretion granules pellet: soluble secretory proteins remain in the 0.9 M sucrose load. After collection, the granule membrane suspension was diluted in lysis medium supplemented with saponin and Na₂SO₄ (at final concentrations of 10 μg/ml and 0.3 M, respectively) and maintained at 0°C for 60 min. Alternatively, sodium carbonate treatment (0.1 M Na₂CO₃-0.1 M KCl)-5 mM EDTA [pH 11.5], 30 min, 4°C) was used in place of saponin-sulfate with no perceptible alteration in polypeptide composition of the membrane subfractions. Purified membrane granules were obtained by subsequent dilution with lysis medium (until the refractive index was below that of 0.15 M sucrose) and sedimentation (120 min at 150,000 g, in a Beckman SW41 rotor).

Chemical and Enzyme Assay of Cell Fractions

α-Amylase, γ-glutamyl transferase (γ-GT), cytochrome c oxidase, rotenone insensitive NADH-cytochrome c reductase, β-N-acetylglucosaminidase, amine oxidase (type A), and protein were determined as described previously (7). Peroxidase activity was determined with the colorimetric di-
Processing of Subcellular Fractions for Electron Microscopy

Secretion granules were fixed in suspension by the addition of 50% glutaraldehyde, 10% formaldehyde, 0.5 M sodium phosphate (pH 7.4) to final concentrations of 3% (vol/vol), 1% (vol/vol), and 0.1 M, respectively. After several buffer rinses sedimented granule fractions were postfixed in 2% OsO₄, stained with uranyl acetate (0.5% [wt/vol] in 50 mM maleate buffer, pH 5.8), dehydrated in ethanol and propylene oxide, and embedded in Epon. All micrographs were taken on a Siemens electron microscope 101.

Radioiodination Procedures

Isolated membrane fractions (50 µg protein resuspended in 200 µl of 50 mM Tris-HCl, pH 7.5) were radioiodinated using either lactoperoxidase-glucose oxidase (8 mU lactoperoxidase, 6 mU glucose oxidase, 10 µM glucose [21]) or chloramidine T (20 µl, 2.5 mg/ml) as catalysts and 1.0 µCi Na¹²⁵I for 30 and 3 min, respectively, at 4°C. With chloramidine T, reactions were terminated by the addition of sodium metabisulfite (40 µl, 2.5 mg/ml); for lactoperoxidase-glucose oxidase, termination of reaction was by addition of sodium azide (0.1%). Parotid secretory proteins purified from granule lysates [7] were radioiodinated using chloramidine T as described for membrane polypeptides. Labeled membranes as well as content were separated from unincorporated iodine by gel filtration on a 6-ml column of Agarose A-0.5 M or Biogel-P4, respectively, both previously equilibrated in 50 mM ammonium bicarbonate.

PAGE and Peptide Mapping

One-dimensional SDS PAGE, used to identify antigens recognized by the anti-membrane antiserum and to resolve membrane and content polypeptides for radiolabeling and peptide mapping, was performed on linear acrylamide gradients prepared in the Laemmli buffer system [24]. Two-dimensional PAGE (isoelectric focusing; SDS) was carried out by a modification (1) of the O’Farrell procedure [30]. Iodinated membrane or content samples were solubilized (3 min at 80°C) in 1.0% SDS, 10% (vol/vol) 2-mercaptoethanol, 10 mM Tris-HCl (pH 8.0) before addition of Nonidet P-40 (8% [wt/vol]), urea (9 M), amphot (pH range 3-10, 2%), 2-mercaptoethanol (10% [vol/vol]), and Tris-HCl (pH 8.0, 10 mM), all final concentrations. First dimension isoelectric focusing was carried out in acrylamide tube gels (0.3 x 10.5 cm), and electrode buffers (degassed) were 10 mM H₃PO₄ and 20 mM NaOH. Electrophoresis was conducted for 18 h at 400 V followed by 30-45 min at 800 V. Gels were removed and either cut in 0.5-cm segments to measure the pH (in I-ml water extracts) or equilibrated in 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.9), 0.01% bromophenol blue before second dimension SDS gel electrophoresis. Resolving gels (16 x 16 x 0.1 cm) were 7-14% linear acrylamide gradients prepared in the Laemmli buffer system [24].

The procedures of Elder et al. [13] as modified by Speicher et al. [37] were used to prepare and analyze chymotryptic and trypsin-digests of individual polypeptides by two-dimensional mapping on cellulose (20 x 20 cm) thin-layer plates (Eastman Kodak Co., Rochester, NY). Individual polypeptides from sodium carbonate-treated membranes were originally separated on one-dimensional SDS gels as indicated above. By increasing the length of the resolving gel to 23 cm and loading 150 µg protein, it was possible to resolve most of the granule membrane polypeptides (shown in two-dimensional patterns in Fig. 4) as individual bands and to detect them by Coomassie Blue staining. Bands were excised and processed for analysis from fixed, stained gels. A 15-cm electrophoretic migration and a 17-cm chromatographic migration of the basic fuchsin tracking dye were used in the two-dimensional analysis of the proteolytic digests. Autoradiograms were exposed at -70°C in the presence of image intensifying screens.

Materials

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and female New Zealand rabbits from Pineacres (West Brattleboro, VT). Complete and incomplete Freund’s adjuvant, lactoperoxidase, and Nonidet P-40 were obtained from Calbiochem- Behring Corp. (La Jolla, CA). Glucose oxidase was from Sigma Chemical Co. (St. Louis, MO). Rhodamine-conjngated IgG (heavy and light chain specific), trypsin, and chymotrypsin were obtained from Cooper Biomedical, Inc. (Malvern, PA). Utrapure urea was obtained from Schwarz-Mann (Spring Valley, NY); Na¹²⁵I from Amersham Corp. (Arlington Heights, IL); carrier ampholines from LKB Instruments, Inc. (Gaithersburg, MD) or Bio-Rad Laboratories (Richmond, CA); other electrophoresis reagents from Bio-Rad Laboratories. All other supplies were from general distributors.

Results

An Antiserum to Rat Parotid Granule Membranes: Immunofluorescent Labeling of Other Exocrine Secretory Tissues

The parotid granule membrane antiserum used in the present study came from a bleed taken from the rabbit 5 wk into the antigen injection program. As judged by the results of an immunoprecipitation experiment (Fig. 1), many membrane polypeptides can be identified as antigens. Although the antigenicity of different species clearly varies and tends to favor the glycoproteins of higher apparent molecular mass, the antiserum was considered to be useful as an easily applied, low resolution probe to test for compositional overlap (and the extent of localization of total antigens to granule membranes) in other secretory tissues. The appearance by indirect immunofluorescence of sections of parotid, lacrimal, pancreatic, and submandibular glands reacted with granule membrane antiserum is shown in Fig. 2. For all four exocrine secretory tissues, immunostaining is concentrated over secretion granule membrane profiles identified on the basis of their apical cytoplasmic location, size, and frequency. Interestingly, staining extends to a varying extent to both the apical (especially prominent in lacrimal and pancreas) and basolateral (generally limited staining) domains of the plasma membrane but does not appear to be significant over basal or perinuclear cytoplasmic regions known to contain membranes of the endoplasmic reticulum, mitochondria, and Golgi complex, respectively. In the case of lacrimal and submandibular glands (Fig. 2, E and F), the staining includes the granule membranes of distinct cell types comprising these tissues. The image observed for all acinar cells can be contrasted with that obtained in parotid after staining with...
Figure 2. Comparative indirect immunofluorescent staining of rat exocrine tissues using heterologous antiserum to parotid secretory granule membranes (A and C-F) and heterologous antiserum to parotid proline-rich secretory proteins (B). (A) Parotid, 4–6-μm thick frozen section. (B) Parotid, 1-μm etched Epon section. (C) Parotid, 0.5-μm thick frozen section. (D) Pancreas, 0.5-μm thick frozen section. (E) Lacrimal, 0.5-μm thick frozen section. (F) Submandibular, 0.5-μm thick frozen section. Note in all cases that staining is concentrated over the stored granule population and is not detected in the basal cytoplasm containing abundant rough endoplasmic reticulum and mitochondria. Primary antiserum dilution for all immunolocalizations was 1:50. Bars, 10 μm.
antibodies to the proline-rich secretory proteins where immunofluorescence extends throughout the content matrix rather than showing peripheral concentration at the bordering granule membrane. Finally, the granule membrane antibody does not exhibit detectable binding to duct cell profiles or within the luminal, secretory space. As well, preimmune serum used in place of primary immune serum (or sole use of secondary rhodaminated goat anti-rabbit IgG) gave, at most, weak fluorescence with no obvious localization. Evidently, this screening study suggests that the composition of granule membranes is distinct from most major cellular membranes and that a more detailed evaluation of the extent of compositional overlap between the granule membranes of different exocrine tissues is warranted.

2. Proline-rich secretory polypeptides were purified from rat parotid as outlined by Muenzer et al. (27) and heterologous antiserum developed according to Papermaster et al. (33). Immunohistochemical analyses show that the antiserum is selective for the entire family of proline-rich polypeptides.

Secretion Granule Fractions from Exocrine Tissues: Morphological and Biochemical Characterization

As an essential step to a comparison of the composition of different types of secretion granule membranes at much higher resolution, we sought highly purified, representative granule fractions from lacrimal, pancreatic, and submandibular tissue. By introducing minor modifications of preexisting fractionation procedures (5, 7), it has been possible in each case to obtain secretion granules with a very favorable yield and a low level of contamination. The results of these efforts are documented morphologically and biochemically in Fig. 3 and Tables I–III.

Representative low magnification electron micrographs of the secretion granule fractions obtained from each tissue are shown in Fig. 3. The majority of components are electron dense secretion granules which, at higher magnifications, are observed to be bounded by continuous unit membranes. Organelle contamination is minimal; only rare mitochon-

Figure 3. Low magnification electron micrographs of purified exocrine secretory granule fractions. Representative micrographs of: (A) top half of submandibular granule fraction pellet; (B) bottom half of a submandibular granule fraction pellet; (C) lacrimal secretion granule fraction; and (D) pancreas zymogen granule fraction. Bars, 1 μm.
Table I. Distribution of Marker Enzymes in Cell Fractionation of Rat Exorbital Lacrimal

| Fraction | Protein | β-N-Acetyl glucosaminidase | Cytochrome c oxidase | NADH-cytochrome c reductase | Peroxidase | γ-GT |
|----------|---------|----------------------------|---------------------|---------------------------|------------|------|
|          | mg      | %H RSA*                    | %H RSA              | %H RSA                    | %H RSA     | %H RSA |
| Homogenate (H) | 167.4 | 100  | 1 | 100  | 1 | 100  | 1 | (9.16) | (30.6) | (412.0) | (454.0) | (0.88) |
| NS       | 122.6  | 73.2 | 93.8 | 1.27 | 79.9 | 1.09 | 80.0 | 1.11 | 80.1 | 1.08 | 56.2 | 0.76 |
| NP       | 44.8   | 26.8 | 6.2  | 0.23 | 20.1 | 0.75 | 20.0 | 0.76 | 19.9 | 0.75 | 43.8 | 1.63 |
| G1       | 15.3   | 9.4  | 6.0  | 0.63 | 2.2  | 0.23 | 3.25 | 0.35 | 22.5 | 2.38 | 8.9  | 0.95 |
| Gradient recovery % | 99.0 | 99.8 | 91.6 | 99.6 | 99.9 | 92.1 |
| G2       | 6.6    | 4.1  | 1.8  | 0.45 | 0.4  | 0.09 | 0.6  | 0.15 | 14.6 | 3.53 | 5.6  | 1.38 |
| Gradient recovery % | 99.6 | 98.7 | 98.9 | 100.1 | 94.2 | 96.5 |

Abbreviations for isolated fractions are as in Materials and Methods. Numbers in parentheses represent total homogenate activities. \* RSA, sp act (per mg protein) relative to that of the homogenate (H).

Table II. Distribution of Marker Enzymes in Cell Fractionation of Rat Pancreas

| Fraction | Protein | β-N-Acetyl glucosaminidase | Cytochrome c oxidase | NADH-cytochrome c reductase | α-Amylase | γ-GT |
|----------|---------|----------------------------|---------------------|---------------------------|------------|------|
|          | mg      | %H RSA*                    | %H RSA              | %H RSA                    | %H RSA     | %H RSA |
| Homogenate (H) | 223.9 | 100  | 1 | 100  | 1 | 100  | 1 | (36.49) | (52.83) | (3020.0) | (19814) | (107.6) |
| NS       | 181.5  | 81.1 | 89.0 | 1.08 | 77.3 | 0.95 | 87.6 | 1.08 | 83.9 | 1.03 | 68.7 | 0.84 |
| NP       | 42.4   | 18.9 | 11.0 | 0.58 | 22.7 | 1.20 | 12.4 | 0.66 | 16.1 | 0.85 | 31.3 | 1.65 |
| G1       | 21.0   | 9.6  | Not detected | 5.3  | 0.35 | 2.2  | 0.23 | 30.5 | 3.17 | 5.6  | 0.58 |
| Gradient recovery % | 101.7 | 98.4 | 100.4 | 99.6 | 96.3 | 97.8 |
| G2       | 10.0   | 4.6  | Not detected | 0.4  | 0.08 | 0.06 | 0.01 | 18.5 | 4.01 | 3.0  | 0.67 |
| Gradient recovery % | 100.6 | –    | 97.3 | 100.7 | 96.8 | 94.5 |

Abbreviations for isolated fractions are as in Materials and Methods. Numbers in parentheses represent total homogenate activities. \* RSA, sp act (per mg protein) relative to that of the homogenate (H).

Pancreatic granules appear uniform and spherical, whereas lacrimal and submandibular granules are heterogeneous in size, shape, and especially in the case of submandibular granules, appearance of packaged content. The images are consistent with those observed in situ and, in part, reflect an origin from distinct cell types within the respective glands (4, 18, 22, 29).

In assessing the biochemical purity of the granule preparations, enzyme and chemical assays for markers of granules as well as of contaminating organelles were conducted on all fractions generated during purification. The results obtained are presented in Tables I—III; for clarity, only data for NS and NP (together comprising the original homogenate) and for G1 and G2 (the granule-enriched fractions) are shown. However, at each step it is possible to account for nearly all (>90%) of the activity associated with the immediately preceding (parent) fraction. In all cases contamination of fractions G2-L, P, S, by mitochondria (cytochrome c oxidase), endoplasmic reticulum (rotenone insensitive NADH-cytochrome c reductase), and lysosomes (β-N-acetyl glucosaminidase) is minimal. The contaminant markers are reduced to <1% of the original homogenate activity (with the exception of β-N-acetylglucosaminidase in the lacrimal granule fraction) and exhibit large decreases in specific activity (signifying depurification) in comparison to homogenate values. Further, all results compare favorably with those obtained for parotid secretion granules (7).

α-Amylase and peroxidase, which have been used as granule content marker activities for pancreas and lacrimal, respectively, are obtained in G2p and G2L in favorable yields (>15% of homogenate activity). As discussed before (7), these yields represent minimum estimates because they are based on the assumption that the activities selected mark granules exclusively, yet other organelles comprising the transport pathway are known to contain active enzyme (5, 18). So far, neither amylase nor peroxidase activities have been detected in homogenates or purified granule fractions from submandibular tissue. Although the activities were expected, based on the results of others (4, 29), their absence here may reflect decreased expression due to developmental regulation (4) or to the presence of inactive forms (29). Finally, as in the case of the parotid gland, γ-GT activity is associated with secretion granules in all three tissues, and...
Table III. Distribution of Marker Enzymes in Cell Fractionation of Rat Submandibular

| Fraction       | Protein | β-N-Acetyl glucosaminidase | Cytochrome c oxidase | NADH-cytochrome c reductase | γ-GT |
|----------------|---------|----------------------------|----------------------|-----------------------------|------|
|                | mg %H   | %H RSA                     | %H RSA               | %H RSA                      | %H RSA |
| Homogenate (H) | 317 100 | 100 1 (11.45)              | 100 1 (100.0)        | 100 1 (588)                 | 100 1 (0.61) |
| NS             | 158 49.8| 73.0 1.46                  | 58.9 1.18            | 71.7 1.44                   | 72.5 1.46 |
| NP             | 159 50.2| 27.0 0.54                  | 41.1 0.82            | 28.3 0.56                   | 27.5 0.55 |
| G1             | 12.3 3.9| 2.6 0.66                   | 0.3 0.08             | 2.2 0.55                    | 4.5 1.15 |
| Gradient recovery % | 97.7 103.5 | 91.5 | 99.3 | 93.4 |
| G2             | 5.9 1.9 | 0.3 0.15                  | 0.03 0.02            | 0.5 0.26                    | 3.4 1.81 |
| Gradient recovery % | 105.5 105.5 | 97.6 | 98.3 | 100.1 |

Abbreviations for isolated fractions are as in Materials and Methods. Numbers in parentheses represent total homogenate activities. RSA, sp act (per mg protein) relative to that of the homogenate (H).

Table IV. Distribution of Marker Enzymes in Pancreatic Zymogen Granule Subfractions

| Fraction       | Protein | Cytochrome c oxidase | Amine oxidase type A | α-Amylase | γ-GT |
|----------------|---------|----------------------|----------------------|-----------|------|
|                | % Lysate | % Lysate RSA        | % Lysate RSA         | % Lysate RSA | % Lysate RSA |
| Lysate-G2p     | 100      | 100 1              | 100 1               | 100 1     | 100 1 |
| (12.6 mg)      |          | (12.6 mg)          | (12.6 mg)           | (12.6 mg) | (12.6 mg) |
| Gradient fractions |        |                     |                     |           |      |
| Buffer overlay | 0.0      | 0.0 0               | 0.0 0               | 0.0 0     | 0.0 0 |
| Membrane interface | 1.8 2.6 | 1.44 0.0            | 0.0 0               | 0.26 0.15 | 53.1 29.83 |
| 0.75 M Layer   | 6.0      | 2.7 0.44            | 0.0 0               | 3.1 0.51  | 21.9 3.65 |
| 0.75/0.90 M Interface | 14.5 | 2.2 0.15           | 0.0 0               | 13.8 0.95 | 4.7 0.32 |
| 0.90 M Layer   | 72.9     | 12.5 0.17           | 15.8 0.22           | 85.1 1.17 | 11.8 0.16 |
| Pellet         | 4.0      | 81.4 20.29          | 87.4 21.78          | 1.1 0.28  | 9.3 3.68 |
| Recovery       | 99.2     | 101.4 103.2         | 103.4 100.8         |           |      |
| Final membranes| 0.4 0.55 | 1.30 0.0            | 0.0 -               | 0.0 -     | 48.0 110.2 |

The results shown are representative of two experiments in which marker enzyme distribution was followed throughout zymogen granule subfractionation. Number in parenthesis represent total lysate protein. RSA, sp act (per mg protein) relative to that of the lysate.

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Comparative Analysis of Granule Membrane Polypeptides

Membrane Purification. In our previous fractionation studies of rat parotid tissue (7) mitochondria were identified as the only organelle present in the purified granule fraction (0.9% of homogenate cytochrome c oxidase activity and 0.3% of homogenate amine oxidase-type A activity) that could potentially contribute significantly as a contaminant to the final membrane preparation. By purifying parotid mitochondria and determining their specific activities of cytochrome c oxidase and amine oxidase-type A (7), we estimated that mitochondria contributed, at most, 5% of the protein present in the purified granule membrane preparation. By purifying parotid mitochondria and determining their specific activities of cytochrome c oxidase and amine oxidase-type A (7), we estimated that mitochondria contributed, at most, 5% of the protein present in the purified granule membrane preparation. Therefore, it has been used as a marker to follow the purification of membranes from granule lysates.

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activity was associated with membrane of higher density that floats out of the load of the original gradient but only partially penetrates the overlying sucrose layer. Where this membrane has been recovered, treated with saponin-sulfate, and ultimately analyzed by SDS PAGE in parallel with the usual membrane fraction, the polypeptide profiles observed were essentially identical. Thus, the incomplete recovery reflects loss as a result of either an unexplained higher buoyant density for this membrane or failure of extremely small membrane vesicles generated during lysis to float to the appropriate density during centrifugation. Even where recovery is decreased relative to that observed before (7), the contribution of mitochondria to total protein is negligible for pancreatic membranes and is estimated to be no more than 7–8% in the other cases. Clearly, these estimates represent an upper limit because they assume no damage is incurred by contaminating mitochondria (and thus no loss of mitochondrial protein) during granule lysis and membrane purification. Since the main purpose of the present study is to identify polypeptides similar to those characterized in parotid granule membranes, the incomplete recovery does not represent a major limitation.

Two-Dimensional PAGE

The capability of obtaining highly purified granule membrane preparations from all four exocrine tissues made possible the detailed comparison of polypeptide composition. Autoradiograms of radioiodinated secretion granule membrane polypeptides resolved by two-dimensional polyacrylamide gel electrophoresis are shown in Fig. 4. Since the pH gradient established during isoelectric focusing exhibited minor variations in different experiments, the polypeptide profiles for lacrimal, submandibular, and pancreas have been matched to those of parotid membranes subjected to electrophoresis in parallel. Each profile comprises ~25 species with electrophoretic mobilities corresponding to apparent molecular masses ranging from 18 to 150 kD. In the case of parotid, the number and distribution by apparent molecular mass are consistent with the one-dimensional profile shown previously using either tyrosine- or amino group–directed labeling (7). Also, polypeptides of low apparent molecular mass are more prevalent than larger species in all cases. As well, the majority of granule membrane polypeptides are acidic, focusing between pH 5 and 7. This observation is consistent with the previous reports for the general nature of the polypeptides of adrenal chromaffin granules (3), pancreatic zymogen granules (34), and parotid granules (9), although in the latter case where residual mitochondrial contamination appears to be significant, the pattern is considerably more complex than the one we have observed.

The most striking observation seen by comparing the profiles for the four types of membranes is the presence of apparent extensive polypeptide homology. Not only are identities suggested by overlapping mobilities for individual species, but also the two-dimensional patterns of spots in a number of regions are similar, if not identical. Specifically, major radiolabeled species (~10 distinct polypeptides) that coincide in pI and molecular mass are located in the range 24–30 kD and, in part, ≥85 kD. Evidently, these common polypeptides do not reflect incompletely removed secretory proteins since as shown in Fig. 4 C, neither radiolabeling nor protein staining of parotid granule content reveals a pattern having comparable isoelectric points and apparent molecular masses.

In addition, at least four to six species in the range 40–70 kD exhibit common mobilities but show much wider quantitative variations in intensity between the different types of membranes. Some of these species are observed in more than one but not all preparations. Finally, each sample contains unique polypeptides for which there is no counterpart in the other patterns. Although some of the latter components could reflect tissue-specific residual secretory contaminants (because we have not conducted a comprehensive analysis for such species as done previously for parotid granule membranes (7)), we consider this possibility unlikely because the polypeptide profiles for parotid and pancreatic specimens (Fig. 4, A and B) are unchanged when the membranes are treated with sodium carbonate. This treatment has been used widely as a means of stripping extrinsic and soluble proteins from membrane fractions (5, 15, 20). Thus, the unique polypeptides are more likely to be integral membrane components.

Comparative Peptide Mapping of Membrane Polypeptides

The observation of an overlapping two-dimensional pattern of at least 10 granule membrane polypeptides from different exocrine secretory cells strongly suggests the presence of common polypeptides, conceivably involved in general secretory function. To increase the certainty of this observation, we checked for structural homology at a still more refined level between selected pairs of parotid and pancreatic polypeptides by performing two-dimensional mapping of chymotryptic as well as trypic peptide digests. All analyses were performed at equal radioactivity/molecular mass ratios (37).

Fig. 5 shows as a representative example, autoradiograms of radioiodinated peptides in chymotryptic and trypic profiles of a major overlapping 29-kD polypeptide prepared from parotid and pancreatic membranes. Comparative examination indicates that the chymotryptic spectra and trypic spectra correspond nearly exactly for each polypeptide source with only minor quantitative differences existing for selected peptide fragments (open arrows in Fig. 5, A–D). These observations are confirmed by mixing digests from the two sources and co-mapping (Fig. 5, E and F). These peptide mapping analyses have been extended in two ways: first, we have examined comparatively the majority of matching parotid and pancreatic granule membrane polypeptides (chymotryptic hydrolysates only) and second, we have examined parotid secretory polypeptides using both chymotryptic and trypic hydrolysis. In the first case, with few exceptions (e.g., certain pancreatic and parotid glycoproteins with high molecular mass), the peptide maps are essentially identical for polypeptides of similar apparent molecular mass from the two types of granule membranes (data not shown). Again, minor quantitative differences in the incidence of several peptide fragments have been noted for all matching peptide profiles. In the second case, the peptide profiles for all parotid secretory proteins (5–56 kD) have been analyzed for possible compositional similarities to polypeptides identified as membrane constituents. Selected examples (either
Figure 4. Comparative two-dimensional SDS PAGE analyses of purified exocrine secretory granule membrane polypeptides. (A and B) Autoradiograms obtained by parallel electrophoresis of radioiodinated (chloramine T) parotid (A) and pancreas (B) secretory granule membrane polypeptides. (C) Two-dimensional SDS PAGE analyses of radioiodinated (chloramine T, top) and silver-stained (bottom) parotid secretory polypeptides obtained from granule lysates (7). (D-F) Autoradiograms of radioiodinated (lactoperoxidase-glucose oxidase) polypeptides subjected to electrophoresis in parallel for secretory granule membranes of parotid (D), submandibular (E), and lacrimal (F). The polypeptide indicated with an asterisk in D represents absorbed radiolabeled lactoperoxidase. The arrowhead in B reflects the position of pancreatic zymogen granule membrane protein GP-2. Note that it is incompletely resolved from higher molecular mass (~85-95 kD) glycoproteins related to γ-GT (10). (pH values are indicated horizontally, molecular mass x 10^{-3} vertically).

Chymotryptic or trypsin hydrolysates (that purposely focus on the 20-35-kD region (where the strongest similarities in exocrine membrane composition have been observed) are shown in Fig. 6. In all cases, secretory polypeptides were found to possess entirely distinct peptide profiles from those obtained for membrane polypeptides. Furthermore, in no case have we observed the pattern of shared peptides observed in all granule membrane maps (see below).

Coupled with the two-dimensional analyses of undigested polypeptides presented in Fig. 4, these results argue strongly that granule membranes from different exocrine cell types have an overlapping polypeptide composition.

The autoradiograms shown in Fig. 7, A-D (tryptic peptide patterns obtained for parotid granule membrane polypeptides of 26, 37, 41, and 44 kD) serve to establish separate points concerning the extent of structural interrelationship between polypeptides of the same granule membrane type. Comparison of these profiles with one another and with that in Fig. 5B reveals that, with the exception of 41- and 44-kD species, each profile shows a unique composition that is ap-
Figure 5. Autoradiograms of two-dimensional peptide maps from a parotid and pancreas secretory granule membrane polypeptide of 29,000 D. Gel slices were radioiodinated and processed as described in Materials and Methods. Electrophoresis was in the horizontal direction.
Figure 6. Autoradiograms of two-dimensional chymotryptic (A–C) and tryptic (D–F) peptide maps from several parotid secretory polypeptides. Parotid secretory polypeptides, purified from granule lysates (7), were resolved by one-dimensional SDS PAGE (24) and excised gel slices were radioiodinated and processed as described in Materials and Methods for membrane polypeptides. Details of mapping are given in Fig. 5. Polypeptide apparent molecular masses (in kilodaltons): (A) 35; (B) 33; (C) 25; (D) 35; (E) 27; (F) 22.

Discussion

One of the special features of exocrine secretory glands, especially the parotid, is the presence of a single secretory cell type in large quantity and containing an unusually large internal compartment of stored secretion. Our previous studies have capitalized on these properties, and we have established that the membrane devoted to the storage function has an unusually low protein concentration and a rather limited spectrum of polypeptides that are probably largely distinct from those of other intracellular membranes (7). As a consequence of the extensive documentation of the purity of isolated parotid granule membranes (7), we felt that they would serve as an excellent compositional standard for examining comparatively the membranes in other cell types where the...
operation of vesicular shuttles between the Golgi complex and the cell surface is particularly prevalent. The underlying motivation for this investigation is the two-part working hypothesis, first, that exocrine storage membranes have a very limited repertoire of functions mostly related to secretion (i.e., packaging and discharge of macromolecules and subsequent membrane retrieval) and second, that these basic operations involve common membrane machinery in all cells. So far our main focus has been on other exocrine glands which, like the parotid, are distinguished by sizable intracellular secretory storage compartments but where the type of secretory cell is less homogeneous.

The initial approach involved indirect immunofluorescence studies using a polyspecific anti-parotid granule membrane antiserum which indicated a uniform and, for the most part, specific granule membrane staining restricted to all acinar cells of all tissues. Although the limited resolution of such a probe must be underscored (especially because selected antigens, particularly the membrane glycoproteins, are disproportionately emphasized in relation to the total polypeptide spectrum), these observations argue simultaneously in favor of both parts of the working hypothesis—

functional specialization of granule membrane yet common composition irrespective of the diverse chemical nature of the stored exocrine secretion.

Previously, a similar approach has been taken to illustrate the widespread occurrence of synaptic vesicle antigens in neural and endocrine secretory tissues (6, 23, 25, 38). Interestingly, no staining of exocrine secretory tissues was observed (23, 25, 38). This finding contrasts with our preliminary efforts in the reverse direction (8) where we have shown low intensity, but specific, immunostaining of both neural and endocrine tissues (e.g., cerebellum, anterior pituitary, adrenal medulla), using the parotid granule membrane antiserum. Evidently, these observations deserve further investigation at higher resolution and sensitivity. They suggest further support for the working hypothesis, but more importantly, they may identify polypeptides that are either identical or antigenically related to some of the species that are shared between different exocrine cells.

The guiding nature of the immunolocalization studies, using the polyspecific anti-membrane antiserum, is clearly surpassed in the fractionation experiments that form the main part of this study and lead to the most definitive results.

*Figure 7. Autoradiograms of two-dimensional tryptic peptide maps from several parotid secretory granule membrane polypeptides. Details of mapping are given in Fig. 5. Polypeptide apparent molecular masses (in kilodaltons): (A) 26; (B) 37; (C) 41; and (D) 44.*
regarding the general composition of exocrine secretory membranes. To obtain granule membranes of sufficient purity for comparative analysis, we wish to emphasize the importance of taking advantage of their characteristically very low buoyant density in relation to potential contaminants, particularly mitochondria. Both Table IV of the present studies and Table IV already reported (7) indicate very clearly that pancreatic and parotid granule membranes can be separated efficiently from mitochondrial marker activities by flotation through 0.75 M sucrose. In studies where such procedures have not been used, especially if the original granule fraction was obtained by differential centrifugation, it is very likely that the final granule membrane fraction is substantially contaminated. Using granule membrane preparations having negligible levels of mitochondrial contamination and obtained in fairly good yield, the two-dimensional electrophoretic analysis identified extensive compositional overlap for all of the cell types investigated. Between 10 and 15 radioiodinated polypeptides present in lacrimal, pancreas, and submandibular preparations are observed to have virtually the same isoelectric point and apparent molecular mass as those found in the parotid standard. Most of the overlap involves species that have a molecular mass <35 kD and are not likely to be extensively glycosylated since they do not exhibit an extended series of stuttered isoelectric points in Fig. 4 and they were not labeled in the limited lectin overlay studies conducted earlier (7). For three major reasons we wish to emphasize that attention is almost certainly focused on integral membrane components belonging to granules. First, the polypeptides of 25–35 kD have been identified previously as major radiolabeled parotid species using both tyrosine- and amino group–directed covalent labeling (7) even though they stain poorly with Coomassie Blue. Their relative prominence among a rather limited spectrum of total polypeptides is not characteristic of other more functionally diverse cellular membranes. Second, the possibility of contributions of residual secretory contaminants to the common spectrum is almost certainly negligible. Neither Coomassie Blue, silver-stained, nor radiolabeled one-dimensional (7) or two-dimensional gel profiles of granule content fractions identify any polypeptide with common mobilities in all granule preparations. This is exemplified for parotid secretory polypeptides shown in Fig. 4. Further, we are unaware of any polypeptide (with the possible exception of carbonic anhydrase) that occur as common, low molecular mass, acidic, acinar cell secretory products of all four glands under consideration. The kallikreins/prekallikreins appear to be ruled out as possible candidates because although they are present in all four tissues, they are detected only in duct cells, and not in acinar cells of rat parotid and exorbital lacrimal glands (31). Similarly, it is possible to rule out major urinary proteins from consideration since they are not found in pancreas and are generally <20 kD (36).

The final reason for justifiably considering the 25–35-kD common polypeptides as granule membrane components results from their refractoriness to extraction by the saponin-sulfate or carbonate (pH 11.5) treatments used during final membrane purification. The procedures are among the most thorough means known for bringing about the selective removal of polypeptides adsorbed to biological membranes (5, 7, 15) and thereby ensuring that contamination by soluble protein is overcome. The latter contention is supported by studies in progress, examining proteinase K digestion of intact parotid granules. These studies indicate partial exposure (fragments $\geq$6 kD) on the cytoplasmic aspect of the granule membrane of the 25–35-kD polypeptides.

Peptide mapping studies represent the most refined comparative analysis we have conducted. Separate maps obtained after chymotrypsin and trypsin digestion show very clearly that membrane and content proteins are structurally unrelated and that one of the principal overlapping species ($\sim$29 kD) in parotid and pancreatic two-dimensional polypeptide profiles is essentially identical. Because the peptide mapping approach has been extended to other higher molecular mass species, it has been possible to establish the extent of structural interrelationships between polypeptides of the same granule membrane type. In most cases, distinct patterns having limited relationships to that of the 29-kD species were observed. Thus it has been possible to argue against the possibility that a membrane composition enriched in low molecular mass polypeptides reflects extensive proteolysis of higher molecular mass species either in situ or during organelle isolation. These limited interrelationships (i.e., overlapping peptides) that do exist between different polypeptides of the same membrane may be significant. Consequently, peptide mapping is currently being carried out on a more comprehensive scale because it may enable the identification of domains that could contribute to the underlying basis for the specific granule membrane composition, which is so clearly suggested by the immunostaining shown in Fig. 2.

Finally, some consideration should be given to polypeptides that show substantial quantitative variations between the granule membrane preparations of the different exocrine tissues (or that are even unique to a particular tissue). Especially in submandibular and lacrimal fractions, some of these species could reflect the presence of more than a single type of granule membrane population. Indeed, mucus granules that package and maintain unusually large quantities of charged proteoglycans in osmotically inactive form (prevalent in mixed glands such as the submandibular) may require increased levels of special membrane machinery. As well, the presence of selected polypeptides in some but not all preparations could be related to an emphasis on a particular second messenger system in bringing about amplified secretory discharge in response to external stimuli.

In all cases integral membrane glycoproteins may exhibit rather substantial quantitative variations in the different preparations. This is clearly the case for a prominent ~78-kD species known as GP-2 (35) that is present in pancreatic zymogen granule membrane profiles (Fig. 4 B) but has no identifiable counterpart in other exocrine tissues. A similar, but not quite so extreme, variation applies to $\gamma$-GT and structurally related antigens (10) of higher molecular mass ($\geq$95 kD). Its enzyme activity has proven to be invaluable in the present study as a general granule membrane marker during cell fractionation, yet the total activity ranges over three orders of magnitude in the exocrine tissues examined. Both the enzyme and related antigens are known to reside in large part at the apical plasma membrane of exocrine glands (10, 16, 26). Thus the amount found in granule membranes not only reflects the total tissue level but also may reflect the extent and regularity with which the granule membrane delivers and exchanges these glycoproteins with the cell sur-
face. In addition, the extent of glycosylation (and thus the isoelectric point and apparent molecular mass) of γ-GT and related antigens have been found to vary among different epithelial tissues (10). Thus unique polypeptides (related to γ-GT or otherwise) identified by comparing the two-dimensional profiles for different granule membrane preparations may only reflect varying extents of glycosylation that are characteristic of a particular tissue.

Even though differences in the polypeptide composition between the storage membranes could reflect a host of different factors, the principal and most significant message resulting from our study is the clear identification of overlapping composition. As a consequence of the functional specialization of granule membranes in the packaging and discharge of exportable proteins, it is very tempting to view the common membrane polypeptides as components potentially involved in basic mechanisms of secretory storage or even the general operation of vesicular carriers between the Golgi complex and the cell surface. However, it should be stressed that we presently have no information concerning the overall subcellular distribution of these polypeptides because they are not the prominent antigens that give rise to the granule-specific staining observed in Fig. 2. Although we consider it very unlikely that common polypeptides, especially those 25–35 kD, are contributed by some contamination source that has gone undetected in our analyses, past and present, this possibility must be addressed. Thus, our studies in the near future will focus on examining the localization, structure, and (ultimately) function of individual components from this common class. As well, we plan to check for structural counterparts to the common polypeptides in the secretory membranes of endocrine and neural systems and to examine epithelial tissues that are specialized for endocytosis (e.g., intestinal mucosa and kidney proximal tubule) to judge whether the same or related species might be candidates for a role in internalization shuffling.

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