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Hypothesis

An N-terminal hydrophobic peak is the sorting signal of regulated secretory proteins

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Abstract Endocrine and exocrine cells each contain a regulated and constitutive secretory pathway. The presence of two distinct secretory pathways in the same cell type requires a sorting step to direct secretory proteins to the correct pathway. It is thought that regulated secretory proteins contain a specific sorting signal. However, this signal has not been identified. Amino acid sequence comparisons have not revealed any significant similarity between different regulated secretory proteins, suggesting that the sorting signal does not consist of a conserved primary sequence. In the present report, we have analyzed the predicted secondary structures of regulated secretory proteins and identified an N-terminal hydrophobic peak (NHP) which is located approximately from amino acids 9–26, overlaps with a predicted α-helix and contains charged amino acid residues. This signal is present in regulated secretory proteins that exhibit an N-terminal sorting sequence, but it is absent from constitutively secreted proteins and proteins where the sorting sequence is not located near the N-terminus. It appears that the NHP is both necessary and sufficient for sorting of many secretory proteins to the regulated secretory pathway.

Key words: Prohormone; Targeting; Regulated secretion; Protein structure; Chromogranin; Proopiomelanocortin

1. Introduction

Protein function is dependent on the correct subcellular or extracellular localization, which is determined by specific signals within the protein structure. As an example, the signal peptide or pre-sequence of secretory and membrane proteins directs the nascent proteins to the endoplasmic reticulum for entry into the secretory pathway. Endocrine, neuronal and exocrine cells each contain two separate pathways for protein secretion: the constitutive secretory pathway, common to all eukaryotic cells, and the regulated secretory pathway. In the regulated pathway, peptide hormones, neurotransmitters and digestive enzymes are stored in secretory granules and released by exocytosis in response to extracellular stimulation [1]. Thus, regulated secretion is critical for the rapid secretion of stored proteins. The presence of two distinct secretory pathways in the same cell type requires a sorting step to direct secretory proteins to the correct pathway. Sorting takes place in the trans-Golgi network or condensing vacuoles but the protein signals and sorting mechanism are not understood. Specific aggregation of regulated secretory proteins [2–4] or their binding to sorting receptors [5] have been implicated in sorting. However, recent evidence suggests that secretory protein aggregation is not sufficient for sorting [6–7] and no sorting receptor has been conclusively identified [8].

Regulated secretory proteins contain the signals necessary for sorting to the regulated secretory pathway, while constitutive secretory proteins lack specific routing signals. Thus, a fusion protein consisting of both a regulated secretory protein and a constitutively secreted protein is sorted to the regulated secretory pathway [9]. The unknown sorting signals in several regulated secretory proteins are recognized by the sorting mechanism in both homologous and heterologous cell types, suggesting that a general mechanism for sorting is present in many cell types. However, amino acid sequence comparisons have not revealed any significant similarity between different regulated secretory proteins, suggesting that the sorting signal does not consist of a conserved primary sequence [10]. Our initial sequence comparisons confirmed this interpretation. In the present report, we have analyzed the predicted secondary structures of regulated secretory proteins and identified an N-terminal hydrophobic peak (NHP) which appears to be necessary and sufficient for sorting of secretory proteins to the regulated secretory pathway.

2. Results and discussion

Peptide sequences that are either necessary or sufficient for sorting to the regulated secretory pathway have been reported for several regulated secretory proteins [11–17]. While some of the published sorting sequences include a large portion of the native protein (>200 amino acids), the sorting sequences identified to date consistently include the N-terminal region of these proteins. We compiled the experimentally tested sorting sequences and, in agreement with earlier reports (e.g. [11,14,16]), we did not detect any sequence similarities in the N-terminal region of the proteins (not shown).

We next tested whether aspects of the secondary structure or amino acid usage were similar in all proteins with known N-terminal sorting sequences. The hydrophilicity and secondary structure predictions for the N-terminal 40 amino acids of these proteins are depicted in Fig. 1. Comparison of the predicted structures indicates the presence of an N-terminal hydrophobic peak (NHP) with adjacent relatively hydrophilic regions. Analysis of these structures (Table 1) allowed us to establish consen-

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Abbreviation: NHP, N-terminal hydrophobic peak.

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sus features for the NHP. The NHP is a hydrophobic domain containing charged amino acid residues that is located between residues 9(± 4) and 26(± 6) of the protein (after cleavage of the signal peptide). The domain overlaps with a predicted α-helix that spans the transition between the hydrophobic domain and a neighboring hydrophilic domain in most proteins (Fig. 1). Specifying boundaries of a predicted structure is necessarily subjective, however, the NHP can be defined as generally beginning after, and ending before, a glycine or proline residue, both of which are α-helix destabilizing amino acid residues. Glycine and proline are absent from within the NHP. About 60% of the amino acids in the NHP are either serine or threonine (S/T), glutamic acid or aspartic acid (E/D) or leucine or isoleucine (L/I). This occurrence is twice that which would be expected if the amino acids were randomly distributed in this region. Phenylalanine is present in the NHP of proopiomelanocortin and proenkephalin while no other aromatic amino acids are found in the NHP of sorted proteins (Fig. 1). Helical wheel analysis of the NHP indicates that this region exhibits amphipathic properties in the majority of the proteins tested. These consensus features are summarized in Table 2.

To determine the specificity of the NHP, we searched for similar structures in the sequences of constitutively secreted proteins. This analysis was limited to proteins that have been shown experimentally to be secreted constitutively from endocrine cells. The rationale for this selection was that proteins that are produced in cells without a regulated secretory pathway may contain sorting signals that become functional in endocrine cells, as has been shown for apolipoprotein [18] and human choriogonadotropin [19]. Rat angiotensinogen [20], truncated G-protein from vesicular stomatitis virus [9], and anglerfish prosomatostatin II [12] each have been shown to be secreted constitutively when expressed in endocrine cell lines. These control proteins did not exhibit hydrophobic peaks as defined above (Fig. 2). These results demonstrate that a defined structure, the NHP, is predicted in eight regulated secretory proteins while this structure is absent from tested constitutively secreted proteins.

Table 1
Comparison of N-terminal hydrophobic peaks

Protein	Flanking residues		# A.A.	% DESTLI
	amino	carboxy		
mPOMC	Q9	P33	23	70%
rPRP	G4	P14	9	78%
hCGA	G10	P29	18	61%
rCGB	N9	P31	21	57%
rPENK	G18	G31	12	58%
rPSS	P5	G20	14	36%
rPSS(Δ13–26)	P5	P22	16	50%
Consensus	G/P9 ± 4	P/G26 ± 6	16 ± 5	59% ± 13%

The N-terminal amino acid sequences of mouse proopiomelanocortin (mPOMC [11]), rat basic proline-rich protein (rPRP [16]), human chromogranin A (hCGA [15]), rat chromogranin B (rCGB [17]), rat proenkephalin (rPENK [14]), rat prosomatostatin (rPSS [12]) and a deletion mutant of rat prosomatostatin (rPSS(Δ13–26) [23]) (Fig. 1) were analyzed for the location and amino acid composition of the NHP. The table lists the residues flanking the NHP at its amino and carboxy terminus (Flanking residues); the total number of amino acid residues in the NHP (excluding the flanking residues) (#A.A.); and the percentage of amino acids represented by Asp (D), Glu (E), Ser (S), Thr (T), Leu (L) or Ile (I) (%DESTLI). The consensus features are depicted as the mean ± 95% confidence interval for each column.

Table 2
Consensus features of the N-terminal hydrophobic peak in regulated secretory proteins

1. The NHP spans approximately amino acids 9–26 of the protein (following cleavage of the signal peptide).
2. The NHP consists of a charged, hydrophobic peak with adjacent hydrophilic regions.
3. The NHP overlaps with a predicted α-helix (generally amphipathic in nature).
4. Helix breaking amino acids (Gly and Pro) are found on either side of the NHP, but Gly and Pro are absent from within the NHP. The amino acid composition of NHP is rich in Glu, Asp, Ser, Thr, Leu and Ile (about 60%) while aromatic amino acids are rarely present.

We next addressed the question of whether this structure plays a role in secretion. Rat basic proline-rich protein is sorted to the regulated secretory pathway in AtT-20 cells. Deletion of residues 5–17, including the NHP (residues 5–13), from this protein causes constitutive secretion of the mutant protein [16], suggesting that the region containing the NHP is necessary for sorting of this protein to the regulated secretory pathway. Similarly, mouse proopiomelanocortin exhibits an NHP at residues 10–32 and residues 2–26 of this protein, including most of the NHP, are necessary for sorting to the regulated secretory pathway [21]. In addition, a chloramphenicol acetyl transferase-fusion protein with amino acid residues 1–26 of proopiomelanocortin is correctly sorted in transfected AtT-20 cells. However, a fusion protein with residues 1–10, which do not contain the NHP, is not sorted to the regulated secretory pathway [11]. Thus, residues 11–26 are necessary for sorting of the fusion protein. These results demonstrate that the NHPs of basic proline-rich protein and proopiomelanocortin are necessary for sorting to the regulated secretory pathway.

Fusion proteins containing chloramphenicol acetyl transferase and small N-terminal fragments of proopiomelanocortin (residues 1–26 [11]) or pro-enkephalin (residues 1–31 [22]), respectively, are sorted to the regulated secretory pathway. These results suggest that the N-terminal fragments, each including the NHP (Table 1), are sufficient for sorting of the fusion proteins to the regulated secretory pathway. We conclude that the NHP appears to be both necessary and sufficient for sorting of secretory proteins to the regulated secretory pathway.

To further determine if the NHP is consistent with known sorting mutants, we analyzed the structure of rat prosomatostatin that contains a sorting signal in the N-terminal 54 amino acid residues [12]. This region exhibits two hydrophobic domains (Fig. 1E). Contrary to expectations, deletion of the NHP (residues 13–26) did not block sorting [23]. However, that deletion relocates the second hydrophobic domain (residues 27–52) to a position compatible with the consensus features for an NHP (Fig. 1G; Table 1) which may allow for the observed sorting of this construct. As expected, deletion of only the second hydrophobic domain (Fig. 1H) did not prevent sorting [23]. These results indicate that a single NHP in the correct location can act as a sorting signal for rat prosomatostatin, and that the primary sequence is not the important part of the signal.

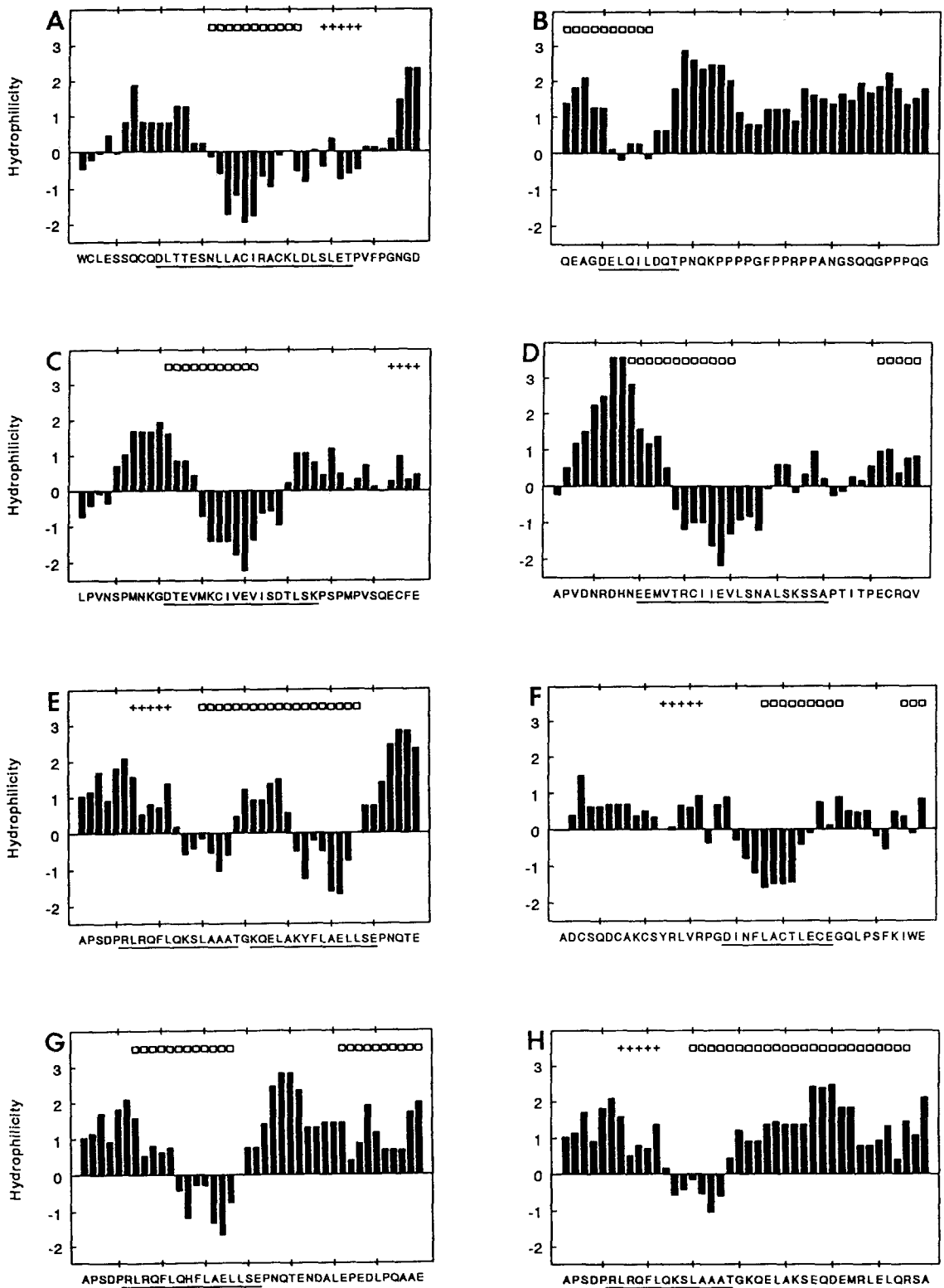

Fig. 1. Hydrophilicity and secondary structure prediction for the N-terminal 40 amino acids of the regulated secretory proteins mouse proopiomelanocortin (A), rat basic proline-rich protein (B), human chromogranin A (C), rat chromogranin B (D), rat prosomatostatin (E), rat proenkephalin (F), rat prosomatostatinΔ(13–26) (G), and rat prosomatostatinΔ(27–52) (H). Each protein contains an N-terminal hydrophobic peak (NHP; underlined) that overlaps with a predicted α-helix. Nucleotide sequences were retrieved from the Genbank and EMBL databases, translated and analyzed using the Wisconsin Genetics Computer Group sequence analysis software package (version 7.0; 1991). The subprogram 'peptidestructure' was used to calculate hydrohilicity and the location of secondary structures [30]. Solid bars represent the relative hydrophilicity of each residue averaged over a window of 7 residues [31], open boxes represent areas of predicted α-helix, and pluses represent areas of predicted β-sheets [32]. The NHP is underlined.

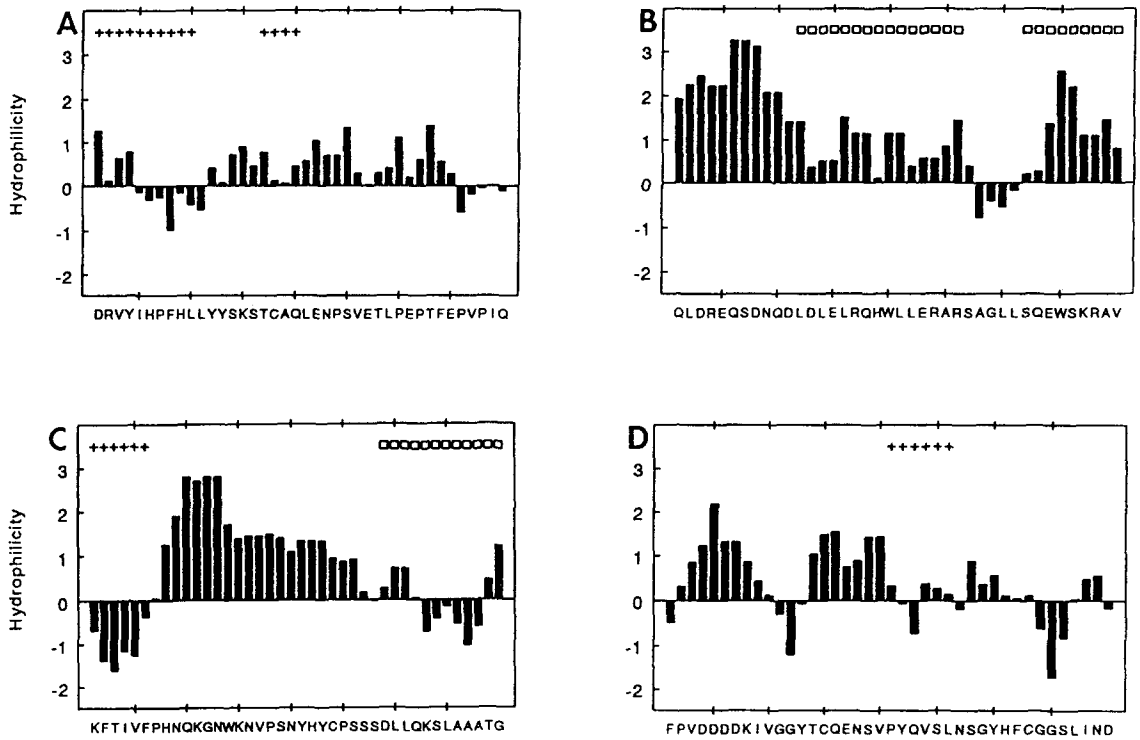

Fig. 2. Hydrophilicity and secondary structure prediction for the N-terminal 40 amino acids of the constitutively secreted proteins rat angiotensinogen (A), anglerfish prosomatostatin II (B), and vesicular stomatitis virus G-protein (C). The sequence of the regulated secretory protein rat trypsinogen is shown in D. Analyses and presentation are as described in Fig. 1.

We propose that the NHP can serve as a sorting signal for the subset of regulated secretory proteins that exhibit N-terminal sorting sequences and that additional sorting signals may function for other regulated secretory proteins. Consistent with this, deletion of the N-terminal pro-peptide from trypsinogen does not affect its sorting to the regulated secretory pathway [24] and trypsinogen does not exhibit an NHP (Fig. 2D). The presence of different sorting signals is consistent with the observation that some endocrine cells selectively sort secretory proteins to separate secretory granules [25,26]. As described above, anglerfish prosomatostatin II is not sorted to the regulated secretory pathway in mammalian endocrine cells [12]. This observation suggests that mammalian cells do not recognize the anglerfish sorting signal, consistent with the presence of different sorting signals in the different cell types. NHP-containing and non-NHP-containing secretory proteins can be sorted to the same subcellular location, since secretogranin II, which lacks an NHP, and chromogranin B are co-stored in many endocrine cells. Interestingly, it was recently demonstrated that chromogranin B, but not secretogranin II, is re-routed to the constitutive secretory pathway in DTT-treated PC-12 cells [17]. The authors suggested that the unique disulfide bond, which involves residues 17 and 38 in chromogranin B, is involved in sorting of this protein. Secretogranin II does not contain a disulfide bond [17]. These observations suggest that separate sorting signals function in the two granins, consistent with the model presented in this report.

To test the predictive value of the consensus rules for NHP, we analyzed the structure of insulin. An NHP was detected in the amino-terminal B-chain, indicating that this region may represent the previously unknown sorting signal for insulin.

While it now seems clear that calcium-induced aggregation is not responsible for sorting of regulated secretory proteins [6,7,27], recent evidence points to a possible mode of action for NHPs in sorting of regulated secretory proteins. Peptides with the NHP of either chromogranin A [28], chromogranin B [29] or human proopiomelanocortin [11] exhibited sequence specific and pH-specific binding to secretory granule membranes or membrane proteins, suggesting the presence of binding proteins. These reports suggest that the NHP mediates binding of regulated secretory proteins to membrane receptor protein(s) that are responsible for the sorting of secretory proteins to the regulated secretory pathway. In addition, the NHP may mediate direct interactions between regulated secretory proteins [8].

In conclusion, we have identified a structural motif that exhibits the properties expected of a sorting signal for regulated secretory proteins. Deletion of the NHP causes missorting of a regulated secretory protein while the NHP is apparently sufficient for sorting of a reporter protein to the regulated secretory pathway.

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