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Secretory granule targeting of atrial natriuretic peptide correlates with its calcium-mediated aggregation

(regulated secretion/AtT-20 cells/calcium binding/peptide precursor)

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ABSTRACT **Atrial natriuretic peptide (ANP) is a 28-aa peptide hormone secreted predominantly from atrial cardiocytes. ANP is first synthesized in the form of a 126-aa precursor (proANP) which is targeted to dense core granules of the regulated secretory pathway. ProANP is stored until the cell receives a signal that triggers the processing and release of the mature peptide (regulated secretion). Various models have been proposed to explain the targeting of selected proteins to the regulated secretory pathway, including specific "sorting receptors" and calcium-mediated aggregation. As potential calcium binding regions had previously been reported in the profragment of ANP, the current study was undertaken in an effort to determine the relationship between the ability of ANP to enter the regulated secretory pathway and its calcium-mediated aggregation. Deletion and site-directed mutagenesis of selected regions of the prosegment demonstrates that acidic amino acids at positions 23 and 24 are critical for both regulated secretion of proANP from transfected AtT-20 cells and calcium-mediated aggregation of purified recombinant proANP *in vitro*. These results demonstrate that the ability of certain proteins to enter secretory granules is directly linked to their calcium-mediated aggregation.**

Atrial natriuretic peptide (ANP) is a peptide hormone whose role is the modulation of salt and water balance in mammals (1, 2). While several tissues have been reported to express the ANP gene, its primary source is the atrial cardiomyocyte, where it is first synthesized as a precursor of 151 aa (Pre-proANP; Fig. 1). Following signal peptide cleavage, most of the 126-aa proANP is targeted to the regulated secretory pathway, where it is stored intact in the dense core secretory granules (3, 4). Increases in either blood volume or blood pressure trigger the proteolytic cleavage of the 28-aa mature ANP peptide from the carboxy terminus of proANP concomitant with the release of the secretory granule contents from the cell (5). Secretory granule targeting of proANP has also been demonstrated in transfected endocrine cells in culture including mouse pituitary AtT-20 cells (6) and rat pheochromocytoma PC12 cells (7).

The mechanism by which proANP enters the dense core secretory granule of the regulated secretory pathway is currently unclear. Evidence suggests that intracellular sorting of proteins to secretory granules is encoded by a dominant signal on the protein itself: fusion of a protein normally secreted constitutively to a protein destined for secretory granules results in the regulated secretion of the fusion protein (8). Although no consensus amino acid sequence has been found to date in proteins targeted to the regulated secretory pathway, two principle models have been proposed to account for secretory granule sorting of proteins. The first involves the binding of the protein to a secretory granule "receptor" localized in the trans-Golgi network (TGN; refs. 9 and 10) or to another acceptor protein that is itself destined for the secretory granule. Indeed, injection of cells with an antibody to chromogranin B (a secretory granule protein) results in the rerouting of the normally constitutively secreted antibody to the regulated secretory pathway (11). The second model involves the preferential aggregation of proteins to be packaged in dense core granules (12–14). This aggregation would serve as a signal for encapsulation and budding off of the nascent granule from the TGN or would serve as a means of retaining selected proteins in the maturing secretory granules (15). Several proteins destined for the regulated secretory pathway do indeed show a preferential tendency to aggregate *in vitro* in the presence of increased levels of calcium and decreased pH, conditions that have been proposed to prevail in the TGN (16, 17). Indeed, as demonstrated by Colomer *et al.* (18), secretory protein aggregation can depend on several individual or combined factors: their origin, protein–protein interactions, pH, and calcium. It is quite conceivable that aspects of both of these models could be operative to different degrees in the secretory granule targeting of various proteins (19).

In vitro, proANP shows a pH- and concentration-dependent binding of calcium, which leads to its aggregation (20). Both pH 6.5 or lower and millimolar concentration of Ca^{2+} were required to obtain optimal aggregation. Between amino acids 11 and 44 (relative to the amino terminus of proANP), proANP contains five aspartic acids and six glutamic acids with a total negative charge of −8 (Fig. 1 and ref. 1). It has been suggested that this cluster of acidic amino acid residues might bind calcium and participate in the aggregation of proANP (20). In the current study, we have tested the role of the acidic region of the rat proANP prosegment in the regulated secretion of proANP from transfected mouse pituitary AtT-20 cells. Our results show a direct link between the ability of proANP mutants to be secreted by the regulated secretory pathway and their calcium- and pH-mediated aggregation *in vitro*.

MATERIALS AND METHODS

Recombinant Plasmid Construction. The expression vector for native rat proANP was constructed by PCR amplification of the protein coding sequence of the rat ANP cDNA from plasmid pANF (a gift from Mona Nemer, Clinical Research Institute of Montreal) using the following oligonucleotides: forward, 5′-CCCAAGCTTCATCAGATCGTGCC-3′; and reverse, 5′-CGGGATCCGCGATCTGCTCGAGC-3′.

Incorporated *Hin*dIII and *Bam*HI restriction sites (underlined) were used to subclone the cDNA into the expression vector RSV-globin (21), the expression of which is driven by

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Abbreviations: ANP, atrial natriuretic peptide; TGN, trans-Golgi network; RIA, radioimmunoassay.

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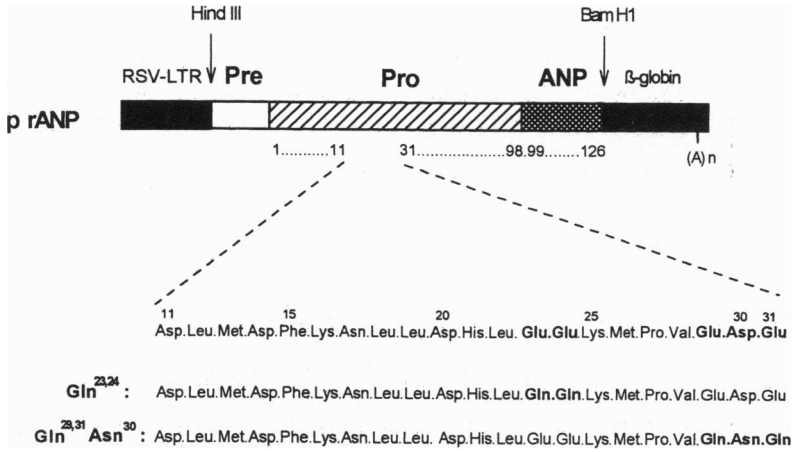

FIG. 1. Diagram of the expression vector for rat pre-proANP (p rANP). Pre, signal peptide; Pro, profragment; RSV, Rous sarcoma virus promoter/enhancer; (A)n, location of polyadenylylation signals; $Gln^{23,24}$, site-directed mutagenesis of $Glu^{23,24} \rightarrow Gln^{23,24}$; and $Gln^{29,31}Asn^{30}$, site-directed mutagenesis of $Glu^{29,31}Asp^{30} \rightarrow Gln^{29,31}Asn^{30}$.

the Rous sarcoma virus LTR promoter/enhancer. A 3′ RNA extension from the rabbit β-globin provided an intron and polyadenylylation signal (p rANP; Fig. 1).

Site-directed mutagenesis of the ANP prosegment was achieved by overlap-extension PCR (22) using the following oligonucleotides (reverse complements are not shown): deletion of residues 11–31, 5′-CGGAGGCATGACTGTGTTGGACACC-3′; $Glu^{23,24}$ to $Gln^{23,24}$, 5′-CGGCATCTTCTGCTGCAGGTGGTC-3′; and $Glu^{29}Asp^{30}Glu^{31}$ to $Gln^{29}Asn^{30}Gln^{31}$, 5′-ATGCCGGTACAAAATCAGGTCATGCCT-3′. All amplified cDNA fragments were sequenced in their entirety using the dideoxy chain termination method.

Cell Culture and Transfection. AtT-20 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 0.1% SerXtend (Irvine Scientific), and 10 μg of gentamycin per ml. Cells were maintained in a 37°C humidified incubator in 10% CO_2/90% air. Before transfection, cells were grown to ≈80% confluence and recovered by trypsinization. Cells were washed twice in Hepes-buffered saline (HBS; 137 mM NaCl/5 mM KCl/1 mM Na_2HPO_4/1 mg of dextrose per ml/20 mM Hepes, pH 7.05) and resuspended at a density of 1.5×10^7 cells/ml in HBS. Cells (667 μl) were mixed with 50 μg of the appropriate plasmid DNA and placed in a 0.4-cm electroporation cuvette (Bio-Rad). Electroporation was carried out at 300 V, 100 μF at room temperature using the ElectroPorator apparatus (Invitrogen). Ten minutes after electroporation, the cells were placed in a 25-cm^2 flask containing culture medium. Twenty-four hours after transfection, cells were replated into 6-well dishes at a density of 10^6 cells/well.

Secretion Assay for ANP and β-Endorphin. Forty-eight hours after transfection, cells were rinsed twice with prewarmed DMEM containing 5% FCS, and 2 ml of prewarmed medium were placed in each well. After 3 hr, this medium was collected and used as the reference supernatant (basal; Fig. 2 and Table 1). Two ml of prewarmed medium containing 10 μM forskolin (Sigma) were placed on the cells for an additional 3 hr. This medium corresponded to the stimulated cell supernatant (forskolin; Fig. 2 and Table 1).

Immunoreactive ANP and β-endorphin (β-end) were quantitated in the supernatants by radioimmunoassay. For ANP, antiserum to the C terminus of ANP was used with [^{125}I]ANP(99–126) as the radioactive tracer as described (23).

For β-endorphin, samples or standard (rat β-endorphin, 1.9–1000 fmol) were incubated overnight at 4°C with 10,000 counts/min of iodinated rat β-endorphin and with rabbit anti-rat β-endorphin antiserum (1:12,000; Peninsula Laboratories) in a total volume of 400 μl in 0.1 M sodium phosphate buffer, pH 7.4/0.15 M NaCl/0.1% BSA/0.1% Triton X-100/0.01% NaN_3. Separation of free from bound β-endorphin was made by addition of 100 μl of goat anti-rabbit immunoglobulin G antiserum (1:25; Immunocorp Sciences, Montreal) and 100 μl of normal rabbit serum (1:50; GIBCO/BRL). After 3 hr at room temperature, 1 ml of 12.5% polyethylene glycol was added, and tubes were centrifuged at $1500 \times g$ for 20 min. The radioactivity in the pellet was measured in a gamma counter.

Purification of Recombinant ANP. For purification of biochemical quantities of ANP, a total of 10^8 AtT-20 cells were transfected by electroporation as described above. Cells were replated at a density of 10^7 cells per 50-cm^2 dish in DMEM containing 10% FCS. Twelve hours after replating, the medium was changed to serum-free DMEM/F12 supplemented with insulin/transferrin/selenium (GIBCO/BRL). Culture

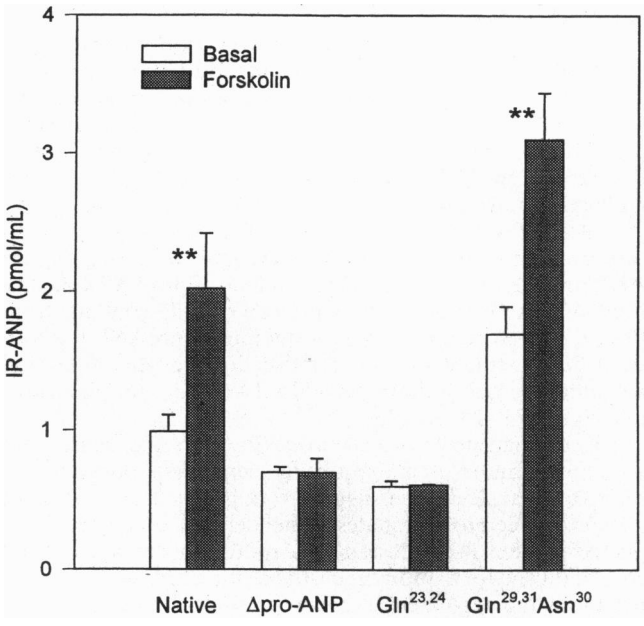

FIG. 2. Effect of mutations on native proANP sorting to the regulated secretory pathway. AtT-20 cells were transfected with the expression vectors for either native proANP or mutants in the profragment region (ΔproANP, $Gln^{23,24}$, and $Gln^{29,31}Asn^{30}$). Forty-eight hours after transfection, cells were incubated for 3 hr in the absence (basal) then in the presence (forskolin) of 10 μM forskolin. ANP was measured by RIA. Results were expressed in pmol/ml and were the mean ± SEM of 16–25 independent transfections and are derived from the results in Table 1. **, $P < 0.001$ (paired Student's t test).

Table 1. Effect of forskolin on secretion of native and mutated proANP

Plasmid	*n*	Forskolin	[proANP], pmol/ml	[β-end], pmol/ml	Secretion index*
Native	16	−	0.99 ± 0.12	1.96 ± 0.56	1.18 ± 0.23
		+	2.02 ± 0.40	3.40 ± 0.92	
ΔproANP	17	−	0.70 ± 0.04	1.05 ± 0.13	0.50 ± 0.12†
		+	0.70 ± 0.10	2.06 ± 0.15	
$Gln^{23,24}$	18	−	0.60 ± 0.04	0.96 ± 0.06	0.56 ± 0.06†
		+	0.61 ± 0.01	1.76 ± 0.17	
$Gln^{29,31}Asn^{30}$	25	−	1.70 ± 0.20	1.55 ± 0.54	0.80 ± 0.15
		+	3.10 ± 0.34	4.03 ± 0.61	

*Secretion index is calculated as fold stimulation of proANP/fold stimulation of β-end.
†$P < 0.01$ relative to index of stimulation of native proANP (two-way ANOVA).

supernatants were collected every 12 hr for 3 days, cleared, and adjusted to 0.3 mg of phenylmethylsulfonyl fluoride (PMSF) per ml and 10 μg of pepstatin per ml, and they were then immediately frozen at −40°C. Supernatants were passed over Sep-Pak C_{18} cartridges (Millipore), and ANP was eluted with 80% acetonitrile/0.1% (vol/vol) trifluoroacetic acid. Eluates were lyophilized and resuspended in 0.1 M acetic acid. Pooled eluates were purified twice by reverse-phase HPLC on Bondapack C_{18} (Waters) and were eluted with a gradient of acetonitrile (25 to 55%)/0.1% trifluoroacetic acid. ANP containing fractions were identified by radioimmunoassay (RIA), pooled, lyophilized, and stored at −40°C.

Aggregation Assays. *In vitro* proANP aggregation assay has been described (20). Briefly, 20 μg of native or mutated proANP were incubated in 0.05 M imidazole buffer (pH 6.5) in the presence or absence of 10 mM $CaCl_2$ in a total volume of 20 μl in a Beckman Airfuge centrifugation tube. After 1 hr of incubation at room temperature, the samples were centrifuged for 30 min at 28 psi (1 psi = 6.89 kPa) in the Airfuge. The supernatant was removed, and the pellet was dissolved in 1 M acetic acid. ProANP in the pellet and the supernatant was quantified by RIA. We previously demonstrated that under these conditions, aggregation was maximal and reached 50–60% (20).

RESULTS

Secretion of Native and Mutated proANP by AtT-20 Cells. Mouse pituitary AtT-20 cells have been used by many groups to study regulated secretion (19). AtT-20 cells express endogenous proopiomelanocortin, which is sorted to the regulated secretory pathway and processed to ACTH and other peptides, such as β-endorphin (24). These peptides are stored in secretory granules until their release is stimulated by secretagogues. To study the determinants of secretory granule targeting of proANP, cultured AtT-20 cells were transiently transfected with expression vectors for either native or mutated rat proANP. ProANP and/or ANP secretion was measured by RIA 48 hr after transfection in two successive 3-hr incubations, first in the absence then in the presence of forskolin, a secretagogue that directly stimulates adenylate cyclase and causes the cyclic AMP-induced release of proteins stored in secretory granules. As an internal control for the function of the regulated secretory pathway, immunoreactive β-endorphin was also measured by RIA in the same culture supernatants.

Incubation of native proANP expressing-AtT-20 cells with forskolin resulted in a 2-fold stimulation of ANP secretion (Fig. 2 and Table 1, native), while secretion of the endogenous β-endorphin was stimulated ≈1.5-fold by the same treatment (Table 1). Analysis of the immunoreactive ANP by HPLC showed that the unstimulated cells released ≈40–60% of the unprocessed form (proANP) of the peptide (data not shown), although we did not test the correlation between processing and regulated secretion in these conditions. This experiment shows that proANP is released in presence of a secretagogue, indicating that it is targeted to the regulated secretory pathway in AtT-20 cells. These results are consistent with those obtained by Shields *et al.* (6). However, it cannot be excluded that a portion of proANP can be secreted through a constitutive pathway. To quantitate the efficiency of proANP targeting to the regulated secretory pathway, we calculated a "secretion index" corresponding to the ratio of fold stimulation of proANP secretion over that of the endogenous β-endorphin. As shown on Table 1, the secretion index for native proANP is close to 1 (1.18 ± 0.23), indicating that proANP and β-endorphin are targeted to the regulated secretory pathway with roughly equal efficiency.

Previous *in vitro* studies using synthetic peptides have implicated the region between amino acids 11 and 31 of the rat ANP profragment in calcium binding (20). To test for a role of this region in the regulated secretion of proANP, AtT-20 cells were transfected with an expression vector in which this region had been selectively deleted (ΔproANP). The results (Fig. 2 and Table 1) show that while ΔproANP was secreted at ≈70% of the level of the native peptide (indicating that the targeted deletion did not have a major effect on the biosynthesis of the protein), its expression was not stimulated by forskolin, while that of the endogenous immunoreactive β-endorphin was stimulated by ≈2-fold, resulting in a dramatically reduced secretion index (0.5 ± 0.12) for the ΔproANP protein. These results support the conclusion that sequences between amino acids 11 and 31 of the rat ANP profragment are implicated in the targeting of proANP to the regulated secretory pathway.

To further delineate the residues responsible for the secretory granule targeting of proANP and to test whether these correspond to acidic amino acids that could bind calcium, site-directed mutations (Glu → Gln and Asp → Asn) were introduced in the ANP profragment (Fig. 1). Analysis of AtT-20 cells transfected with the corresponding expression vectors demonstrated that neutralization of the acidic pair $Glu^{23}Glu^{24}$ abolished regulated secretion (Fig. 2 and Table 1, $Gln^{23,24}$), while neutralization of the acidic triplet $Glu^{29}Asp^{30}Glu^{31}$ had no such effect (Fig. 2 and Table 1, $Gln^{29,31}Asn^{30}$). Neither mutation had a major effect on either the level of biosynthesis of the proANP peptide or on the regulated secretion of the endogenous β-endorphin. As compared with native proANP, the secretion index was significantly decreased for $Gln^{23,24}$ (0.56 ± 0.06), while it was not significantly changed for $Gln^{29,31}Asn^{30}$ (0.8 ± 0.15). Taken together, these results indicate that the pair of glutamic acid residues at positions 23 and 24 of the ANP profragment is necessary for the regulated secretion of proANP by AtT-20 cells.

Calcium-Mediated Aggregation of Recombinant Native and Mutated proANP Peptides. To test whether the observed effect of mutations on regulated secretion of proANP correlated with a preferential ability of the corresponding protein to aggregate *in vitro*, native and mutated proANP proteins were purified from transfected cell supernatants and were incubated at a protein concentration of 1 mg/ml in the presence of 10 mM calcium at pH 6.5, conditions which were found to promote optimal aggregation of proANP. ProANP was quantitated in

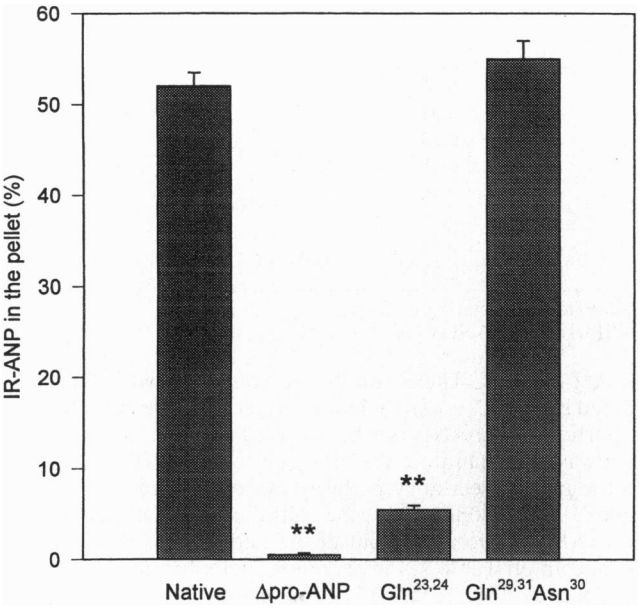

FIG. 3. Effect of mutations on native proANP aggregation in vitro. Native proANP or mutants in the profragment region (ΔproANP, $Gln^{23,24}$, and $Gln^{29,31}Asn^{30}$) were incubated at the concentration of 1 mg/ml in the presence of 10 mM calcium at pH 6.5. After centrifugation, ANP in the pellet and the supernatant was measured by RIA. Results were expressed as the percent of ANP in the pellet and were the mean ± SEM of duplicate determinations from two independent experiments. **, $P < 0.001$ (two-way ANOVA).

the pellets and supernatants following high-speed centrifugation. Under these conditions, ≈50% of the native proANP was found in the pellet after centrifugation (Fig. 3), confirming its reported tendency to aggregate under these conditions (20). While the $Gln^{29,31}Asn^{30}$proANP precipitated to a similar extent, precipitation of both the ΔproANP and the $Gln^{23,24}$ proANP was severely reduced (Fig. 3). These results demonstrate that the glutamic acid residues at positions 23 and 24 of the ANP prosegment are necessary for the calcium-mediated aggregation of proANP *in vitro*.

DISCUSSION

Results from the present study show that whereas rat proANP has the capacity to aggregate *in vitro* and is targeted to the regulated secretory pathway in transfected AtT-20 cells, the two glutamic acid residues at position 23 and 24 in the profragment are required for both *in vitro* aggregation and regulated secretion. These results extend our previous data (20) indicating that the highly acidic portion (amino acids 11–30) of the proANP profragment is capable of interacting with calcium and demonstrate a direct correlation between calcium-mediated aggregation and protein sorting. Comparison of the protein sequences of the human, canine, bovine, rabbit, rat, and mouse ANP profragments shows absolute conservation of an acidic pair (either Glu-Glu or Glu-Asp) at positions 23 and 24, lending further support for their importance in ANP biosynthesis.

The transient nature of proANP aggregation during its transit through the secretory pathway and the apparently high concentrations of calcium present in the TGN (25) are conditions that would favor a low-affinity interaction of calcium with proANP. Indeed, proANP binds calcium with an affinity of ≈8 mM (20). While our results implicate the glutamic acid residues at positions 23 and 24 in proANP aggregation and regulated secretion, they are most probably not the only residues involved in calcium binding, as the $Gln^{23,24}$ mutant still binds calcium *in vitro* (data not shown). These results could suggest that other residues in addition to those at positions 23 and 24 are involved in a calcium-mediated conformational change, which leads to aggregation. Alternatively, calcium binding to proANP may serve other functions unrelated to aggregation, such as the inhibition of the prosegment cleaving protease until release of the secretory granule.

Condensation of proteins in the TGN was first described by Palade (26) and led to the suggestion that protein condensation might initiate secretory granule biogenesis. The finding that pH decreases and calcium concentration increases in the transition from the Golgi to immature granules further led to the hypothesis that secretory proteins destined for the regulated secretory pathway and which have the capacity to form aggregates are selectively retained in the immature granules, whereas soluble material is secreted by the constitutive secretory pathway (12). The aggregation-sorting model is supported by studies on a number of proteins (13, 18), including the chromogranin family, the major constituents of secretory vesicles in endocrine cells (27). These proteins have a high proportion of acidic residues and have been shown to form homo- or heteroaggregates at a low-pH and/or high-calcium environment. As such, one of their proposed biological roles is to facilitate the sorting of proteins to the regulated secretory pathway (27). Although chromogranins are colocalized with proANP in the secretory granules of atrial cardiocytes (28), dense core secretory granules are absent from the atrial cells of mice in which the gene coding for proANP has been deleted by homologous recombination (29). Thus, while chromogranins may interact with proANP to synergize its aggregation and secretory granule targeting in atrial cardiomyocytes, they are clearly unable to direct the biogenesis of secretory granules in this cell type in the absence of proANP.

A comparative analysis of the amino acid sequences and secondary structures of 15 prohormones and propeptides that are sorted to AtT-20 cell secretory granules has led to the proposal of a consensus motif, which consists of an amphipathic α-helix with a serine or a threonine located on the same side of the helix as two leucines (30). More recently, Gorr and Darling (31) have analyzed the predicted secondary structure of proteins for which the N-terminal region of their profragment had been shown to be involved in sorting to the regulated secretory pathway. They identified an N-terminal hydrophobic peak, which contains several charged amino acids and overlaps with a predicted α-helix. The N-terminal hydrophobic peak was located between amino acids 9 (±4) and 26 (±6) of the different profragments and 60% of the amino acids were either serine, threonine, glutamic acid, aspartic acid, leucine, or isoleucine. Both Chou–Fasman and Garnier–Osguthorpe–Robson analysis predict the presence of an α-helix in rat proANP profragment corresponding to residues 11–34 (data not shown). Nevertheless, this region is neither amphipathic nor does it contain an apparent N-terminal hydrophobic peak.

Because not all calcium binding sites are implicated in protein aggregation and because little is known about the actual protein structures required for calcium-mediated targeting to the regulated secretory pathway, it is not yet possible to determine its contribution to the regulated secretion of other proteins by sequence comparison alone. In addition, the possible contribution of other targeting mechanisms (such as protein–protein contacts or others) make it essential to extend more refined structure–function studies to additional secretory proteins to better define the molecular mechanisms involved in intracellular targeting to dense core secretory granules and regulated secretion.

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