Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Biochimie (1994) 76, 265–270

Identification of a sorting signal for the regulated secretory pathway at the N-terminus of pro-opiomelanocortin

DR Cool, Y Peng Loh

Section on Cellular Neurobiology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Building 49/Room 5A38, Bethesda, MD 20892, USA

(Received 18 February 1994; accepted 31 May 1994)

Summary — The N-terminal 26 amino acids of the prohormone pro-opiomelanocortin (POMC) were investigated to determine whether this region has the capacity to act as a sorting signal for the regulated secretory pathway. Constructs were made using the N-terminal 101, 50, 26 or 10 amino acids of POMC fused to the chloramphenicol acetyltransferase (CAT) reporter protein and expressed in AtT20 cells to show that at least the first 26 amino acids were required to sort CAT to the regulated secretory pathway. Full length POMC was mutated by deleting amino acids 2–26 from the N-terminal region. Analysis of Neuro-2a cells expressing this mutation compared to wild type POMC indicated that these 26 amino acids contain information essential for sorting POMC to the regulated secretory pathway. The results presented here suggest the presence of a conformation-dependent signal in the N-terminal 26 amino acids of POMC responsible for sorting POMC to the regulated secretory pathway.

prohormone / sorting signal / conformation / regulated secretion

Neuroendocrine cells, such as those found in the anterior pituitary, contain two major secretory pathways by which all proteins are secreted. The first of these is the constitutive secretory pathway, which is present in all cells and is responsible for the continuous secretion of proteins from the cell, such as the glycoprotein laminin [1]. Constitutive secretion has a very short transit time (~10–15 min) [2] and is also responsible for maintenance of the plasma membrane resident proteins [1]. In contrast, some specialized cells contain a second 'regulated' secretory pathway in which proteins to be secreted are segregated away from the constitutive proteins and are packaged in high concentrations into vesicles budding off the *trans*-Golgi network (TGN). These vesicles are then stored and many are aligned on the cytoplasmic surface of the plasma membrane where they await hormonal or neuronal stimuli which cause a Ca^{2+}-dependent exocytosis of the vesicle contents [1, 3, 4].

Within the past decade, molecular signals have been identified which act to target or retain proteins to specific subcellular compartments. Most notable of these are the KDEL signals found in the primary amino acid sequence of proteins to be retained in the endoplasmic reticulum (ER) [5] and the mannose-6-phosphate receptor signal for targeting proteins to the lysosomes [6–9]. Although the regulation of translation, processing and secretion of prohormones has been extensively studied [10–17], very little has been learned about the requirements for sorting prohormones into the regulated secretory pathway. However, since the constitutive secretory pathway is considered the default secretory pathway of the cell and thus would not require a sorting signal, it is thought that a signal would be required for directing proteins to the regulated secretory pathway. Comparison of the amino acid sequences of the numerous proteins destined for the regulated secretory pathway show that they lack a primary amino acid consensus sequence capable of acting as a sorting signal [18]. The lack of a consensus sequence in the primary structure suggests that the conformation of the protein may be involved in the sorting to the regulated pathway. By computer analysis, a degenerate motif (SXLL) consisting of specific regions of a homologous conformation (α-helix) in 15 different prohormones was identified and proposed to have a function in sorting to regulated secretory vesicles [18]. However, for POMC, this motif is not conserved across species suggesting that it is not a common sorting signal (fig 1A) [19–22].

In our laboratory, we have attempted to identify a sorting signal of the regulated secretory pathway for

the prohormone, pro-opiomelanocortin (POMC). POMC is synthesized in the corticotroph cells of the anterior pituitary, in the intermediate pituitary, as well as in the brain. It is processed in the TGN and acidic secretory granules of these cells by endoproteases [10, 23, 24], to yield ACTH, β-lipotropin (β-LPH), β-endorphin, N-POMC$_{1-49}$ and γ-MSH [13, 25–28]. POMC contains a unique conformational domain which is very highly conserved across species [29]. This conformational domain is represented as a hairpin loop region consisting of 11 amino acid residues between cys 8 and cys 20 (C_8QDLTTESNLLAC$_{20}$) stabilized by two disulfide bridges [30] (fig 1B). This motif may also be present in other prohormones such as met-enkephalin [31], pro-oxytocin [32] and pro-vasopressin [33] which have cysteine residues in similar positions and thus have the potential to form and stabilize a hairpin loop structure. Likewise, one of the resident proteins of the regulated secretory pathway, chromogranin B, also contains a 20 amino acid hairpin loop structure stabilized by a single disulfide bridge [34]. We have therefore focussed on the N-terminal domain of POMC to identify a sorting signal for the regulated pathway.

Two requirements for such a sorting signal are that it be both sufficient and necessary for sorting of the prohormone to the regulated secretory pathway. One region or several domains of a protein may be sufficient to enable sorting without the rest of the molecule being present. However, a particular region may also be required for sorting, and therefore sorting may not occur in a protein lacking that region. To determine if the N-terminus of POMC is sufficient for sorting, this region has been fused to a distinct reporter protein, chloramphenicol acetyl transferase (CAT) and the constructs were expressed in cells that contain the endogenous prohormone. Specifically, cDNA encoding the signal peptide and 101, 50, 26 or 10 amino acids (aa) of the N-terminus of POMC were ligated in frame to the CAT gene and expressed in AtT20 cells, a mouse anterior pituitary cell line which endogenously synthesizes, stores and secretes POMC in response to a secretagogue [35]. The fate of the N-POMC-CAT fusion protein in these cells was examined first by immunocytochemistry which showed that when the hairpin loop and disulfide bridges were present (101, 50 and 26) the CAT immunoreactive material (CAT$_i$) was localized in small punctate granules throughout the cytoplasm and extending into the neurites (fig 2). Immunoelectron microscopy confirmed that the CAT$_i$ for the 26 aa construct was localized in dense core secretory granules of the regulated secretory pathway. In contrast, the CAT$_i$ for the 10 aa construct was not present in the punctate or dense core granules at either the light (fig 2) or the electron microscope level [35].

Another characteristic feature of the regulated secretory pathway is the release of proteins upon stimulation with a secretagogue such as forskolin. Thus, the immunocytochemical observations were further supported by secretion studies in which forskolin stimulated the release of N-POMC-CAT fusion proteins containing 26 and 101 amino acids, but not the shortest, 10 amino acid fusion protein, which was secreted constitutively [35]. Finally, subcellular fractionation of the cells expressing 26 and 101 N-POMC-CAT fusion proteins provided further evidence that these fusion proteins were localized in secretory granules.

These findings showed that the first 26 N-terminal amino acids of POMC were sufficient to target POMC to the regulated secretory pathway. In order to establish that these 26 amino acids were necessary for sorting POMC to the regulated pathway, a POMC cDNA construct was made in which the nucleotides encoding amino acids 2 through 26 were deleted (NTD-POMC). As a control, wild type POMC was expressed in Neuro-2a cells, a cell line that does not make endogenous POMC but does have both a regulated and constitutive pathway [36, 37], and was found to be localized in punctate granules in the main cell body, extending out along the neurites and

Figure 1A

Human POMC	C Q D L T T E S N L L E C
Rat POMC	C Q D L T T E S N L L A C
Guinea pig POMC	C Q D L T T E R H L L E C
African clawed frog POMC	C A D L S S E D G V L E C
Rana ridibunda	C T D L S S E D G I L E C
Trout A POMC	C H D L S S E N N L L E C
Ostrich POMC	C Q D L T T E A G V L A C

Figure 1B

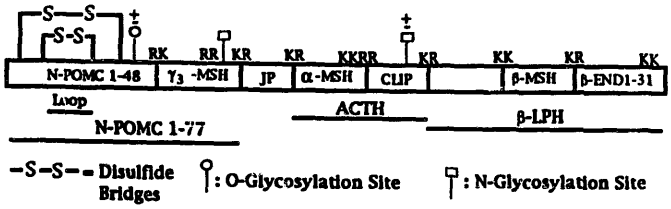

Fig 1. A. Amino acid sequence comparison of the hairpin loop region of N-POMC for human, rat, ostrich, *Xenopus laevis* and *Rana ridibunda*. **B.** Structure of human/bovine pro-opiomelanocortin. The diagram shows paired basic residue cleavage sites where processing of the prohormone occurs. Disulfide bridges and the location of the hairpin loop are shown in N-POMC$_{1-48}$. MSH, melanocyte stimulating hormone; JP, joining peptide; CLIP, corticotropin-like intermediate lobe peptide; LPH, liprotropin; END, endorphin; ACTH, adrenocorticotropin.

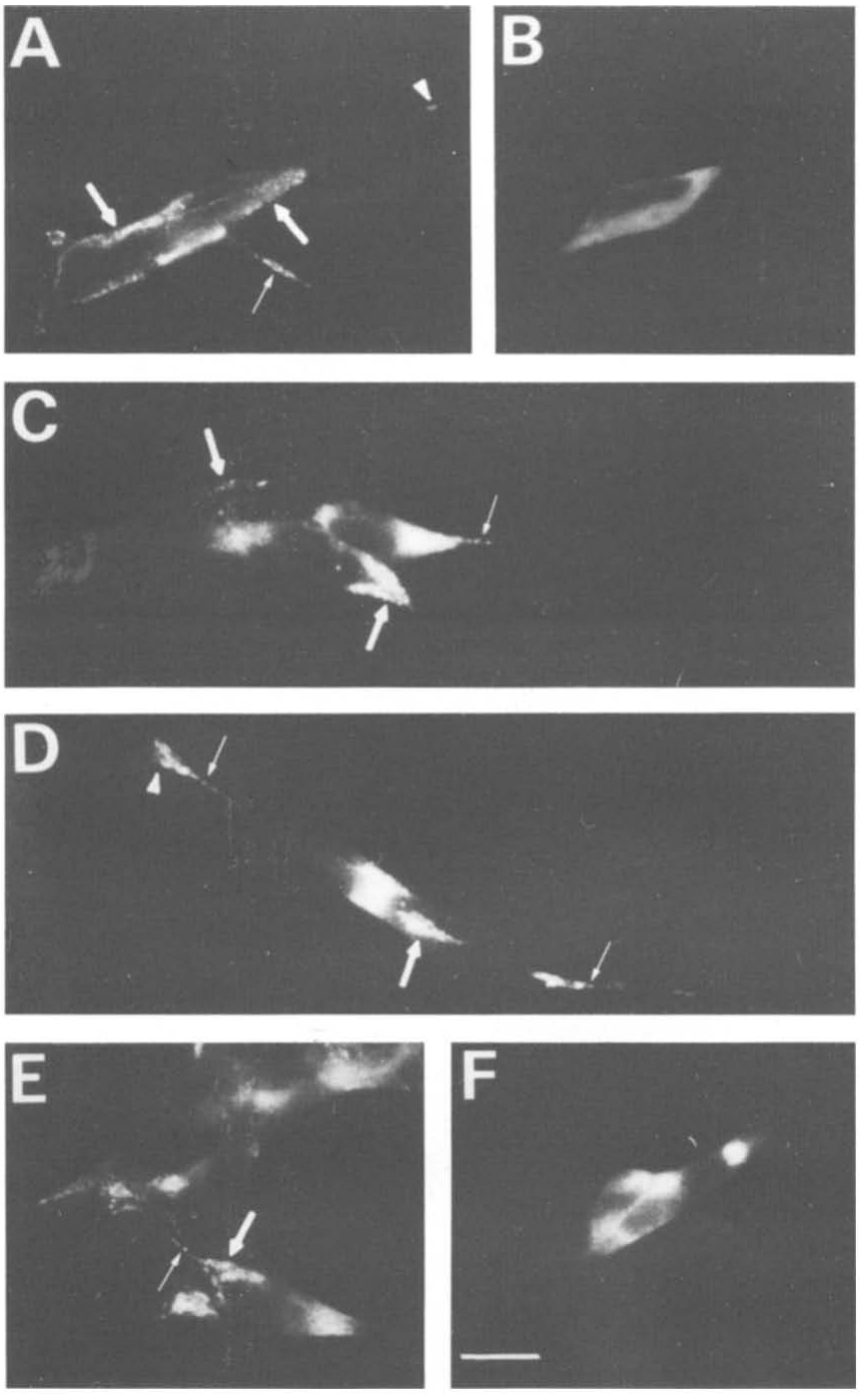

Fig 2. Immunocytochemical localization of POMC and N-POMC-CAT fusion proteins transfected into AtT20 cells [35]. AtT20 cells were stained with ACTH antibody (**A**), or transiently transfected with plasmids encoding: CAT (**B**), 101 amino acid-CAT construct (**C**), 50 amino acid-CAT construct (**D**), 26 amino acid-CAT construct (**E**), and 10 amino acid-CAT construct (**F**) and immunostained with CAT antibody. Punctate granules are indicated in the cell bodies (large arrows), processes (small arrows) and the tips of processes (arrowheads). Bar 10 μm (figure reprinted with permission from Wissenschaftliche Verlagsgesellschaft mbH).

appered to be accumulated in the tips of the neurites (fig 3A). On the contrary, when the NTD-POMC mutant was transfected and expressed in Neuro-2a cells, the ACTH immunoreactive material was localized to the Golgi and perinuclear region, but not in secretory granules (fig 3B). Cells expressing NTD-POMC showed no stimulation of secretion of immunoreactive ACTH with K^+/Ca^{2+}, (0.74 fold; fig 4), when compared to cells expressing POMC (5.0-fold; fig 4), but constitutive secretion was observed suggesting that this region was not only sufficient but necessary for sorting POMC to the regulated secretory pathway. Further studies expressing site-directed mutagenized POMC cDNAs suggest that the hairpin

loop and at least one disulfide bridge are necessary for sorting POMC to the regulated pathway (DR Cool, M Fenger, CR Snell, YP Loh, in preparation). Recent studies by Roy *et al* [36] also suggest involvement of the disulfide bridges in targeting POMC to the regulated pathway. A requirement for the disulfide bridge has also been shown for sorting chromogranin B to the regulated secretory pathway [38]. In these experiments, PC12 cells (a rat pheochromocytoma cell line) were treated with DTT and pulse-labeled with [^{35}S]-sulfate followed by a chase. This study showed that the DTT caused the labeled chromogranin B to be missorted to the constitutive secretory pathway. Another regulated secretory protein which does not have disulfide bridges, secretogranin II, was not affected by DTT and was targeted correctly to the regulated pathway. These experiments suggest that the disulfide bridges play a key role in the sorting process of the proteins examined. Since disulfide bridges are not present in all prohormones or secretogranins targeted to the regulated pathway, the question arises as to the role they play in the sorting of these specific prohormones? One possibility is that the relevant sorting signal conformational loop structure is unstable in some of these molecules and requires the close proximity of a disulfide bridge for stabilization.

Other events such as pH- and Ca^{2+} sensitive aggregation of chromogranin B molecules in the TGN of GH_4Cl and PC12 cells have been shown to be required for sorting to the regulated pathway [39, 40]. Aggregation of POMC has not yet been shown, however the N-terminal 76 amino acids were found to bind to the lumenal but not to the cytoplasmic side of secretory granule membranes or to the extracellular side of the plasma membrane of AtT20 and BSC40 cells in a pH-dependent manner [35].

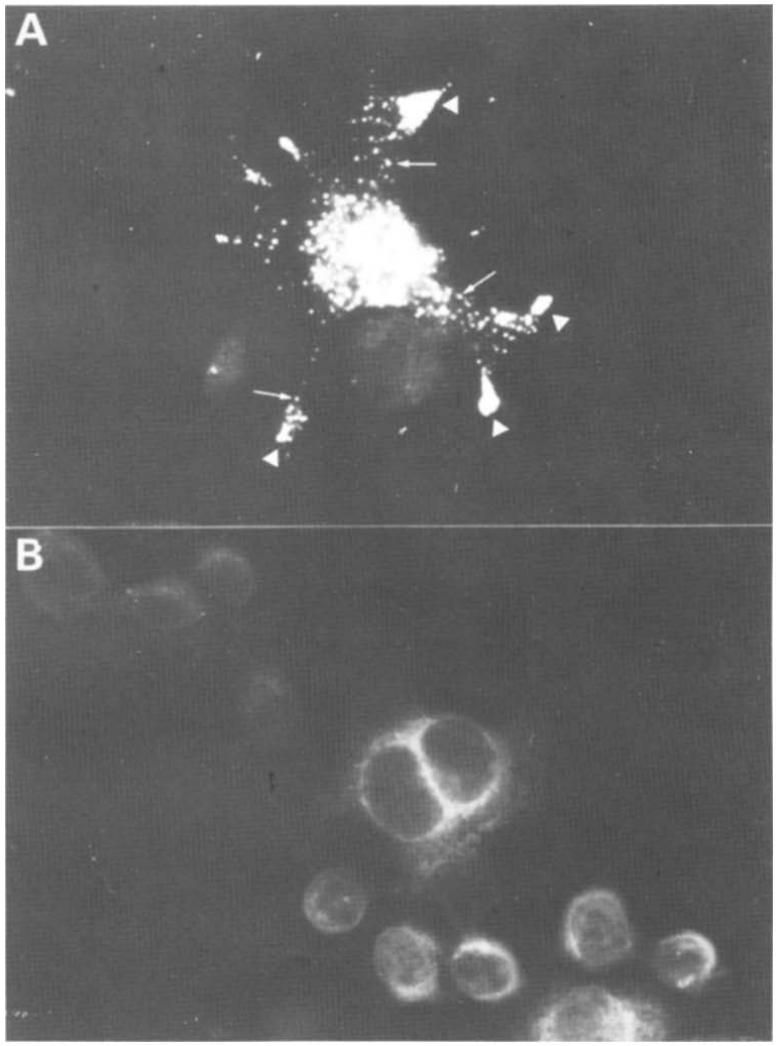

Fig 3. Immunocytochemical localization of ACTH immunoreactive material in Neuro-2a cells transiently transfected with POMC (**A**) and N-terminal deleted POMC (NTD-POMC) (**B**). Arrows indicate punctate secretory granules and arrowheads indicate granules in the tips of processes. Methods. Lipofectin (Gibco BRL, Grand Island, NY) was used to transiently transfect the plasmids containing POMC or NTD-POMC. The transfected cells were grown on poly-L-lysine coated coverslips. Expression of the cDNA was stimulated for 24 h with 100 nM dexamethasone. The cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum in PBS and incubated 16 h with antibody to ACTH$^{1-39}$ (DP4; [43]) at 1:2500 dilution. The cells were stained using a goat anti-rabbit serum conjugated with rhodamine.

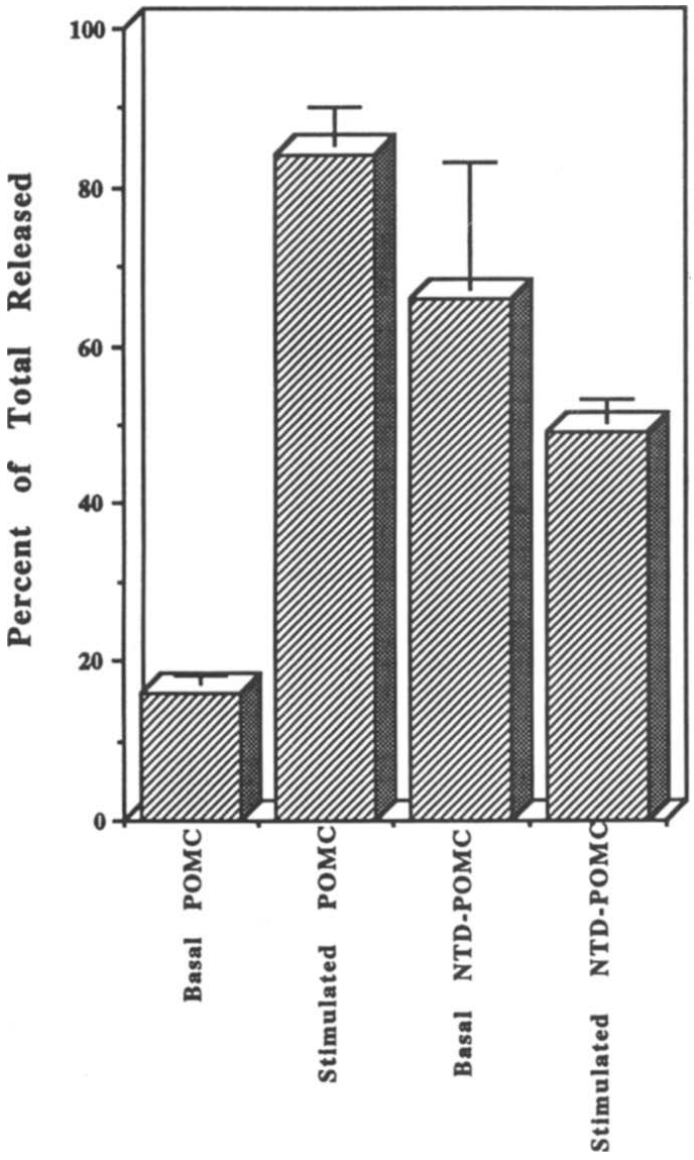

Fig 4. Effects of K^+/Ca^{2+} on the secretion of POMC and NTD-POMC from Neuro-2a cells. Methods. Transiently transfected Neuro-2a cells were exposed for 3 h to buffer containing either normal concentrations of K^+ (5 mM) and Ca^{2+} (1 mM) (basal). This buffer was then replaced either by the same volume of basal buffer or a stimulation buffer containing high concentrations of K^+ (51 mM) and Ca^{2+} (5.4 mM). The amount of ACTH immunoreactive material secreted into the media was assayed by radioimmunoassay. The values represent the mean and SEM for two experiments. The values for percent of total released were calculated by dividing the $ACTH_i$ released during the second 3 h by the total $ACTH_i$ (*ie* $ACTH_i$ in released during the first 3 h + $ACTH_i$ released during the second 3 h + the amount in the cell extract).

What then is the role for the N-terminal region of POMC, specifically the loop, in sorting? As mentioned before, several models for the sorting of prohormones have been proposed, including a receptor-mediated mechanism [35, 41] and a self-aggregation mediated mechanism [40, 42]. The hairpin loop region of N-POMC may be involved in intermolecular aggregation of POMC molecules in the more acidic environment of the TGN. Since N-POMC also binds to the lumenal side of the secretory granule membranes, it is tempting to speculate that the N-terminal regions on the surface of the aggregate may bind to an as yet undiscovered receptor. Alternatively, due to the acidic and hydrophobic nature of the amino acid residues in the loop region, binding of this region to the lumenal side of the secretory granule membranes may occur *via* ionic or hydrophobic interaction with specific lipids. In both of these mechanisms the aggregated complex could cause a localized or clustered binding of the N-terminal regions to the membrane and thus originate specific areas for budding.

The regulated secretory pathway in which prohormones are synthesized, processed, stored and secreted from neuroendocrine cells represents a complex series of steps in the intracellular life of the prohormone. Thus it is not surprising that at a major junction (the TGN) where the prohormone could be routed either to the constitutive or regulated secretory pathways, there should be a mechanism for maintaining the continuity of the regulated secretory pathway by ensuring that the prohormone reaches the compartment where it can be processed and stored (the regulated secretory granule). The evidence presented here suggests the existence of a conformation-dependent signal responsible for sorting the prohormone POMC away from the constitutive pathway and to the regulated secretory pathway.

Acknowledgments

We would like to thank Ms Winnie Tam for her technical and intellectual contributions and Drs Theodore Friedman and Ana-Maria Bamberger for their critical reading of the manuscript.

References

1 Gumbiner B, Kelly RB (1982) Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell* 28, 51–59

2 Burgess TL, Craik CS, Matsuuchi L, Kelly, RB (1987) *In vitro* mutagenesis of trypsinogen: role of the amino terminus in intracellular protein targeting to secretory granules. *J Celi Biol* 105, 659–658

3 Tooze SA, Chanat E, Tooze J, Huttner WB (1993) Secretory granule formation. *In: Mechanisms of Intracellular Trafficking and Processing of Proproteins* (Loh YP, ed) CRC Press, Inc, Boca Raton, Fl 158–177

4 Castro MG, Gusovsky F, Loh YP (1989) Transmembrane signals mediating adrenocorticotrophin release from mouse anterior pituitary cells. *Mol Cell Endocrinol* 65, 165–173

5 Munro S, Pelham H (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48, 899–907

6 Sahagian GG, Distler JJ, Jourdian GW (1982) Membrane receptor for phosphomannosyl residues. *Methods Enzymol* 83, 392–396

7 Jourdian GW, Sahagian GG, Distler J (1981) The role of carbohydrates in the recognition and uptake of glycoproteins by mammalian cells. *Biochem Soc Trans* 9, 510–512

8 Sahagian GG (1984) The mannose 6-phosphate receptor: function, biosynthesis and translocation. *Biol Cell* 51, 207–214

9 Willingham MC, Pastan IH, Sahagian GG, Jourdian GW, Neufeld EF (1981) Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *Proc Natl Acad Sci USA* 78, 6967–6971

10 Gumbiner B, Kelly RB (1981) Secretory granules of an anterior pituitary cell line, AtT20 contain only mature forms of corticotropin and β-lipotropin. *Proc Natl Acad Sci USA* 78, 318–322

11 Heisler S, Reisine T (1984) Forskolin stimulates adenylate cyclase activity, cyclic AMP accumulation and adrenocorticotropin secretion from mouse anterior pituitary tumor cells. *J Neurochem* 42, 1659–1666

12 Seger MA, Bennett HPJ (1986) Structure and bioactivity of the amino-terminal fragment of pro-opiomelanocortin. *J Steroid Biochem* 25, 703–710

13 Loh YP (1992) Molecular mechanisms of beta-endorphin biosynthesis. *Biochem Pharmacol* 44, 843–849

14 Matsuuchi L, Kelly RB (1991) Constitutive and basal secretion from the endocrine cell line, AtT20. *J Cell Biol* 112, 843–852

15 Schnabel E, Mains RE, Farquhar MG (1989) Proteolytic processing of pro-ACTH/Endorphin begins in the Golgi complex of pituitary corticotropes and AtT20 cells. *Mol Endocrinol* 3, 1223–1235

16 Schwartz J, Gibson S, White A (1991) Regulation of ACTH secretory pathways in cultured pituitary cells. *Am J Physiol* 261, C793–C798

17 Castellano F, Heuser J, Marchetti S, Bruno B, Luini A (1992) Glucocorticoid stabilization of actin filaments: a possible mechanism for inhibition of corticotropin release. *Proc Natl Acad Sci USA* 89, 3775–3779

18 Kizer JS, Tropsha A (1991) A motif found in propeptides and prohormones that may target them to secretory vesicles. *Biochem Biophys Res Commun* 174, 586–592

19 Hilario E, Lihrmann I, Vaudry H (1990) Characterization of the cDNA encoding pro-opiomelanocortin in the frog *Rana ridibunda*. *Biochem Biophys Res Commun* 173, 653–659

20 Wong M, Rius RA, Loh YP (1991) Characterization of *Xenopus laevis* proenkephalin gene. *Brain Res Mol Brain Res* 11, 197–205

21 Salbert G, Chauveau I, Bonnec G, Valotaire Y, Jego P (1992) One of the two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol Endocrinol* 6, 1605–1613

22 Naude RJ, Litthauer D, Oelofsen W, Chretien M, Lazure C (1993) The production of the Ostrich NH2-terminal POMC fragment requires cleavage at a unique signal peptidase site. *Peptides* 14, 519–529

23 Loh YP, Gritsch HA (1981) Evidence for intragranular processing of propiocortin in the mouse pituitary intermediate lobe. *Eur J Cell Biol* 26, 177–183

24 Tooze J, Tooze SA, Fuller SD (1987) Sorting of progeny coronavirus from condensed secretory proteins at the exit from the trans-Golgi network of AtT20 cells. *J Cell Biol* 105, 1215–1226

25 Eipper B, Mains, RE (1980) Structure and biosynthesis of pro-adrenocorticotropin hormone. *Endocrinol Rev* 1, 1–27

26 Estivariz FE, Friedman TC, Chikuma T, Loh YP (1992) Processing of adrenocorticotropin by two proteases in bovine intermediate lobe secretory vesicle membranes. A distinct acidic, tetrabasic residue-specific calcium-activated serine protease and a PC2-like enzyme. *J Biol Chem* 267, 7456–7463

27 Estivariz FE, Birch NP, Loh YP (1989) Generation of Lys-γ3-melanotropin from pro-opiomelanocortin by a bovine intermediate lobe secretory vesicle membrane-associated aspartic protease and purified pro-opiomelanocortin converting enzyme. *J Biol Chem* 264, 17796–17801

28 Benjannet S, Rondeau N, Day R, Chretien M, Seidah NG (1991) PC1 and PC2 are proprotein convertases capable of cleaving pro-opiomelanocortin at distinct pairs of basic residues. *Proc Natl Acad Sci USA* 88, 3564–3568

29 Douglass J, Civelli O, Herbert E (1984) Polyprotein gene expression: generation of diversity of neuroendocrine peptides. *Annu Rev Biochem* 53, 665–715

30 Seger MA, Bennett HPJ (1986) Structure and bioactivity of the amino-terminal fragment of pro-opiomelanocortin. *J Steroid Biochem* 25, 703–710

31 Comb M, Seeburg PH, Adelman J, Eiden L, Herbert E (1982) Primary structure of the human met and leu-enkephalin precursor and its mRNA. *Nature* 295, 663–666

32 Ruppert S, Scherer G, Shutz G (1984) Recent gene conversion involving bovine vasopressin and oxytocin precursor genes suggested by nucleotide sequence. *Nature* 304, 554–557

33 Land H, Schutz G, Schmale H, Richter D (1982) Nucleotide sequence of cloned cDNA encoding bovine arginine vasopressin neurophysin II precursor. *Nature* 295, 299–303

34 Benedum UM, Lamouroux A, Konecki DS, Rosa P, Hille A, Baeuerle PA, Frank R, Lottspeich F, Mallet J, Huttner WB (1987) The primary structure of human secretogranin I (chromogranin B): comparison with chromogranin A reveals homologous terminal domains and a large intervening variable region. *EMBO J* 6, 1203–1211

35 Tam WHH, Andreasson KA, Loh YP (1993) The amino-terminal sequence of pro-opiomelanocortin directs intracellular targeting to the regulated secretory pathway. *Eur J Cell Biol* 62, 294–306

36 Roy P, Chevrier D, Fournier H, Racine C, Zollinger M, Crine P, Boileau G (1991) Investigation of a possible role of the amino-terminal pro-region of pro-opiomelanocortin in its processing and targeting to secretory granules. *Mol Cell Endocrinol* 82, 237–250

37 Ross J, Olmsted JB, Rosenbaum JL (1977) The ultrastructure of mouse neuroblastoma cells in tissue culture. *Tissue Cell* 7, 107–136

38 Chanat E, Weiss U, Huttner WB, Tooze SA (1993) Reduction of the disulfide bond of chromogranin B (secretogranin I) in the *trans*-Golgi network causes its missorting to the constitutive secretory pathway. *EMBO J* 12, 2159–2168

39 Gerdes HH, Rosa P, Phillips E, Baeuerle PA, Frank R, Argos P, Huttner WB (1989) The primary structure of human secretogranin II, a widespread tyrosine sulfated secretory granule protein that exhibits low pH and calcium induced aggregation. *J Biol Chem* 264, 12009–12015

40 Chanat E, Huttner WB (1991) Milieu-induced, selective aggregation of regulated secretory proteins in the *trans*-Golgi network. *J Cell Biol* 115, 1505–1519

41 Kelly RB (1987) From organelle to organelle. *Nature* 326, 14–15

42 Arvan P, Castle D (1992) Protein sorting and secretion granule formation in regulated secretory cells. *Trends Cell Biol* 2, 327–331

43 Andreasson KI, Tam WWH, Feurst TO, Moss B, Loh YP (1989) Production of pro-opiomelanocortin (POMC) by a vaccinia virus transient expression system and *in vitro* processing of the expressed prohormone by POMC-converting enzyme. *FEBS Lett* 248, 43–47