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Association of newly synthesized pro-opiomelanocortin with secretory granule membranes in pituitary pars intermedia cells

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The prohormone, pro-opiomelanocortin (POMC) is synthesized on ribosomes, subsequently routed to the Golgi apparatus and finally packaged into secretory granules where it is processed to various biologically active hormones (α-melanotropin, adrenocorticotropin, β-endorphin and β-lipotropin). We report here that in frog and mouse pars intermedia cells, newly synthesized [^{3}H]Arg-labeled POMC is associated with the secretory granule membrane prior to processing. This association with the secretory granule membrane may be related to the intracellular transport and packaging of POMC and/or the facilitation of processing of the prohormone within the organelle.

Prohormone packaging *Secretory vesicle* *Pituitary intermediate lobe* *Prohormone conversion*

1. INTRODUCTION

Pro-opiomelanocortin (POMC) is a prohormone which is synthesized in the intermediate lobe of the pituitary (pars intermedia). This prohormone is cleaved in the pars intermedia to yield several biologically active peptides: α-melanotropin (α-MSH), adrenocorticotropin (ACTH), β-lipotropin (β-LPH) and β-endorphin [1–3]. Studies on the mouse and frog pituitary pars intermedia suggest that newly synthesized POMC is intracellularly routed from the rough endoplasmic reticulum through the Golgi apparatus and subsequently packaged into secretory granules [3–6]. Thus, the processing of POMC occurs intragranularly. The mechanism by which POMC is sorted within the cell and directed for packaging into secretory granules is not known. Binding of POMC to specific granule membrane receptors may be involved in such intracellular sorting, as has been shown for the targeting of lysosomal enzymes into lysosomes [7–9]. Furthermore, it has been demonstrated that the POMC converting enzyme which processes the prohormone to its hormone products [10] is associated with the secretory granule membrane. These observations led us to investigate the possibility that newly synthesized POMC may be associated with the secretory granule membrane.

2. MATERIALS AND METHODS

2.1. *Animals*

Xenopus laevis frogs, 40–70 g, were purchased from Nasco Biologicals (Ft. Atkinson, WI). NIH strain white mice were obtained from the National Institutes of Health (Bethesda, MD) animal facilities.

2.2. *Incubation of neurointermediate lobes with [^{3}H]arginine*

Twenty neurointermediate lobes (neural lobe plus intermediate lobe) from frogs or mice were incubated for 30 min in amphibian [2] or mouse incubation buffer [3] containing 18.2 μM [^{3}H]arginine (specific activity 18.4 Ci/mmol, New England

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Nuclear, Boston, MA). The lobes were then homogenized in (1 ml) 0.23 M sucrose (frogs) or 0.32 M sucrose (mice) buffered with 10 mM Tris-Cl (pH 7.4). Secretory granules were isolated as described below. Previous studies have shown that POMC is transported into secretory granules by approx. 15 min after synthesis [6].

2.3. *Preparation of secretory granules*

Neurointermediate lobe homogenates were centrifuged at 1000 × *g* for 30 s to remove the nuclei and cell debris. The supernatant was then centrifuged at 12 000 × *g* for 15 min to yield a mitochondria enriched fraction (P_2). The supernatant from the P_2 fraction was centrifuged at 24 000 × *g* for 15 min to yield a secretory granule enriched fraction (P_3) and the supernatant then centrifuged for 1 h at 100 000 × *g* to yield a microsomal fraction (P_4). · Details of the procedure and characterization of the fractions with respect to different markers (monoamine oxidase for mitochondria, α-MSH for secretory granules and β-glucuronidase for lysosomes) have been reported elsewhere [3]. Although the P_2 fraction also contained secretory granules, the P_3 secretory granule enriched fraction was used to analyse for POMC binding to secretory granule membranes.

2.4. *Analysis of newly synthesized [^{3}H]Arg-labeled POMC in the secretory granule*

Secretory granules (P_3 fraction) were suspended in 10 mM Tris-Cl (pH 7.4) and lysed by freezing and thawing 5 times. The lysed secretory granules were spun at 100 000 × *g* for 30 min in a Beckman airfuge (Palo Alto, CA). The supernatant and pellet were analysed for [^{3}H]Arg-labeled POMC. In some experiments, further sequential extraction of the membrane pellet was carried out. The pellet was extracted with 1 M NaCl, spun at 100 000 × *g* for 30 min and the NaCl supernatant was analysed for [^{3}H]Arg POMC. The pellet was then re-extracted with 1.5 M KSCN, spun and the supernatant and pellet analysed for [^{3}H]Arg POMC. 0.1 N HCl was also tested for its ability to extract POMC from secretory granules. Samples were acidified and made up in 0.9 M acetic acid-5 M urea and the [^{3}H]Arg POMC detected by acid-urea gel electrophoresis [2]. Immunoprecipitation of [^{3}H]Arg POMC was carried out using excess ACTH antiserum and the immuno-complex precipitated with *Staphylococcus aureus* Cowan I cells (The Enzyme Center, Boston, MA) as described previously [11]. The immunoprecipitate was analysed by acid-urea gels.

3. RESULTS

Association of newly synthesized POMC with the secretory granule membrane was examined in the frog and mouse pars intermedia. Fig.1 (upper

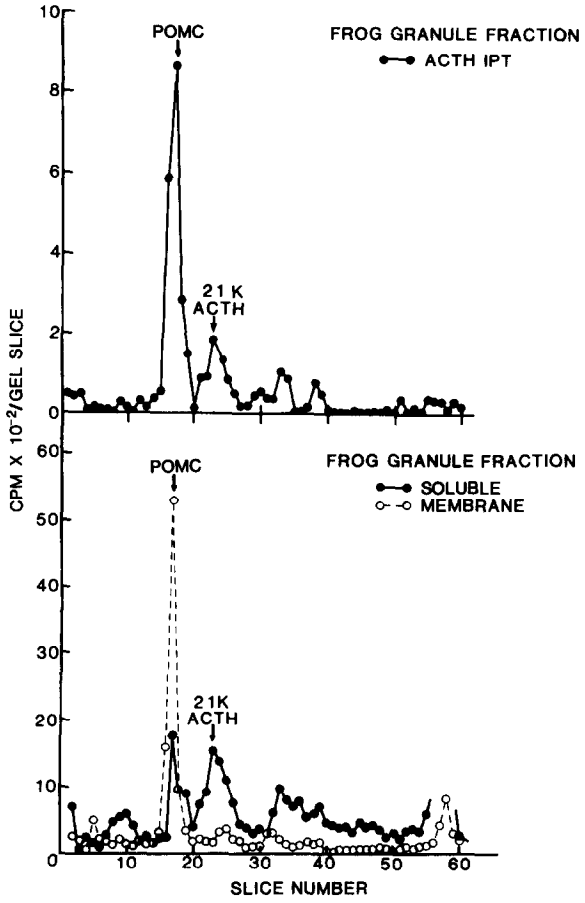

Fig.1. Upper: acid-urea gel profile of anti-ACTH immunoprecipitated [^{3}H]Arg-labeled proteins in the frog secretory granule fraction. The arrows show the POMC peak and the 21-kDa ACTH peak. Lower: acid-urea gel profiles of [^{3}H]Arg-labeled proteins in the frog secretory granule soluble (●—●) and membrane (○---○) fractions. The arrows indicate the POMC peak and the POMC-derived product, 21-kDa ACTH. The large (cut-off) peak between slice 55 and 60 in the profile of the soluble fraction was due to free [^{3}H]Arg.

panel) shows the acid-urea gel profile of anti-ACTH immunoprecipitated [^{3}H]Arg POMC and products present in the total P_3 secretory granule fraction isolated from frog neurointermediate lobes. The major ACTH-related peak was POMC, identified by its previously determined R_f on acid-urea gels and immunological properties [2]. A small amount of ACTH-related processed products was also observed in the gel profile. Fig.1 (lower panel) shows that most of the POMC in the secretory granule fraction was found in the membrane fraction. However, the intermediate, 21-kDa ACTH, generated by the cleavage of POMC between ACTH and β-LPH was found primarily in the soluble fraction. Likewise, as shown in fig.2, the major ACTH-related peak in the total mouse secretory granule fraction was POMC (upper panel). Fig.2 (lower panel) shows that POMC was present primarily in the secretory granule membrane fraction.

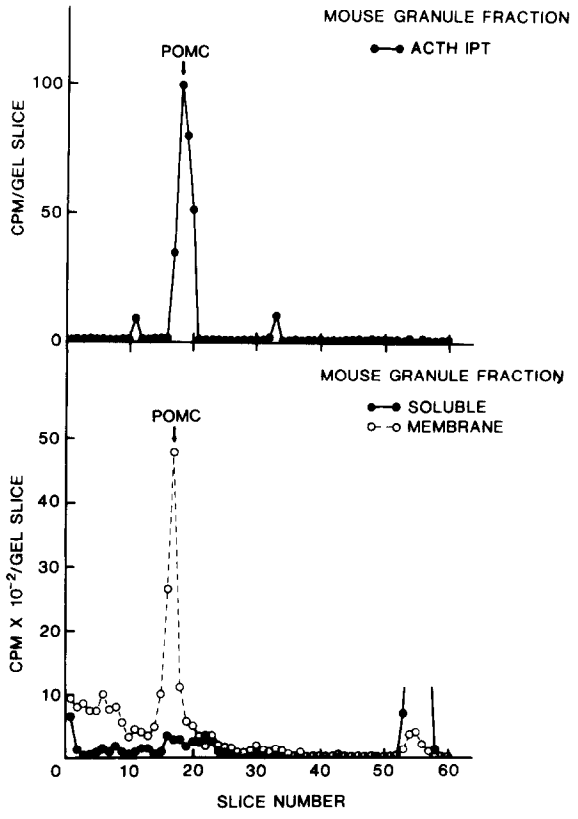

Fig.2. Upper: acid-urea gel profile of anti-ACTH immunoprecipitated [^{3}H]Arg-labeled proteins in the mouse secretory granule fraction. The arrow shows the POMC peak. Lower: acid-urea gel profile of [^{3}H]Arg-labeled proteins in the mouse secretory granule soluble (●—●) and membrane (○---○) fractions. The arrow indicates the POMC peak. The large (cut-off) peak between slice 54 and 58 in the profile of the soluble fraction was due to free [^{3}H]Arg.

Quantitation of the amount of newly synthesized POMC that was associated with the granule membrane, under different extraction conditions, is shown in table 1. After lysis of the frog secretory granules in 10 mM Tris-Cl, 73.6% of the POMC was associated with the membrane fraction. 1.0 M NaCl did not extract significant amounts of POMC from the membrane. However, when a more chaotropic agent, 1.5 M KSCN was used, 82% of the POMC was extracted from the granule membrane. Only 20% of the POMC was present in the remaining pellet after KSCN extraction (table 1). The newly synthesized secretory granule membrane associated mouse POMC showed similar extraction characteristics (table 1). 0.1 N HCl was also as effective as 1.5 M KSCN in extracting POMC from the secretory granule membrane (not shown).

4. DISCUSSION

The results presented indicate that most of the newly synthesized POMC was associated with the secretory granule membrane, both in amphibian and mammalian pars intermedia cells. After initial

Table 1

Extraction of [^{3}H]Arg POMC from frog and mouse neurointermediate lobe secretory granules

Extraction conditions	% total cpm[a] [^{3}H]Arg POMC	
	Pellet	Supernatant
Frog		
10 mM Tris-Cl (pH 7.4)	73.6 ± 3.7	26.4 ± 3.7
1.0 M NaCl	71.6 ± 2.3	28.4 ± 2.3
1.5 M KSCN	20.1 ± 2.8	79.9 ± 2.8
Mouse		
10 mM Tris-Cl (pH 7.4)	77.8 ± 4.8	22.2 ± 4.8
1.0 M NaCl	87.5 ± 3.9	12.5 ± 3.9
1.5 M KSCN	16.1 ± 1.5	84.0 ± 1.5

[a] Values shown are the mean ± SE, n = 4

lysis of the granules, the amount of prohormone associated with the secretory granule membranes was 74–78% (table 1). The repeated freezing and thawing to disrupt the granules may have caused some of the prohormone to be dislodged from the membrane. It is therefore likely that the amount of POMC that is membrane associated may be higher in vivo. Interestingly, in the case of the frog where some processing of POMC has occurred during the 30 min pulse incubation in [^{3}H]Arg, it was apparent that the product peaks (e.g., 21-kDa ACTH, see fig.1) were not membrane associated. Sequential extraction of the granule membrane indicated that 1.5 M KSCN was necessary to displace most of the POMC from the membrane. 1.0 M NaCl was ineffective. It appears, therefore, that POMC is tightly bound to the granule membrane, via ionic and/or hydrophobic interactions.

Similar results have been reported by Noe and Moran [12] for proglucagon, pro-somatostatin and pro-insulin in anglerfish islet cells. These prohormones were associated with the secretory granule membrane, but the hormones were not, with the exception of insulin which showed some membrane association. Furthermore, they showed that the binding of radioactively labeled forms of these prohormones to the granule membrane was saturable and competitively inhibited by adding excess unlabeled prohormone, indicating selective affinity. The association of newly synthesized prohormones to the secretory granule membrane therefore seems to be a general phenomenon and may involve specific membrane receptors. Such membrane association may be important in the intracellular sorting and directing of prohormones to their appropriate destination, i.e., the secretory granule. An example of receptor-mediated intracellular sorting is the targeting of lysosomal enzymes to the primary lysosome via binding to phosphomannosyl receptors [7–9]. Direct evidence for the involvement of specific receptors in the intracellular transport and sorting of prohormones awaits the identification of the binding region on the prohormones and ultimately the isolation of such receptors.

The association of prohormones to the granule membrane may have yet another physiological significance. Several prohormone converting enzymes have been shown to have activity residing in the secretory granule membrane [10,11,13,14]. It is also known that there is a strict order in which the several pairs of basic residues in POMC are cleaved. The association of POMC and its processing enzyme with the granule membrane may provide a juxtaposition favorable for conformational matching between enzyme and substrate and hence promote more efficient conversion of the prohormone.

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