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AGGREGATION AND CONCENTRATION-DEPENDENT SORTING OF EXOCRINE REGULATED SECRETORY PROTEINS

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Summary: Sorting between the regulated and the constitutive secretory pathway in exocrine cells is thought to involve aggregation of regulated secretory proteins. This study demonstrates that, unlike endocrine secretory proteins, exocrine secretory proteins, including amylase, do not undergo homotypic aggregation under the conditions found in the sorting organelles. Also, unlike other exocrine proteins, amylase does not aggregate with chondroitin sulfate. Since amylase exhibits heterotypic aggregation, the role of protein concentration in amylase sorting was tested in AR42J cells. Secretion was stimulated with substance P and cholecystokinin from both untreated and dexamethasone-treated cells, with more efficient stimulation from dexamethasone-treated cells. These results indicate that amylase sorting is enhanced when its expression is stimulated by dexamethasone treatment

Exocrine and endocrine cells exhibit both a regulated and a constitutive secretory pathway (1,2). Sorting of secretory proteins involves specific signals (3) and takes place in the trans-Golgi network, condensing vacuoles and maturing secretory granules (4), although the exact mechanism is not known. Many endocrine secretory proteins aggregate under the conditions of low pH and high calcium concentrations found in the sorting organelles and it is thought that this aggregation plays a role in the sorting process (5-10). Exocrine secretory proteins in crude zymogen extracts also exhibit low pH and calcium dependent aggregation, (11-14), and chymotrypsinogen aggregates in the presence of glycosaminoglycan (15). However, it is not known if exocrine proteins form homotypic aggregates, similar to those of endocrine secretory proteins.

Protein aggregation is concentration dependent (8,9) and cholecystokinin (CCK)-stimulated secretion of amylase, a measure of sorting efficiency, is increased when amylase expression and secretory granule biogenesis are induced with dexamethasone in AR42J cells (16-18). These findings are consistent with the proposed role of protein aggregation in the sorting of exocrine

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proteins to the regulated secretory pathway. However, since dexamethasone-treatment also induces expression of CCK-receptors (17), it was tested if the increased stimulated secretion in dexamethasone-treated cells is concentration dependent, independently of the role of specific receptors. Together with earlier data, the present results indicate that amylase forms heterotypic aggregates and that sorting efficiency of amylase to the regulated secretory pathway is improved by increased protein expression, independent of receptor expression.

Methods

Aggregation experiments: Individual secretory proteins and chondroitin sulfate were purchased from Sigma Chem. Co., St. Louis, MO and dissolved at 10 mg/ml in 10 mM Hepes, pH 8.0. Protein aggregation was quantitated by recording the absorbance of each protein (1mg/ml) at 340 nm in 50 mM HEPES, pH 7.4 or 50 mM MES, pH 5.9 in the presence or absence of 15 mM $CaCl_2$. Chondroitin sulfate was added to achieve a protein:glycosaminoglycan ratio of 20:1. Aggregation of insulin was verified by centrifugation of the samples for 10 min at 16,000 x g. The pellets and 25% of the supernatant fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with Coomassie blue.

Secretion experiments: Rat pancreatic acinar AR42J cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY) containing 10% fetal calf serum (Hyclone, Logan, UT), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Life Technologies) at 37°C and 5% CO_2 in a humidified atmosphere. For hormone-treatment, the cells were plated in 6-well plates at a concentration of 10^5 cells/well and incubated with hormones as indicated in the text. Cells were incubated in Krebs-Ringer buffer containing 15 mM Hepes, pH 7.4 for 60 min at 37°C and secretion was stimulated with 100 nM cholecystokinin or substance P, as indicated in the figure legends. Control samples were incubated in the absence of secretogogues. Cell extracts were prepared in Krebs-Ringer buffer by freezing and thawing the cells, followed by homogenization through a 26 gauge needle. Amylase activity was determined with the Phadebas assay (Kabi-Pharmacia Diagnostics, Piscataway, NJ). The relative secretion of amylase was calculated as the ratio of amylase activity in the secretion medium divided by the total amylase activity in medium and cell extract. The data were analyzed by Student's t-test or ANOVA and $p < 0.05$ was considered significant.

Results

In initial experiments, the heterotypic aggregation of exocrine secretory proteins in zymogen granule extracts (11-13) was confirmed using zymogen granule extracts from porcine pancreas (data not shown). Homotypic aggregation was tested, using purified exocrine and endocrine secretory proteins (Fig. 1). None of the exocrine proteins exhibited aggregation, while the endocrine control proteins insulin and prolactin exhibited low pH-induced aggregation, as previously reported (8, 10). The addition of calcium (15 mM) had no effect on exocrine protein aggregation but in some experiments enhanced insulin and prolactin aggregation at pH 7.4 (not shown). The constitutive secretory protein ovalbumin did not aggregate in this assay (Fig. 1). The aggregation of insulin was confirmed by centrifugation followed by SDS-gel electrophoretic

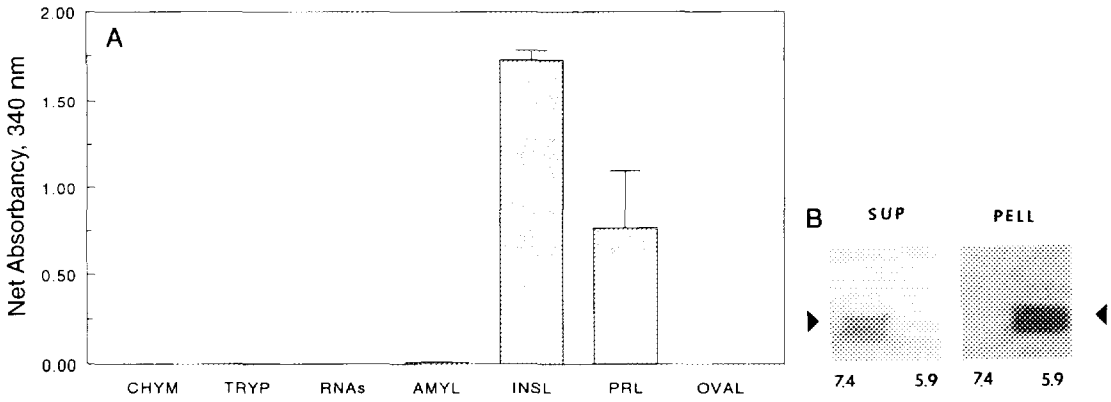

Figure 1. Low pH-induced aggregation of secretory proteins. (A) Chymotrypsinogen (CHYM), trypsinogen (TRYP), RnAse (RNAs), amylase (AMYL), insulin (INSL), prolactin, (PRL) or ovalbumin (OVAL) were incubated for 1 h at pH 5.9 or pH 7.4. The data are the mean ± range of two independent samples expressed as Net absorbance (340 nm) = absorbance (pH 5.9)-absorbance (pH 7.4). Similar results were obtained in at least three other experiments. (B) Insulin was incubated at pH 5.9 or 7.4 followed by centrifugation and gel electrophoresis of the supernatant and pellet fractions. The gel was stained with Coomassie blue and the region containing insulin (arrowheads) shown.

analysis of the supernatant and pellet fractions (Fig. 1). LeBlond et al. (12) reported that in zymogen granule extracts, amylase aggregated in the presence of 100 mM or higher concentrations of KCl but not at lower KCl concentrations. In the present analysis of the pure protein we did not detect an aggregating effect of 250 mM KCl.

Chymotrypsinogen aggregates in the presence of chondroitin sulfate (15). To determine if other exocrine proteins exhibit similar aggregation, trypsinogen, amylase, and RNAse were incubated in the presence of chondroitin sulfate (Fig. 2). Chymotrypsinogen, trypsinogen and RNAse were aggregated by the glycosaminoglycan while amylase did not aggregate under these conditions.

While we have shown that amylase does not aggregate homotypically or in the presence of chondroitin sulfate, the enzyme does aggregate in the presence of other zymogen granule proteins (e.g. 14), suggesting that amylase may aggregate in vivo. The pancreatic acinar cell line AR42J was used to test if increased protein concentration affects the sorting and storage of amylase in the regulated secretory pathway. The relative secretion of amylase from dexamethasone-treated and untreated cells was calculated to determine if the stimulation of amylase expression affected storage and secretion of the enzyme. Fig. 3 shows that about 40% of total amylase activity was secreted from both untreated and dexamethasone-treated cells, respectively, suggesting that amylase expression and storage are induced in parallel by dexamethasone-treatment. Hormone-treatment with a combination of estradiol, insulin and epidermal growth factor has been shown to stimulate secretory granule biogenesis and

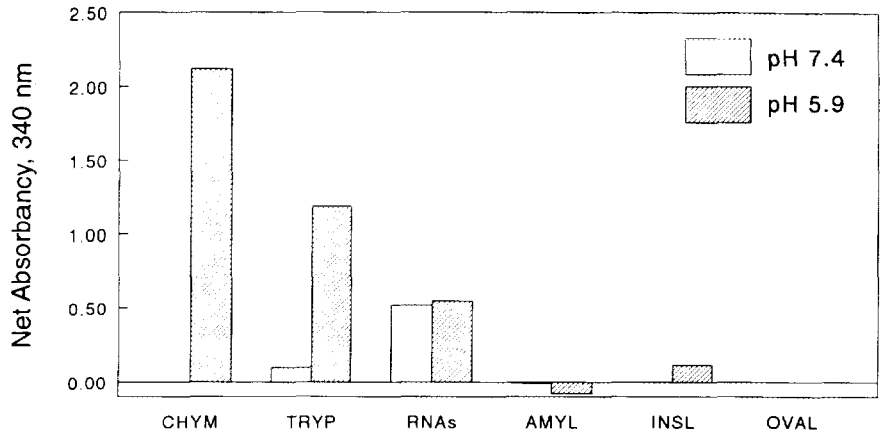

Figure 2. Chondroitin sulfate-induced aggregation of secretory proteins. The proteins (see Fig. 1 for abbreviations) were incubated for 1 h at pH 5.9 or pH 7.4, followed by 1 h incubation in the presence of chondroitin sulfate. The data are expressed as Net absorbance (340 nm) = absorbance (+ChSO4)-absorbance (-ChSO4). Similar results were obtained in two other experiments.

intracellular storage of prolactin in the rat pituitary cell line GH_4C_1 (19). Since estrogen does not affect amylase expression in AR42J cells (16), it was tested if the combination hormone-treatment could induce intracellular storage of amylase, independent of its expression. The combination hormone treatment did not stimulate amylase expression and it had no effect on the relative secretion of amylase (Fig. 3).

To differentiate effects on secretion due to protein sorting and secretogogue receptor expression, two secretogogues were tested. Substance P strongly stimulates secretion from untreated cells (20) but the substance P receptor is down regulated by dexamethasone (21). Conversely, the cholecystokinin receptor is upregulated by dexamethasone (17) and stimulated secretion in response to cholecystokinin stimulation has only been reported consistently for dexamethasone-treated cells (see 18). In the present studies, both substance P and cholecystokinin significantly stimulated secretion from both dexamethasone-treated and untreated cells. Secretion was stimulated nearly 2-fold from untreated cells and about 3-fold from dexamethasone-treated cells (Fig. 4). Similar results were obtained when the cells were simultaneously stimulated with substance P, CCK and phorbol ester (not shown). These results suggest that sorting of amylase to the regulated secretory pathway occurs at both high and low levels of amylase expression, and that the stimulated secretion of amylase is more efficient at high amylase concentrations.

Discussion

Exocrine regulated secretory proteins do not form homotypic aggregates under the calcium and pH conditions that are found in the trans-Golgi network. This finding is in contrast to the results

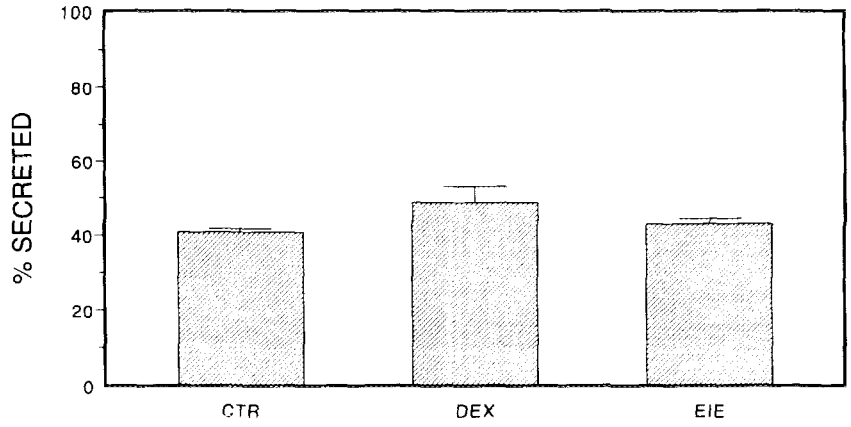

Figure 3. Relative amylase secretion from AR42J cells. The cells were cultured for 4 days in the presence of 100 nM dexamethasone (DEX) or 1 nM estradiol, 300 nM insulin and 10 nM EGF (EIE). Control cells (CTR) were cultured without hormone treatment. Amylase secreted in 1 h was expressed relative to total (medium and cellular) amylase. Samples from two independent experiments were analyzed and the data presented as mean ±SEM (n=6-7).

for endocrine secretory proteins that readily aggregate under these conditions (this report; 5-10). Since pancreatic zymogen granules store a full complement of 15 or more different zymogens and digestive enzymes that exhibit isoelectric points from acidic to basic (13), it is likely that the formation of heterotypic aggregates is favored. In contrast, endocrine secretory granules store a limited number of secretory proteins that may require more specific interactions.

The failure of amylase to form homotypic aggregates or aggregate with chondroitin sulfate suggests that the protein must form heterotypic protein aggregates if its sorting is aggregation

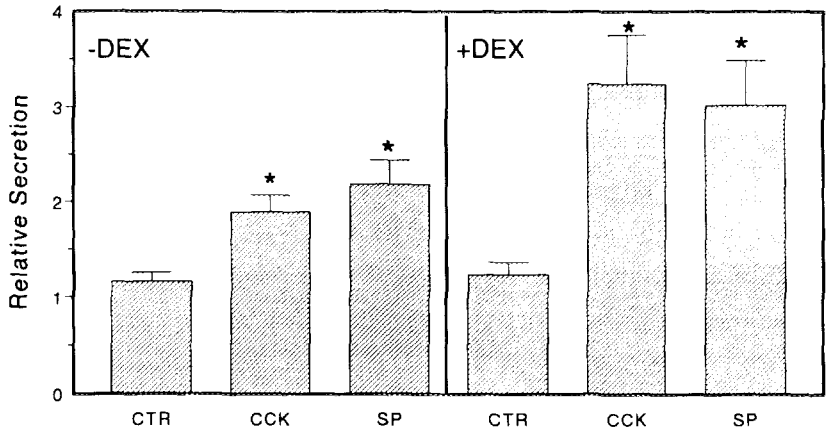

Figure 4. Stimulated amylase secretion. Secretion from AR42J cells was stimulated by incubating the cells with 100 nM CCK_8 or 100 nM substance P for 1 h. Data from 5-6 independent experiments were normalized to the control samples and presented as mean ± SEM (*, different from control, $p<0.002$, n=10-12).

dependent. Consistent with this, Colomer et al. (14) have shown that amylase co-aggregates with zymogen granule proteins and it is sorted to the regulated secretory pathway in exocrine cells. In contrast, amylase is not sorted in pituitary cells (14). GP2 is also not sorted in pituitary cells and forms neither homotypic aggregates nor co-aggregates with pituitary proteins (14). Trypsinogen likewise fails to form homotypic aggregates. However, this protein is sorted in endocrine cells (22) and binds endocrine regulated secretory proteins (23). Thus, it appears that sorting of amylase, but not trypsinogen, depends on a zymogen granule specific aggregating factor.

The effect of amylase expression on sorting was tested in AR4-2J cells. Dexamethasone-treatment led to an approximate doubling of the proportion of amylase in the regulated pathway after subtraction of the constitutive component of secretion. This difference is consistent with early reports on amylase secretion from AR4-2J cells (16-17), while recent reports indicate that stimulated secretion is absent from untreated cells (18). Cholecystokinin and substance P produced similar stimulation of amylase secretion despite the opposite effects of dexamethasone on receptor numbers (20-21). These results suggest that the differences in amylase stimulation observed with cholecystokinin are due to differences in sorting efficiency, rather than differences in receptor numbers.

Since protein aggregation is concentration dependent, the present data are consistent with a role for heterotypic aggregation in the sorting of amylase to the regulated secretory pathway. This aggregation may include a specific sorting protein or receptor. Thus, it can not be excluded that dexamethasone-treatment induces the expression of such a sorting receptor, although an amylase-binding protein has not yet been identified in the pancreas (23).

Since both amylase expression and secretory granule biogenesis were induced by dexamethasone, we attempted to separate the two effects. A combination of estradiol, insulin and EGF has been shown to preferentially induce biogenesis of prolactin storage granules in pituitary GH_4C_1 cells (19) and estradiol does not induce amylase expression (16). The combination hormone-treatment did not increase amylase storage suggesting that secretory granule biogenesis is differentially regulated in pituitary and pancreatic cells.

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