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Sorting of an exocrine secretory protein to the regulated secretory pathway in endocrine cells

Darrin J. Cowley,[1] Maylene Chu,[2] and Sven-Ulrik Gorr*

Department of Periodontics, Endodontics and Dental Hygiene and Department of Biochemistry and Molecular Biology, University of Louisville Health Sciences Center, Louisville, KY 40292, USA

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Abstract

Regulated secretory proteins are stored in secretory granules. While the sorting and storage process appears similar in endocrine and exocrine cells, the extent of overlap of sorting between endocrine and exocrine cell types is not clear. It is predicted that exocrine regulated secretory proteins that are stored with high efficiency in exocrine granules would also be stored efficiently in endocrine granules. To test this hypothesis, parotid secretory protein (PSP), which is stored efficiently in parotid acinar cells, was expressed in the endocrine cell lines GH4C1 and PC12. PSP undergoes stimulated secretion in both cell types. Secretion is similar to that of the endocrine regulated secretory protein chromogranin A but distinct from secreted alkaline phosphatase, a marker for the constitutive secretory pathway in endocrine cells. Subcellular fractionation of GH4C1 cells revealed that PSP co-fractionates with chromogranin A but not with secreted alkaline phosphatase.

Endocrine and exocrine cells store regulated secretory proteins in secretory granules that undergo exocytosis in response to extracellular stimulation [1,2]. Constitutive secretory proteins, on the other hand, are not stored in secretory granules but are directly secreted by vesicles leaving the trans-Golgi network. Alternatively, secretory proteins can initially enter immature secretory granules but are later released by the constitutive-like (or paragranular) secretory pathway [3,4]. The signals and mechanisms responsible for sorting secretory proteins to the correct secretory pathway have been the subject of intense studies in the past 20 years [5–10]. While much progress has been made for individual proteins and cell types, a comprehensive model for sorting of secretory proteins is still lacking [9]. Indeed, the extent of overlap of sorting between endocrine and exocrine cell types is not clear.

Early data suggested that sorting mechanisms are similar in endocrine and exocrine cells. Thus, the exocrine secretory protein trypsinogen is sorted to secretory granules in endocrine cells [11]. Similarly, a variety of secretory proteins can enter exocrine secretory granules; including endocrine regulated secretory proteins [12] and secreted alkaline phosphatase (SEAP). This enzyme is a marker for the constitutive secretory pathways in endocrine cells [13–15] but is sorted to and stored in exocrine secretory granules [16]. More recent studies, however, suggest that endocrine cells are more restrictive in sorting of secretory proteins than exocrine cells. Thus, endocrine cells may use different sorting mechanisms for individual endocrine proteins [17,18] and exocrine secretory proteins are not always stored in endocrine secretory granules [19]. Indeed, the sorting of trypsinogen to endocrine secretory granules may be the results of selective interaction with endogenous secretory proteins [20,21].

In a search for sorting patterns in endocrine and exocrine cells, it was noted that the relative inefficient storage of amylase and PRP in exocrine secretory granules [22] correlates with their exclusion from mature secretory granules in endocrine cells [19]. Based on this,

* Corresponding author. Fax: 1-502-852-1317.
E-mail address: sven.gorr@louisville.edu (S.-U. Gorr).
[1] Present address: Diagnostic Division, Abbott Laboratories, 200 Abbott Park Road, Abbott Park, IL 60064, USA.
[2] Present address: Department of Oral and Maxillofacial Surgery, Medical University of South Carolina, Charleston, SC 29425, USA.

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it is predicted that exocrine regulated secretory proteins that are stored with high efficiency in exocrine granules would also be stored efficiently in endocrine granules. To test this hypothesis, we have tested the sorting of parotid secretory protein (PSP), which is stored efficiently in parotid acinar cells [22], in the endocrine cell lines GH4C1 and PC12. PSP is sorted to the regulated secretory pathway in both endocrine cell types, with efficiency similar to that of the endocrine regulated secretory protein chromogranin A (CgA).

Materials and methods

Cell culture. The cDNA for rat PSP (a gift from Dr. Lily Mirels, University of California at Berkeley) [23] was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) using standard techniques. PSP, CgA, and secreted alkaline phosphatase (SEAP) were expressed in GH4C1 and PC12 cells and their secretion was analyzed as previously described [17,18]. PSP was identified in cell extracts and secretion media by immunoblotting using an antiserum to rat PSP (a gift from Dr. William Ball, Howard University).

Subcellular fractionation. All procedures were conducted at 0–4 °C. Transfected cells from two wells of a 6-well culture plate were scraped into 1 ml/well of 285 mM sucrose and homogenized by passing the cell suspension through a $26\frac{1}{2}$ gauge needle. The cell homogenate was centrifuged for 5 min at 500*g* to pellet unbroken cells and debris. The supernatant fraction was centrifuged for 30 min at 10,000*g*. The pellet was saved and resuspended in 1 ml of 285 mM sucrose and centrifuged for 5 min at 500*g*. The supernatant was again centrifuged for 30 min at 10,000*g* to obtain the 10K pellet (crude granule fraction). The supernatant from the first 10,000*g* centrifugation was centrifuged for 1 h at 100,000*g* to separate the 100K supernatant (soluble fraction) and the 100K pellet (microsomal fraction). The 10K pellet, 100K supernatant, and 100K pellet were analyzed by immunoblotting and quantitated by densitometric scanning. SEAP activity was quantitated by alkaline phosphatase assay (Tropix, Bedford, MA). The amount of PSP, CgA, or SEAP in each fraction was expressed as a fraction of total in the three fractions. The data were analyzed by one-way ANOVA and the pellet fractions were compared to the 100K supernatant with Dunnett's multiple comparisons post-test. $P < 0.05$ was considered statistically significant.

Detergent extraction. GH4C1 cells were homogenized through a $26\frac{1}{2}$ gauge needle and frozen/thawed in PBS with 1 mM PMSF, 1 mM TLCK, and 5 mM EDTA (homogenization buffer). The homogenate was centrifuged for 2 min at 200*g* to remove cellular debris. The supernatant was centrifuged for 30 min at 16,000*g*. The pellet was washed with homogenization buffer and re-centrifuged. The final membrane pellet was resuspended in homogenization buffer with or without 1% Triton X-100. The samples were again centrifuged for 30 min at 16,000*g* and the pellet and supernatant fractions were analyzed by SDS–PAGE and immunoblotting.

Results and discussion

The endocrine cell lines GH4C1 and PC12 store endocrine secretory proteins in secretory granules. Previous data suggest that PC12 cells sort and store endocrine regulated secretory proteins more efficiently than GH4C1 cells [17,18]. Thus, we initially tested the sorting of rat PSP in PC12 cells. PSP exhibited low basal secretion and strong stimulated secretion, consistent with sorting to the regulated secretory pathway (Fig. 1). In fact, secretion of PSP was similar to that of the endocrine secretory protein chromogranin A (CgA), a marker for the regulated secretory pathway in endocrine cells. Secreted alkaline phosphatase (SEAP), a marker for the constitutive secretory pathway in endocrine cells [13,14], was secreted at a high basal rate and exhibited essentially no stimulated secretion in PC12 cells (Fig. 1).

To test if sorting of PSP was dependent on the highly efficient sorting machinery in PC12 cells, PSP was also expressed in GH4C1 cells, which exhibit less efficient sorting than PC12 cells [17]. Fig. 2 shows that basal and stimulated secretion of PSP were again similar to those of CgA. Thus, in both cell types, the basal and stimulated secretion of PSP were similar to those of CgA, the marker for the regulated secretory pathway in endocrine cells.

To test if the regulated secretory proteins PSP and CgA were stored in a different subcellular compartment than the constitutive secretory protein SEAP, GH4C1 cells were homogenized and subjected to subcellular fractionation. PSP and CgA were predominantly retained in the membrane fractions (less than 25%

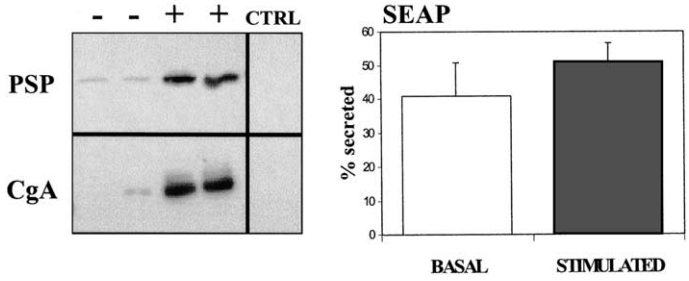

Fig. 1. Stimulated secretion from PC12 cells. PSP, CgA, and SEAP were transiently expressed in PC12 cells. Control cells (Ctrl) were transfected with the pcDNA3 vector alone. Basal (−) and stimulated (+) secretion media were collected and analyzed by immunoblotting (PSP and CgA) or alkaline phosphatase assay (SEAP). The PSP data were repeated with similar results. The quantitation of multiple CgA blots (including the one shown here) has previously been reported [18]. SEAP data are shown as means ± SEM ($n = 3$). % secreted = (SEAP in medium/SEAP in medium + SEAP in cell extract) × 100%.

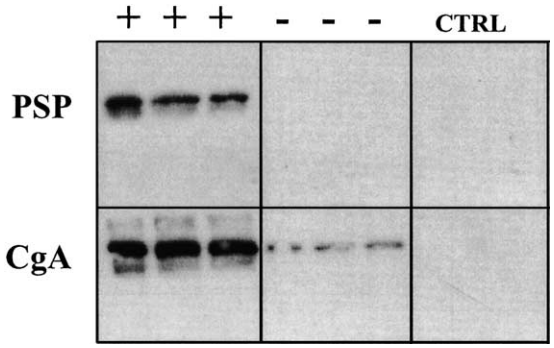

Fig. 2. Stimulated secretion from GH4C1 cells. PSP and CgA were transiently expressed in GH4C1 cells (triplicate wells). Control cells were transfected with the pcDNA3 vector alone (duplicate wells). Basal (−) and stimulated (+) secretion media were collected and analyzed by SDS–PAGE and immunoblotting for PSP and CgA, respectively. Similar results were obtained in four (PSP) and two (CgA) independent experiments.

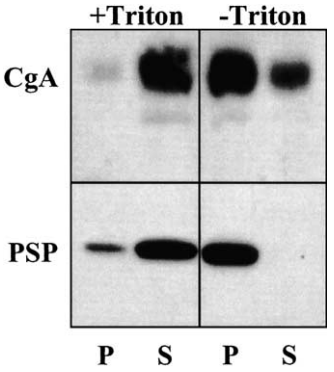

Fig. 4. Triton X-100 extraction of GH4C1 cells. GH4C1 cell membrane fractions were incubated in the presence (+Triton) or absence (−Triton) of 1% Triton X-100 followed by centrifugation. The supernatant (S) and pellet (P) fractions were analyzed by immunoblotting with antisera to PSP or CgA. The data are representative of three independent experiments.

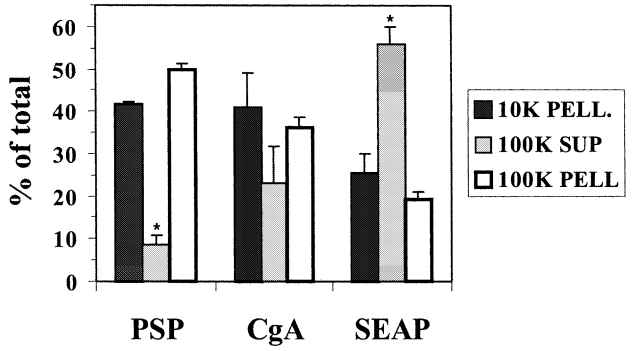

Fig. 3. Subcellular fractionation of GH4C1 cells. PSP, CgA, and SEAP were separately expressed in GH4C1 cells. Cell homogenates were centrifuged at 10,000*g* (10K) and 100,000*g* (100K) and the pellet and supernatant fractions were analyzed by immunoblotting (PSP and CgA) or SEAP assay. Results from three experiments are shown as means ± SEM ($n = 3$). (*) Different from the pellet fractions, $P < 0.01$.

released). SEAP on the other hand was extensively released (55% released), suggesting that it is located in a different membrane compartment than PSP and CgA (Fig. 3). To test if PSP and CgA were stored in a membrane compartment, as opposed to forming an insoluble complex, the cell homogenates were treated with the non-ionic detergent Triton X-100. Fig. 4 shows that both PSP and CgA were efficiently released by the detergent, suggesting that both proteins were located in membrane compartments (secretory granules, membrane vesicles).

The sorting mechanisms for secretory proteins in exocrine and endocrine cells remain an area of conflicting evidence. Based on the original finding that trypsinogen is sorted to secretory granules in endocrine cells [11], it was thought that similar sorting mechanisms operate in both endocrine and exocrine cell types. Subsequent findings that the exocrine proteins amylase and proline-rich proteins are not retained in endocrine secretory granules [19] contributed to the current view that different sorting mechanisms operate in different cell types [9]. Indeed, it appears that trypsinogen and chymotrypsinogen have a unique ability to bind endocrine secretory proteins that could account for the sorting of trypsinogen in endocrine cells [20]. As a complement to these studies, we have now tested the sorting of PSP in endocrine cells. PSP is stored with high efficiency in exocrine acinar cells; and this protein is sorted to the regulated secretory pathway in two different endocrine cell lines. In each case, sorting of PSP was similar to that of the endocrine marker protein CgA but markedly different from the constitutive secretory protein SEAP. The difference in secretion and localization of PSP and SEAP suggests that PSP does not passively enter secretory granules and later exits via the constitutive-like secretory pathway. This conclusion is consistent with the close similarity in stimulated secretion and localization of PSP and CgA, a marker for endocrine secretory granules.

The present results suggest that the efficient sorting of PSP in parotid acinar cells [22] translates to efficient sorting in endocrine cells. Thus, it appears that exocrine proteins are not inherently unable to be sorted in endocrine cells. Instead, sorting efficiency may be a "portable" property of PSP that is not cell-type specific. PC12 and GH4C1 cells use different sorting mechanisms for chromogranin [17,18]. Indeed, GH4C1 cells employ calcium-induced aggregation for sorting of CgA [18], but not sulfated proteoglycans [14]. Parotid acinar cells, on the other hand, use sulfated proteoglycans for sorting of PSP [24]. Interestingly, calcium-induced aggregation does not appear to play a role in protein storage in parotid secretory granules [25] (Venkatesh, Cowley and Gorr, submitted). Which, if any, of these mechanisms are responsible for sorting of PSP in endocrine cells remains to be established.

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