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

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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-POMC1-49 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 (C4QDLTTESENLAC20) stabilized by two disulfide bridges [30]. This motif may also be present in other prohormones such as met-enkephalin [31], pro-oxytocin [32] and provasopressin [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, DNA 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 (CATI) was localized in small punctate granules throughout the cytoplasm and extending into the neurites (fig 2). Immunoelectron microscopy confirmed that the CAT, for the 26 aa construct was localized in dense core secretory granules of the regulated secretory pathway. In contrast, the CAT, 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

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**Figure 1A**

Human POMC: CQDLTTESENLAC

Rat POMC: CQDLTTESENLAC

Guinea pig POMC: CQDLTTESENLAC

African clawed frog POMC: CADLSSEDGVEAC

Rana ridibunda: CTDLSSEDGVEAC

Trout A POMC: CQDLTTEAGVEAC

Ostrich POMC: CQDLTTEAGVEAC

**Figure 1B**

- **N-POMC 1-49**: Loop
- **N-POMC 1-77**: Loop
- **Disulfide Bridges**: O-Glycosylation Site
- **N-Glycosylation Site**: γ

**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. γ-MSH, melanocyte stimulating hormone; JP, joining peptide; CLIP, corticotropin-like intermediate lobe peptide; LPH, lipotropin; END, endorphin; ACTH, adrenocorticotropic.
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+/Ca²⁺, (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, MFenger, 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 DTI" and pulse-labeled with [35S]-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 Ca2+ sensitive aggregation of chromogranin B molecules in the TGN of GH4C1 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 ACTH1-39 (DP4; [43]) at 1:2500 dilution. The cells were stained using a goat anti-rabbit serum conjugated with rhodamine.
Fig 4. Effects of K+/Ca2+ 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 Ca2+ (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 Ca2+ (5.4 mM). The amount of ACTH immunoreactive material secreted into the media was assayed by radioimmunooassay. The values represent the mean and SEM for two experiments. The values for percent of total released were calculated by dividing the ACTH released during the second 3 h by the total ACTH, (i.e. ACTH released during the first 3 h + ACTH 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 luminal 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 luminal 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.

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