Comparison of the Protein Content of Three Different Bovine Secretory Granule Membrane Types: A Search for Exocytosis-specific Shared Proteins

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ABSTRACT A two-dimensional polyacrylamide gel analysis of three types of bovine exocytotic granule membranes has been undertaken. Great care was taken to purify the membranes of biochemical homogeneity with minimal contamination from other membrane sources. The goal was to identify proteins that were present in all three membrane types. Although a number of minor components were observed that co-migrated for two membrane types, no proteins were detected that were present in all three granule membranes. We therefore conclude that such exocytosis-specific proteins do not exist or that they represent <0.1% of the total membrane protein present in a given isolated membrane preparation.

The process of exocytosis involves the movement of preformed exocytotic granules from a given intracellular position to the cell cortex, fusion of the plasma membrane with the granule membrane, and subsequent release of the granule contents. This sequence of events is thought to be common to all classical exocytotic cell types. As such, it is reasonable to hypothesize that exocytosis-specific granule membrane proteins exist and are common to all exocytotic granules within a given species. These proteins could represent a substantial portion of the granule membrane content. Their function might include, for example, binding to F-actin or myosin, and/or facilitation of the membrane-to-membrane fusion reaction. One of the difficulties in any attempt to identify such proteins is the complexity of the protein content of such granule membranes and the problem of contamination from the granule contents, other organelles, and soluble cytosol proteins. A review of the literature reveals few reports of any such attempts and they employed one dimensional electrophoretic resolution technologies. As the sensitivities and resolution of protein analytical methods improve, it becomes increasingly probable that such common proteins can be found. In this report we describe our efforts to use state-of-the-art protein separation and detection methods for the analysis of the membranes of three different exocytotic granule types from the same organism.

MATERIALS AND METHODS

Membrane Isolation: Bovine chromaffin granule membranes were isolated and their purity was assessed as previously described (1). Zymogen granule membranes were isolated by a modification of a previously published method (2). 40 g of bovine pancreas was minced and washed and then homogenized with three passes using a loose fitting teflon-glass homogenizer in 10 vol of 0.3 M sucrose, 0.025 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), 10 μl phenylmethylsulfonyl fluoride (from a 0.1 M stock solution) at pH 6.9 in an ice-water bath. Higher concentrations of these inhibitors and others such as leupeptin and EGTA were initially employed. However, we monitored the proteolytic degradation with two-dimensional (2D) gels by taking a preparation, splitting it into many aliquots, and serially increasing the number and concentration of inhibitors. The resulting membrane preparations were then run on 2D gels. Proteolysis was observed as the appearance of numerous low molecular weight spots and the reduction in amount of high molecular weight spots. As the concentrations and number of inhibitors were increased these artifacts were eliminated and a pattern was observed that did not change with additional inhibitors at higher concentrations. The inhibitors and concentrations finally chosen were those that gave this basic pattern at one quarter the concentrations listed above. The homogenate was then strained through cheese cloth and centrifuged at 500 *g* for 6.5 min. The supernatant was then centrifuged at 17,300 *g* in a Sorvall SS-34 rotor for 10 min. The top layer of the biphasic pellet was swirled off in homogenization solution and the underlying pellets were pooled and resuspended in 30 ml of this solution and centrifuged again at 17,300 *g* for 10 min. The resulting pellet was resuspended in 5 ml of homogenization solution and layered onto linear sucrose gradients of densities from 1.03 to 2.07. Protease inhibitors were included. These gradients were centrifuged at 100,000 *g* for 2 h at 4°C. The fraction from a density of 1.20 to 1.22 was collected and spun again at 100,000 *g* for 30 min. The pellet was lysed in 5 ml of cold 0.2 M Tris HCl, pH 8.0 containing the initial concentrations of protease inhibitors. This was then centrifuged at 100,000 *g* for 60 min and the resulting pellet was resuspended in 4 ml of 1.0 M sucrose plus protease inhibitors, overlaid with 0.5 ml of homogenization solution, and spun at 100,000 *g* for 60 min. The membranes were collected at the interface diluted 10 × with water plus protease inhibitors and spun at 100,000 *g* for 60 min. The pellet was then solubilized for 2D gel electrophoresis.

Bovine alpha serotonin granule membranes were isolated by the method of Van der Meulen, Furuya, and Grinstein (3). And the purity of the preparation was determined by following [3H]serotonin-labeled alpha granules added to the whole cell preparation just prior to lysis.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 JULY 1984 356-360

Catecholamine was used as a marker for chromaffin granules and assayed fluorometrically (4). Succinic dehydrogenase and monoamine oxidase were used as mitochondrial markers (5, 6) as was 2D PAGE (see Results). Glucose-6-phosphatase was used for a marker of the smooth endoplasmic reticulum and the ratio of 280/260 absorbance used for the rough endoplasmic reticulum (7). The plasma membrane markers employed were 5′ nucleotidase (8) and acetylcholinesterase (9).

2D PAGE: The 2D gel system employed is a modification of the O'Farrell technique (10, 11) in which the sample is prepared in SDS plus urea and nonionic detergent. The first dimension is a small cylindrical focusing gel in which the SDS electroelutes off the protein during the focusing step. The second dimension is a 10% thin slab gel in SDS and is the sieving dimension. This system has been used to resolve over 200 polypeptides from the erythrocyte membrane (12). These gels are routinely run over a period of 24 h, stained, photographed, and then dried for radioautography using a commercial gel drier. We use the silver stain method (13), which can detect as little as 0.1 ng per spot. These gels are still sufficiently cohesive to handle the dry down for radioautography. The gels are then put at −78°C with Kodak X-Omat X-ray film. Radioautographic sensitivity can be enhanced 10-fold by rare earth screens (14). To enhance sensitivity still further, we now routinely combine rare earth screen radioautography with fluorography (15) and use the product "Enlightning" (New England Nuclear, Boston, MA). This makes use of the emissions from ^{125}I that are of the weak beta type and ordinarily not utilized. We have been able to increase sensitivity another 10-fold with this technique.

Rough estimates of the relative amount of a given protein in a given preparation were obtained in the following manner. Samples were serially diluted and the dilution at which a given spot of interest was no longer detectable under standard radiofluorographic conditions was noted. Thus, if a particular spot was no longer detectable at dilution x and the most prevalent spot was no longer detectable at dilution $0.1x$ then we would estimate that this spot represented an amount of protein equal to one tenth of the most prominent protein and <5% of the total protein in the sample.

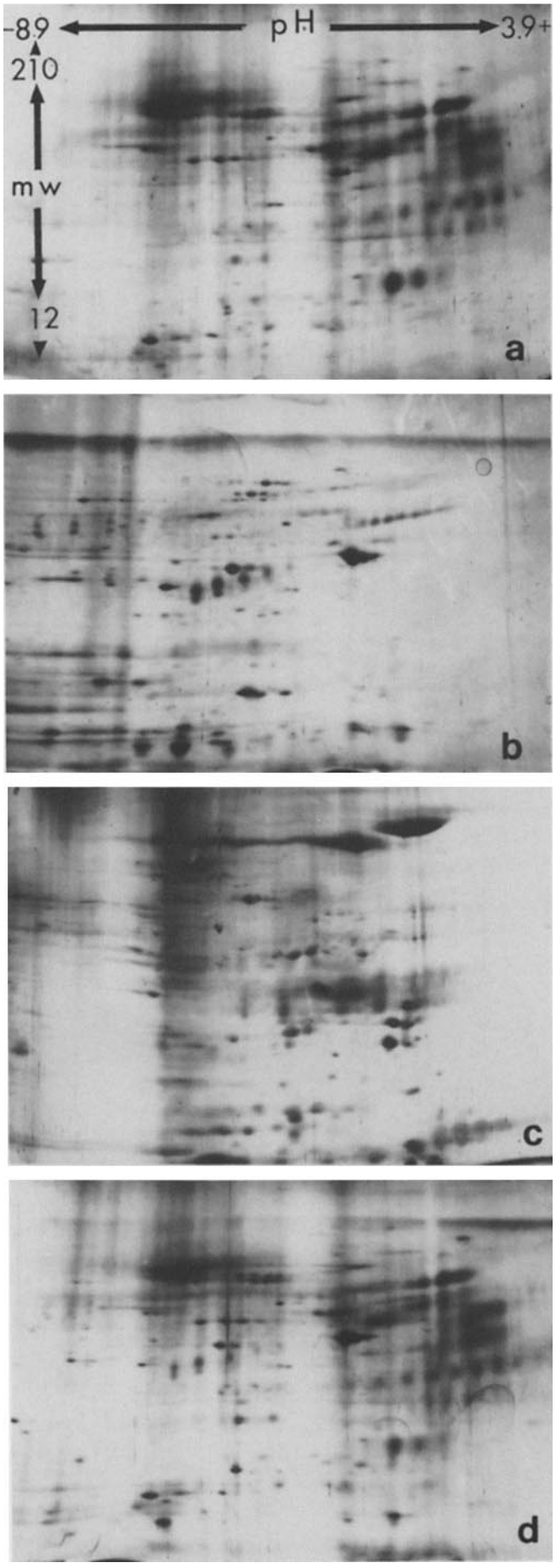
Iodination: In cases in which only small membrane pellets were available the following iodination procedure was developed to iodinate the membrane preparations to completion. A 20-μl high speed pellet of a given membrane was resuspended in 50 μl double deionized water, which had previously been run through a reverse osmosis apparatus then passed through two activated charcoal columns and finally distilled. 10 μl of mercaptoethanol was then added along with 1 mg of ultra pure (Bio-Rad Laboratories, Richmond, CA) SDS. The now solubilized sample was then taken up in a Hamilton syringe and placed in a 0.8-ml glass tube coated with iodogen (16). 40–100 μCi of ^{125}I were then added and the tube was shaken for 30 min after which no more trichloroacetic acid–pelletable counts were observed. 57 mg of ultra pure urea (Schwarz/Mann) were then added and the sample was made up to 100 μl with water. This then was taken as 1 vol of stock grind solution and 30 μl of lysis supplement solution and 120 μl of lysis buffer was then added as previously described. One third to one half of the sample was then run directly on 2D PAGE. It was unnecessary to remove the unbound counts since the unreacted iodine was electrophoresed off the end of the first dimension gel.

Electron Microscopy: Granule pellets were suspended in isolation media and mixed with an equal volume of 3% gluteraldehyde prepared in the respective isolation media. The preparations were then washed and postfixed in 1% OsO4, embedded in Epon, sectioned, and then examined with a Phillips 300 electron microscope.

RESULTS

We have chosen the bovine for this study because methods for the isolation of relatively large quantities of different varieties of granules were available. Three granule types were chosen: chromaffin granules of the adrenal medulla, zymogen granules of the pancreas, and serotonin alpha granules of the bovine platelet. The purity of the preparations was determined by enzyme analysis as previously described (see Materials and Methods), direct electron microscopic examination of the final preparation, and 2D gel analysis. One of the most likely contaminants was presumed to be mitochondria. We were able to evaluate this potential problem by isolating bovine liver mitochondria (which gave the same 2D gel pattern as adrenal medula mitochondria and were much easier to obtain in bulk and high purity) running 2D gels of the preparation and comparing the patterns with those of our other three preparations. Whole mitochondria have an easily recognized spot pattern in which three proteins predominate (1). Any preparation in which these three spots appeared following the silver stain procedure were discarded. All preparations contained <5% contamination from the cytosol or other membrane organelles as determined by enzymatic analysis. By this we mean that no preparation was used that had an contaminating enzymatic activity of 5% or more as referenced to the specific activity of the original whole cell homogenate. Thus if the whole cell homogenate contained 100 U of activity per microgram of potential contaminant, we would only use the final purified whole granule preparation if it contained <5 U of the same activity per microgram of granule protein. In addition, each granule type was examined under the electron microscope for contamination by other organelles or free membrane fragments. In all cases less than three membrane fragments per one hundred granules was observed and the only contaminating, recognizable, intact organelle was an occasional mitochondrion. This contaminant was monitored biochemically as described above. Even though we were able to obtain large amounts of starting material we frequently found that we had insufficient final membrane material to carry out all of the co-electrophoretic runs necessary for a complete comparison of a given preparation. This was especially true for the platelet serotonin granule membrane isolation which required up to three full days of processing. For this reason, and for reasons of ease of comparison, two approaches were taken in the search of homologies. In the first, membranes were prepared and solubilized for 2D gel analysis, and gels were run of each granule membrane type by itself and mixed with one other granule membrane type. The resulting gels were then silver stained and photographed. The results of one such analysis are shown in Fig. 1. In the best gels, over 70 spots could be detected for each membrane type. This made a direct visual comparison difficult and acetate overlays were traced and combined in the various permutations to determine homologies. One representative analysis of this type is shown in Fig. 2.

It immediately became apparent from this type of approach that very few spots were homologous for any pair of membrane types. To facilitate our ability to compare any two complicated gels, we developed an ^{125}I labeling procedure that allowed us to greatly improve both our sensitivity of detection and ability to compare any two patterns (see Materials and Methods). The combination of fluorography and rare earth screens allowed us to obtain radioautographs in a few days, which could then be superimposed over prints of photographs of stained gels in which a very small amount of radiolabeled reference membrane (too little to be seen with silver stain) could be mixed and co-electrophoresed with a much larger sample of test membrane that could be easily stained and photographed. One such radiofluorograph is shown in Fig. 3. Of some concern in these experiments was the possibility that the radioiodination procedure produced altered spot patterns by affecting the apparent molecular weight or isoelectric point of the various proteins. As published previously (1), this was tested directly for the chromaffin granule and zymogen granule membrane preparations by pooling preparations, iodinating them, and then staining with Coomasssie Blue. The gels were photographed and the stain was removed from the spots by extensive washing in methanol; the gels were then restained with silver and rephotographed prior to fluorography. In virtually all cases every spot visible by either staining method was detected in the fluorograms. Also, as reported previously,



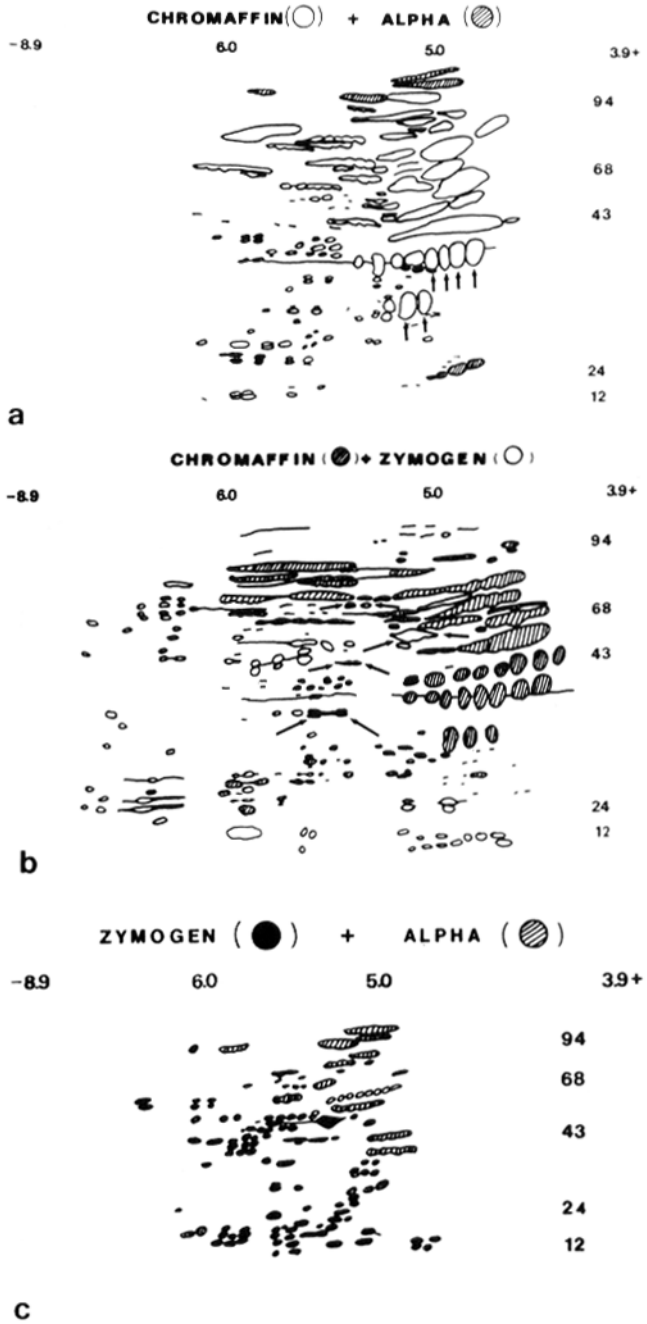


FIGURE 2 Tracings of 2D gels of mixed membrane preparations. Molecular weight values are given on right hand margin ($\times 10^{-3}$) and pH values are at the top. All alpha membranes were radioiodinated and loaded on the gels at protein concentrations that were too low to detect by silver staining. The second membrane type was loaded with high amounts of protein for staining. A tracing was first made of the radiofluorograph and then the acetate sheet was placed over a photograph of the stained gel taken prior to fluorography and the second pattern was copied over the first. Arrows denote co-migrating spots.

FIGURE 1 Silver staining patterns of 2D gels of (*a*) purified chromaffin granule membranes, (*b*) zymogen granule membranes, (*c*) alpha granule membranes, and (*d*) a representative gel of *a* and *b* preparations mixed together.

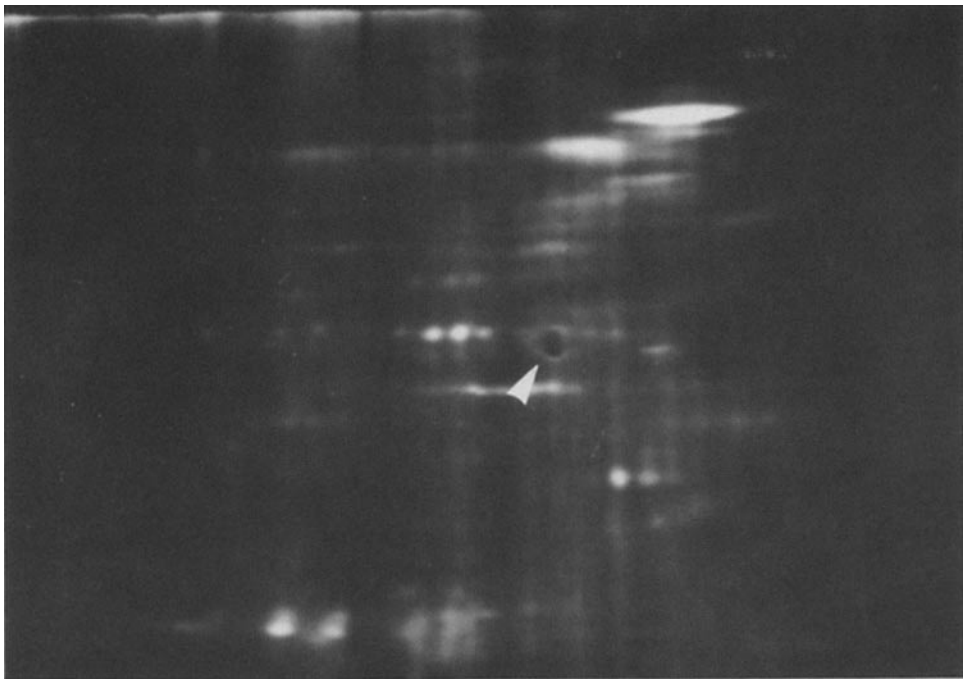


FIGURE 3 Photograph of radiofluorograph of a 2D gel of a ^{125}I-labeled alpha granule membrane preparation mixed with a zymogen membrane preparation. Note the outline of the large spot in the center of the gel which is seen (upper right arrow) in Fig. 2*b*. This protein is shared by chromaffin and zymogen granule membranes, but is obviously not present in alpha membranes as the spot (arrowhead) has actually displaced the background radioactivity from the labeled alpha preparation.

occasionally minor spots could be seen in the fluorogram that were not detectable with staining. Having large amounts of chromaffin granule membranes, we were able to confirm their presence by heavily overloading a replicate 2D gel and detecting these spots with silver stain. Even the shape of the spots was identical when comparing the fluorograph with the staining pattern.

The results of these comparative evaluations can be summerized in rather simple terms. Absolutely no proteins were found that were present in all three granule membrane types. Eight minor components were observed to co-migrate when chromaffin and zymogen membranes were compared (Fig. 2). Two groups of multiple charged species, one of four components and one of two, (a total of six polypeptides representing charge variants of two presumptive sialoglycoproteins) were consistently observed to co-migrate when alpha and chromaffin granule membranes were compared (Fig. 2). No homologies were observed between alpha and zymogen membranes.

Rough estimation of the relative amounts of these components (see Materials and Methods) indicated that only one component represented as much as 1.0% of the total membrane protein. This component possessed a molecular weight of ~50 kd and an isoelectric point of 5.3. Although it is a prominent constituent of zymogen granules, it is a very minor component in chromaffin membranes, and was not observed in any alpha membrane preparation. Another minor shared component co-migrated with actin at 43 kd and may represent a contaminant (see Fig. 3). All other co-migrating proteins taken together represent <0.1% of the total protein in any given preparation.

Of further interest are the homologies between chromaffin and zymogen and alpha and chromaffin membranes which appear to be part of multicharged families. Previous experiments employing enzymes to remove sialic acid from membrane preparations gave rise to multicharged serial spots which increase in apparent molecular weight in the acid direction. When these preparations are treated with neuraminidase these same spots are selectively shifted toward the basic end (17) suggesting that these are sialoglycoproteins.

DISCUSSION

Our original goal was to determine whether common proteins exist that function exclusively in the process of exocytosis per se regardless of the function of the secretory cell type. The recent demonstration that exogenously introduced plastic micro-beads travel unidirectionally in axons with kinetics similar to granules (21) strongly implies that the binding sites for motility on the surface of vesicles are of a general type. This implies that there should be a common protein(s) at least on the exterior of granules that move in cells. We, however, have not been able to find them.

We must now conclude that either such proteins do not exist in the systems studied or that they represent such a small percentage of the total protein in our preparations that they are not detectable by our current methods. This latter possibility would mean that these protein(s) represent less than 0.1% of the total membrane protein in any granule type. We believe that we are using the most sensitive and highest resolution methods available. Even so, there are possible reasons for our results that are due to the nature of the methodology. For example, contaminating granule contents could obscure common proteins. In fact, the presence of chromogranin A as a major protein in our purified chromaffin granule preparations shows that such protein contaminants do exist. Conversely, there may be common proteins but they are modified post translation by the addition of carbohydrate or other charged groups. The use of a wide spectrum of granule specific antibodies will be required to answer this question more definitively. Thus, our conclusion must be tempered by caution and limited to the technology utilized. Within this constraint we are still confident that identical proteins do not represent major constituents of these granule membranes.

The possibility of contamination of our granule membrane preparations by other organelles, soluble nongranule mem-

brane proteins, and extraneous membrane fragments was a major concern in this study. As described in the methods section, great care was taken to minimize this potential problem. However, some contamination is inevitable. We argue, nonetheless, that this does not affect our conclusions. This is because of the nature of our results. Any contamination would tend to produce a false positive result (the discovery of common proteins). Since we conclude that there are no detectable common proteins we consider that this issue is not of major concern.

It is difficult to explain how there can be shared zymogen-chromaffin and alpha-chromaffin homologies but no alpha-zymogen homologies. There are regions on the gels in which "families" of spots exist for all three membrane types (see Fig. 1, *a* and *b* for example). These may represent homologies or near homologies for which the genes are homologous but the gene products have been slightly altered enzymatically.

A review of the literature has not revealed studies comparable to the present report. MacDonald and Ronzio (2) undertook a one-dimensional gel analysis of zymogen granule membrane proteins from rat, cow, dog, and pig sources and found 10 to 15 bands with only one PAS positive band. They reported that the protein pattern of these granule membranes was quite different from either mitochondria or total microsomal membranes. This is consistent with the present report and a recent analysis of chromaffin granules membranes from our laboratories (1). A recently published report analyzed the membrane protein distribution on the alpha granule membrane and found one major and up to ten minor bands. The major component was PAS positive and most of the proteins appeared asymetrically distributed (3). The protein composition of zymogen granule membranes have also been studied (18, 19), but we are not aware of any comparative electrophoretic analysis. A very recent and potentially relevant paper has described a 2D gel analysis of isolated chromaffin granule membranes and reported the presence of alpha-actinin on the outer surface (20). This is exactly the type of protein that one would expect to be present in all granule membranes. In their study, this protein is present in very low amounts and resolves poorly. It runs in an area in our gels that contains numerous more prevalent components and we could have missed it.

In our study we find that although several homologies between membrane types were detected, the overall conclusion is that the most prevalent secretory granule membrane proteins are generally unique to the cell type of origin. The data also imply that either the process of exocytosis per se requires a very small amount of total membrane protein (below our levels of detection) or each granule type possesses a unique set of proteins for exocytosis.

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