Histol Histopathol (1997) 12: 1179-1188

Histology and Histopathology

Invited Review

Prohormone and proneuropeptide synthesis and secretion

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Summary. Hormones and neuropeptides in eukaryotic cells, are synthesised as large precursor molecules in the rough endoplasmic reticulum (RER), from where they are translocated to the Golgi apparatus. The sorting of proteins destined for the regulated secretory pathway from those which will be released constitutively takes place in the trans-Golgi network (TGN). In both these pathways, vesicles need to be transported to the plasma membrane before their contents can be released by exocytosis.

Hormones and neuropeptides need to be secreted from the cells in which are synthesised to exert their biological actions, although they can also play paracrine and autocrine actions. Prohormones and proneuropeptides must undergo post-translational modifications which occur in determined subcellular compartments within eukaryotic cells and are carried out in a strict succession of intracellular events, which give rise to biologically active products.

The biosynthesis of prohormones/proneuropeptides is mediated by the action of endoproteolytic enzymes and other post-translational modifying enzymes within the secretory pathway. The major focus of this review will be the biosynthetic pathway, sorting and intracellular trafficking of prohormone and proneuropeptide precursors within the secretory pathway of eukaryotic cells.

Key words: Prohormone, Proneuropeptide, Sorting, Trafficking, Secretory pathway, Post-translational processing

Introduction

Eukaryotic cells possess an efficient biochemical machinery for protein synthesis and secretion. Membrane surrounded compartments within these cells, allow a fine regulation of metabolic reactions such as sorting mechanisms which separate proteins destined for the regulated or constitutive secretory pathways from proteins destined to lysosomes, mitochondria or plasma membrane. How are proteins targeted to specific organelles or compartments within eukaryotic cells? This can sometimes be explained because particular amino acid sequences (sorting domains) within proteins can convey the capacity to recognise and interact with a specific transporter/carrier protein which can in turn target the protein in question to its cellular destination. However, in most cases, these transporter/carrier systems or defined sorting domains have not been identified, therefore, other intracellular mechanisms must be postulated.

Neuronal and endocrine tissues are constituted by highly specialised secretory cells, which synthesise and release hormones and neuropeptides in a regulated fashion. Thus, the correct trafficking and sorting of hormones/neuropeptides within the secretory pathway of these cells have profound physiological implications, not only for single cell physiology, but also for the integration of crucial physiological responses within higher organisms. Functions such as reproduction, response to stress, food intake, immune response, onset of labour are all mediated by these peptide messengers.

We will review published evidence which relates to biosynthesis, intracellular sorting and trafficking of prohormones/proneuropeptides within the regulated and constitutive secretory pathways of neuronal and endocrine cells. We will also discuss evidence from work done in our laboratory and work done by other groups which suggests, contrary to what was believed until recently, that biosynthesis, trafficking and sorting events affecting prohormones/proneuropeptides are not only dependent on their particular amino acid sequences, but also determined by cell-type specific factors which at present are unknown.

1. Prohormone/proneuropeptide processing: its role in hormone stability, biological activity and sorting mechanisms

It has been previously demonstrated that hormones

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and neuropeptides are synthesised as precursor proteins, e.g., prohormones and proneuropeptides. Until recently, it was believed that higher molecular weight protein precursors were not biologically active. Work done in our laboratory has shown that the full length precursor for corticotrophin-releasing hormone (CRH) is biologically active, i.e., it elicits the release of adrenocorticotrophin (ACTH) from primary cultures of rat anterior pituitary cells (Morrison et al., 1995) and it induces cell proliferation of AtT20 cells, a corticotrophic tumour cell line (Castro et al., 1995b). Biologically active peptides are released from their precursor proteins by enzymatic endoproteolytic cleavages. Most prohormones/proneuropeptides contain within their amino acid sequence, peptides with different biological activities. Proopiomelanocortin (POMC) is endoproteolytically processed to yield ACTH, ß-endorphin and α-melanocyte stimulating hormone (Nakanishi et al., 1979). Proenkephalin contains multiple copies of the same peptide, i.e., six copies of Met-enkephalin and one copy of Leu-enkephalin (Comb et al., 1982). The precursor of FMRF-amide synthesised by the neurons of the mollusc, *Aplysia Californica* contains this tetrapeptide repeated 28 times within its amino acid sequence and a single copy of the related peptide FLRF-amide (Taussing and Scheller, 1986).

Prohormones and proneuropeptides undergo several modifications before they are released. These modifications can be co-translational (i.e., removal of the signal peptide) or post-translational (after they are synthesised within the rough endoplasmic reticulum (RER)). Post-translational modifications are divided into two main categories: 1) proteolytic modifications which involve endoproteases, carboxypeptidases and aminopeptidases, and 2) chemical modifications done by enzymes which covalently modify the lateral chain of amino acids within peptides as: glycosylation, amidation, acetylation, phosphorylation and sulfation. Many of these enzymes have been biochemically characterised, localised to specific tissues or cellular compartments, and in most cases they have been cloned (Seidah et al., 1990; Smeekens and Steiner 1990; Eipper et al., 1992).

Endoproteolytic cleavages are one of the key steps in the generation of many hormones/neuropeptides from their protein precursor molecules. After initial cleavage of the signal peptide in the lumen of RER (Walter et al., 1984) further proteolytic events of most prohormones/proneuropeptides start within late compartments of the Golgi complex i.e., the trans Golgi network (TGN) (Sossin et al., 1990) and continue in the secretory vesicles (Castro et al., 1989). Neuronal and endocrine cells possess cell-type specific endoproteases which cleave hormone/neuropeptide precursor proteins to generate biologically active peptides. The sites of cleavage are paired basic amino acid residues, such as lysine-arginine (KR), RR, RK or KK, although endoproteolysis at tri- and tetra-basic amino acid residues has also been reported (for review, see Smeekens 1993). Cleavage at single basic amino acid residues may also occur, e.g., the endoproteolytic cleavage of rat prodynorphin by the prohormone convertase 1 (PC1) to yield an 8 kD prodynorphin derived-product and the C-peptide (Dupuy et al., 1994). Although, the presence of basic amino acids is essential to confer an aqueous environment at the site of cleavage, the secondary structure of the surrounding amino acids also seems to play an important role (Rholam et al., 1986). To date, several endoproteases involved in the endoproteolytic processing of prohormones/proneuropeptides have been identified and cloned, and are known as precursor converting enzymes (PCEs) (for review see Smeekens, 1993). These belong to the family of subtilisin/Kex2-serine proteases and exhibit similar amino acid sequences at different domains such as furin/PACE, PC1, PC2, PACE4, PC4, PC5/PC6 and PC7. However, differences have been attributed with respect proprotein specificity as well as tissue localisation for these enzymes (Seidah et al., 1994). An aspartyl endopeptidase has been purified from bovine intermediate lobe secretory vesicles, which possesses endoproteolytic activity on POMC *in vitro* (Loh et al., 1985); this enzyme cleaves mouse and toad POMC *in vitro* generating POMC-derived peptides found in the intermediate lobe of the pituitary gland, *in vivo* (Castro et al., 1989).

If cells need only one PCE or a particular combination of them to cleave a specific prohormone/proneuropeptide still needs to be elucidated; altough the wide distribution of these enzymes in tissues of neuronal/endocrine origin suggests that they might be able to endoproteolytically process a wide number of propeptides. The cellular machinery for endoproteolytic processing and secretion has been conserved during evolution, even in cells so evolutionary distant as yeast and neurons (Bennett and Scheller, 1993). This has been demonstrated by Jung et al., 1993, who showed that the endoproteolytic products of the egg-laying hormone (ELH) which is a prohormone synthesised within the bag cell neurons of the marine mollusc, *Aplysia californica*, are similar when this prohormone is expressed in AtT20 cells. The expression of heterologous proproteins within cells which do not possess a regulated secretory pathway and therefore do not synthesise the endoproteolytic enzymes found in neuroendocrine cells such as PC1 or PC2, does not always yield endoproteolytically processed products from the precursors, e.g., rat proenkephalin (Lindberg et al., 1991), mouse prorenin (Hosaka et al., 1991) and rat procorticotrophin-releasing hormone (proCRH) (Castro et al., 1995b) in spite of these cells expressing furin-like endopeptidases and the precursors containing furin-like cleavage sites. Furin (also called PACE), PC1 and PC2 have been widely employed in prohormone/proneuropeptide processing studies (Hosaka et al., 1991; Zhou et al., 1993; Paquet et al., 1996). Transfection experiments within cells expressing the cDNAs encoding for both a prohormone or proneuropeptide and PC1 or PC2 have shown

differences in the processing pattern of such precursors. For example, the expression of exogenous PC2 cDNA within AtT20 cells enhances the synthesis of POMC-derived peptides, such as $ACTH_{1-13}NH2$ and CLIP, which are poorly synthesised in wild type AtT20 cells expressing low levels of endogenous PC2. Furthermore, production $POMC_{1-49}$, $Lys_{0-\gamma 3}$-MSH and ß-endorphin$_{1-27}$ were only observed in AtT20 cells expressing PC2 (Zhou et al., 1993). Also, human proenkephalin shows different endoproteolytic processing when it is co-expressed with either PC1, PC2 or furin within GH4C1 cells (Breslin et al., 1993). The corticotrophin releasing hormone (CRH) precursor protein is also processed by PC2 within stably transfected CHO cells co-expressing PC2 and pre-proCRH (Perone et al., 1996). It has also been observed that PC2 is capable of endoproteolytically processing proneuropeptide Y within primary cultures of rat superior cervical ganglion neurons (Paquet et al., 1996). It has been shown that endogenous factors *in vivo*, which may be cell- and species-specific, might affect the cleavage specificity of these endopeptidases; these factors can either increase or inhibit processing of prohormones or proneuropeptides. It has been demonstrated that the intact form of the neuroendocrine-specific polypeptide 7B2 is a potent inhibitor of PC2 but not PC1 (Martens et al., 1994). On the other hand, processed 7B2 stimulates POMC cleavage *in vitro*, probably activating PC2 (Braks and Martens, 1995). Variations on intracellular factors might modulate endoproteolysis, like pH and Ca^{2+} concentration. The autocatalytic activation of proPC1 and proPC2 requires defined pH and Ca^{2+} concentrations within the TGN (Shennan et al., 1995; Guest et al., 1997).

Deletion of a particular processing site within a prohormone by site direct mutagenesis may affect cleavage at other sites and also the fate of their prohormone derived-peptides as was reported for ELH prohormone expressed in AtT20 cells. Deletion of two endoproteolytic sites within ELH prohormone causes the H_2N-terminal products to be sorted to the regulated secretory pathway in contrast with the wild type prohormone, where the H_2N-terminal peptide products are released via the constitutive route (Jung et al., 1993). This suggests that a strict succession of endoproteolytic events are necessary to reach the complete and correct maturation and targeting of prohomones and proneuro-peptides. This has also been demonstrated for POMC, whereby a concerted action of PC1 and PC2 is needed in order to generate the correct peptide products (Zhou et al., 1993).

Carboxypeptidases specifically remove basic amino acids (R or K) from the C-terminus of peptides. Carboxypeptidase E (CPE), also known as CPH and enkephalin convertase is thought to be the unique intracellular carboxypeptidase capable of processing neuropeptides (Fricker, 1991). Recently, a CPE-like enzyme called CPD has been purified and characterised from bovine pituitary (Song and Fricker, 1995a). Based on their localisation within secretory granules, the acidic pH and high levels of Ca^{2+} requirements, both enzymes have been suggested as putative physiological carboxypeptidases involved in hormones/neuropeptides processing (Song and Fricker, 1995b).

Aminopeptidases catalyse the hydrolysis of amino acid residues from the H_2N-terminus of peptides. It is well known that aminopeptidases take part in degradation of neuropeptides (Turner, 1987). They can also trim remaining H_2N-terminal basic amino acid residues of peptides generated after endoproteolytic cleavage of prohormones. The role of aminopeptidases in hormone/neuropeptide biosynthesis is crucial, since they modifies the biological activity of neuropeptides/ hormones.

Several chemical modifications of peptides/hormones have been described, but only in a few cases the physiological significance of them has been elucidated. Acetylation at the H_2N-terminus and its significance with respect to biological activity has been studied for a number of neuropeptides/hormones. Two peptides derived from POMC undergo N-terminal acetylation; i.e., ß-endorphin and α-MSH. The presence of the acetyl group conveys changes in the biological activities of these peptides. Acetylated forms of ß-endorphin show a drastic decrease in their binding capacity to the opiod receptors μ and δ, and therefore, they are inactive in their central analgesic effects (Akil et al., 1985). In contrast, acetylated α-MSH increases its stimulating activity on melanocytes (Guttman and Boissonnas, 1961) when compared to non acetylated α-MSH. Interestingly, H_2N-terminal acetylated forms of POMC peptide products such as monoacetylated and di-acetylated MSH and acetyl-ß-endorphin are found in the mammalian intermediate pituitary lobe but not in the anterior pituitary (Glembotski, 1982). These findings reflect tissue-specific differences in the content and probably in the activity of the enzyme/s responsible for the acetylation of prohormones/proneuropeptides peptide products. The acetylation process seems to occur during late stages of the secretory pathway, i.e., within secretory vesicles; this has been described for bovine intermediate pituitary cells (Glembotski 1982).

It is common to find amidated neuropeptides and hormones at their carboxy-terminal amino acid; often the amide group confers full biological activity. Amidation occurs if glycine is present at the carboxy terminal end of peptides to be amidated (Bradbury et al., 1982). Generation of amidated peptides needs the concerted action of two enzymes: the peptidylglycine α-hydroxylating monooxygenase (PHM) and the peptidyl-hydroxyglicine α-amidating lyase (PAL), both generated after endoproteolytic cleavage from the same precursor molecule (Perkins et al., 1990). Amidating activity takes place principally within secretory granules; although recent work has demonstrated that it might also take place in the ER (Yun and Eipper, 1995).

Glycosylation is another common chemical modification found in prohormones/proneuropeptides. The physiological role of carbohydrate side-chains attached

to prohormones/proneuropeptides remains uncertain and the existing data, sometimes reflects conflicting results. This could be due to the use of high levels of tunicamycin, the most commonly used inhibitor for studying protein glycosylation *in vitro*, which may also affect protein biosynthesis. There is evidence that glycosylation of precursor proteins might affect endoproteolysis. The endoproteolytic processing of the H_2N-terminal portion of POMC ($POMC_{1-74}$) provides an example for this. In the anterior pituitary, $POMC_{1-74}$ is glycosylated at both Thr45 and Asn65; on the other hand in the intermediate pituitary, $POMC_{1-74}$ is only glycosylated at Asn65. It has been shown that $POMC_{1-74}$ is not endoproteolytically processed in the anterior pituitary; whilst in the intermediate pituitary, $POMC_{1-74}$ is cleaved to generate γ_3-MSH and $POMC_{1-48}$, the latter without any attached glycosylated chain at Thr45 (Birch et al., 1991). These findings suggest that the carbohydrate-chains cause a steric hindrance for the endoproteolytic activity responsible for the cleavage of $POMC_{1-74}$ to yield $POMC_{1-48}$ and γ_3-MSH. *In vitro* experiments using purified bovine $POMC_{1-77}$ and prohormone endoproteases support this hypothesis (Birch et al., 1991). A similar inhibitory role was attributed to glycosylation in the processing of prorenin; it was found that N-linked glycosylation may reduce proteolysis of mouse prorenin in transfected AtT20 cells (Ladenheim et al., 1991). An alternative approach was used to assess the influence of glycosylation on endoproteolysis of POMC, by replacing Thr45 by Ala45 using site directed mutagenesis (Noel et al., 1991). This experiments showed that glycosylation does not play a critical role in $POMC_{1-74}$ cleavage. However, the basic amino acid residues are important in determining the extent of endoproteolysis. Additional structural features may also play an important role in POMC cleavages. The high degree of conservation in the glycosylation pattern of POMC amongst several species (Uhler and Herbert, 1983), suggests that the oligosaccharide moieties could play essential intracellular functions such as: allow proper folding of proproteins and/or to provide adequate "sorting signals" for targeting to appropriate intracellular compartments. In that respect, some nonglycosylated proteins seem to aggregate irreversibly in association with BiP and are retained in the ER (Hurtley et al., 1989). On the other hand, glycosylated-proteins could have crucial extracellular roles by modifying the interaction with their receptor or altering the half life of the glycosylated peptide. Further research in eukaryotic cell lines which are deficient in the enzymes responsible for oligosaccharide assembly or trimming will provide new insights into the physiological role of glycosylation in endoproteolytic processing, sorting and biological activity of pro-hormones/proneuropeptides.

Prohormones/proneuropeptides undergo further modifications like phosphorylation and sulfation. Phosphorylated peptides derived from POMC (Eipper and Mains, 1982) and proenkephalin (Watkinson et al., 1989) have been identified. The physiological role of phosphorylation remains obscure. Moreover, one can not assign any possible organelle sorting role to phosphorylation due to the ubiquitous intracellular distribution of kinase activities, i.e., within Golgi complex, ER and secretory granules. It has been shown that the intracellular site where sulfation takes place is principally within the Golgi compartment. Carbohydrate residues of glycoproteins, proteoglycans and proteins become sulphated at this stage within the secretory pathway (Lee and Huttner, 1985). It is also known that most sulfated proteins at Tyr residues are secreted which might suggest some function of this chemical modification in sorting events (Tooze and Huttner, 1990).

2. Hormone/neuropeptide secretion: regulated and constitutive secretory pathways

Sorting and secretion of hormones and neuropeptides is a carefully controlled mechanism within endocrine/neuronal cells, since hormones and neuropeptides need to be exported from the cells in which they are synthesised in order to reach their target tissues and exert their biological effects.

Two main routes have been proposed for protein secretion from endocrine and neuronal cells, i.e., constitutive and regulated secretion (Gumbiner and Kelly, 1982). One of the main differences between these two pathways is that proteins which exit the cell using the constitutive route are secreted inmediately after they have been synthesised and exocytosis takes place at a constant rate without any external stimulus; although, constitutive secretion is also regulated at multiple intracellular levels (reviewed by Mostov, 1995). Constitutive secretion may be blocked by inhibiting protein synthesis (Schmidt and Moore, 1994). On the other hand, within the regulated secretory pathway, secreted proteins can be stored at high concentration for long periods of time within secretory vesicles, before they are released. In cells which possess a regulated secretory pathway, the exocytosis step, even in the abscence of protein synthesis, is triggered by an external stimulus, which can in turn change cytoplasmic second messenger levels. Immunohistochemistry at the light microscopy level, makes it possible to distinguish if a protein is located within the regulated or constitutive secretory pathway, due to its prescence within secretory vesicles which are visualized as a fine immunoreactive punctuate pattern (Fig. 1). In contrast, cells which do not have a regulated secretory pathway, do not show this punctate pattern, instead they show a reticular distribution of the immunoreactivity, which is typical of the localisation of the prohormone within the RER (cells with constitutive secretory pathway: fibroblasts, Fig. 2) (Castro et al., 1995b). At the electron microscopic level the secretory granules appear with an electron-dense core which might correlate with the high concentration of the secretory product within these vesicles (Orci et al.,

1987).

Nascent secretory proteins, lysosomal proteins and constitutive membrane proteins share the first steps in their vectorial transport across the intracellular membranous compartments within eukaryotic cells. They must first be targeted to the RER and then, translocated towards the lumen of the RER by means of complex interactions amongst the ribosome-signal sequence, the signal recognition particle (SRP) and SRP receptor. Translocation usually takes place cotranslationally. The targeting of the nascent protein to the ER is regulated by three GTPases, which work as adapters between the cytosol and the protein translocation apparatus in the ER membrane (Bacher et al., 1996). Signal sequences of prohormones and proneuropeptides are usually not more than 30 amino acids in length with strong hydrophobic characteristcs. The signal sequence is cleaved off by a resident endopeptidase as the protein is being synthesised. Although, most of the secretory proteins known so far possess signal peptides, the fact that some proteins lacking a conventional signal sequence such us IL-1α, IL-1ß, bFGF and blood coagulation factor XIIIa are released extracellulary,

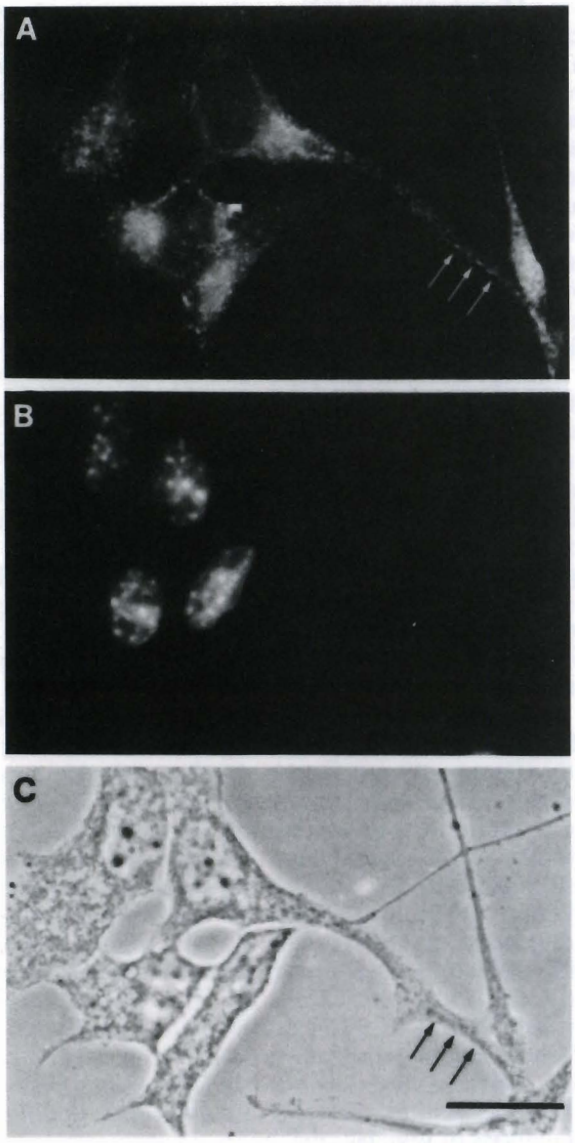

Fig. 1. Immunofluorescence detection of immunoreactive (IR)-ACTH within the neuroendocrine cell line AtT20. **Panel A,** shows IR-ACTH within the cytoplasm and processes (arrows) of AtT20 cells. Note the punctuate distribution of IR-ACTH within these cells. **Panels B and C**, show the nuclei stained with DAPI and the phase contrast micrograph respectively of the cells shown in panel A. Bar: 30 µm.

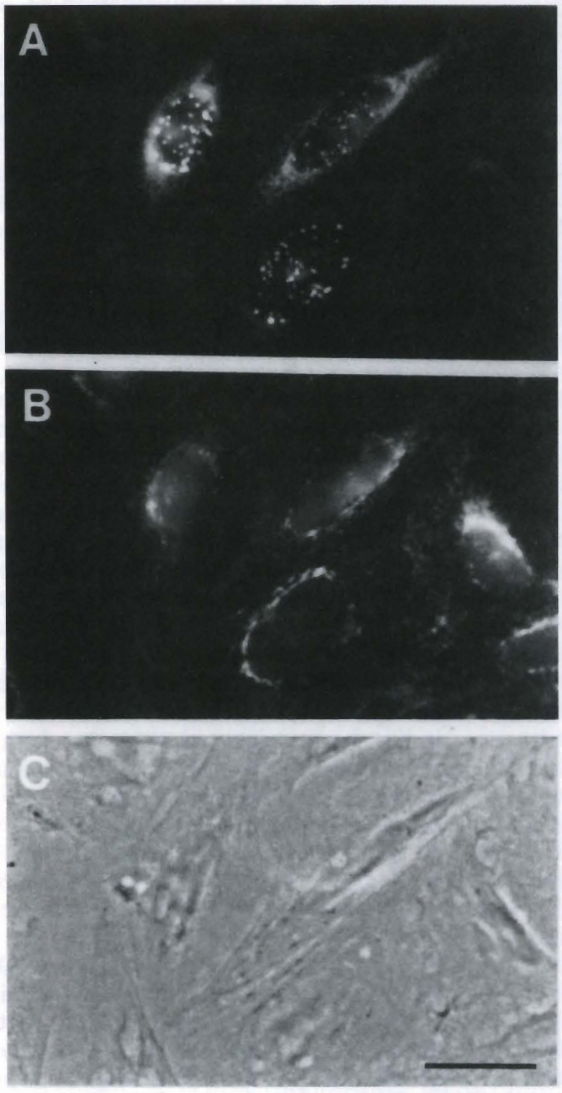

Fig. 2. Immunocytochemical staining of CHO-K1 cells expressing rat pre-procorticotrophin releasing hormone (CRH). **Panel A**, shows immunoreactive (IR)-CRH within the nuclei and the cytoplasm of stably transfected CHO-K1 cells expressing rat pre-proCRH. **Panel B**, shows the yuxtanuclear staining of the Golgi apparatus using wheat germ agglutinin. Comparison between IR-CRH shown in panel A and the Golgi staining shown in panel B, accounts for the localisation of IR-CRH within the Golgi apparatus in transfected CHO cells. **Panel C. P**hase contrast micrograph of the cells shown in panel A. Bar 30 µm.

suggests that an alternative pathway for protein secretion might exist (Rubartelli et al., 1990). It has been described that these proteins can exert extracellular functions. The possibility that they could be released by cell lysis must be excluded, since for their biological activity they require post-translational modifications only performed by living cells. Cleves et al. (1996) have shown an alternative pathway for protein secretion in yeast. This novel secretory mechanism is independent of a signal peptide and does not involve the yeast multidrug resistance-like transporter Ste 6p. The isolation of the genes involved in this alternative secretory mechanism, may provide the tools for the identification of their mammalian counterparts.

The trans-most cisternae of the Golgi complex, a clathrin-coated compartment, is the site where sorting between secretory and constitutive proteins takes place (Orci et al., 1987; Tooze and Huttner, 1990). The presence of distinct secretory pathways within the same cell requires a sorting mechanism to transport secretory proteins to the correct pathway from the ER to Golgi apparatus and finally to secretory granules destined for regulated secretion. Neuronal and endocrine cells, as highly specialized secretory cells, which must separate hormones and neuropeptides from other proteins such as membrane proteins or lysosomal hydrolases. To date, the mechanism implicated in sorting of proteins destined to the regulated secretory pathway is poorly understood. It has been proposed that sorting domains of most regulated secretory proteins reside in their H_2N-terminal region, immediately after the signal sequence responsible for ER translocation. Comparison of amino acid sequences within this region from different secretory proteins does not show a consensus sequence; however, their hydrophobic characteristics and secondary structures appear to be the key elements for sorting, and these are absent in constitutively secreted proteins (Gorr and Darling, 1995). Consistent with this hypothesis, it has been reported that the domain responsible for the targeting of POMC to the regulated secretory pathway resides within its H_2N-terminal region which contains an amphipatic loop of 13 amino acids. Moreover, this conformational signal is dependent on the integrity of the disulfide bridge between amino acids Cys8/Cys20 (Cool et al., 1995). Within pro-somatostatin, the information for targeting to the regulated secretory pathway in transfected mammalian endocrine cell lines is located at the amino-terminal region (Sevarino et al., 1989). Sorting domains within precursor molecules could also be recognised by a membrane-anchored receptor/carrier at the trans-most cisternae of the Golgi complex and this binding could mediate sorting to the regulated secretory pathway. In that respect, a group of 25 kD proteins called «sortases» that bind selectively prolactin, insulin and human growth hormone but did not bind proteins which do not enter the regulated secretory pathway, were thought to be the sorting receptor (Chung et al., 1989). Work done by Gorr and co-workers has demonstrated that the previously characterised sortases were chymotrypsinogen A or B (Gorr et al., 1992). Recently, it has been shown that sorting of proopiomelanocortin (POMC) to the regulated secretory pathway occurs by binding to a sorting receptor, which has been identified as a membrane-associated carboxypeptidase E (also known as carboxypeptidase H) (Cool et al., 1997). These authors have also demonstrated that this receptor specifically binds proteins which are secreted via the regulated secretory pathway.

The term protein "maturation" within secretory granules is employed to described a complex process whereby the clathrin-coat of the secretory granules is lost. Maturation implies, moreover, progressive acidification, prohormone post-translational modifications, changes in calcium concentration, correct protein folding and aggregation within the vectorial route from RER towards the secretory granules. It has been demonstrated that protein aggregation might be a mechanism capable of sorting proteins to the regulated pathway from resident and constitutively secreted proteins (Tooze et al., 1989b); moreover, quality control mechanisms seem to recognise and retain in the early compartments of the secretory pathway, misfolded and partially folded proteins in order to prevent further transport (Hammond and Helenius, 1994).

Endoproteolytic processing of prohormones might affect sorting and intracellular fate of their cleavage-derived peptides as was demonstrated in the case of ELH prohormone in the bag cell neurons of *Aplysia*. The amino- and the carboxy-terminal derived peptides from ELH prohormone were targeted to and packaged into different dense core secretory vesicle within the regulated secretory pathway (Sossin et al., 1990). This results in different subsets of secretory vesicles containing different peptide products derived from the same precursor which exert different biological activities.

Prolactin and growth hormone are both packaged into different secretory vesicles within somatomammotrophic cells (Fumagalli and Zanini, 1985). Different sorting domains within both hormones might be recognised by the protein sorting machinery of eukaryotic cells, whereby these peptides are packaged within different vesicle populations. The corticotrophic neuroendocrine cell line AtT20, is also able to differentially release endogenous ACTH and heterologous immunoreactive-CRH molecules after an extracellular stimulus (Perone and Castro, unpublished results). This finding suggests that ACTH and IR-CRH molecules are packaged into different sets of secretory vesicles within AtT20 cells. Also, different intracellular mechanisms involving second messengers could mediate the release of different populations of vesicles containing secretory peptides.

The Golgi apparatus in neuroendocrine cells and neurons possesses a polarised juxtanuclear localisation (Lowenstein et al., 1994). Therefore, it is posible to assume that the direction of hormone secretion is governed by the Golgi apparatus in these cells. Secretory

vesicles containing hormones might travel towards the cell surface associated and perhaps, guided by microtubules and or microfilaments. This assumption is sustained by experiments employing drugs which disrupt the microtubule-microfilament network of cells in culture. To date, the role of cytoskeleton in the secretion of prohormones/proneuropeptides is unclear. However, our studies in AtT20 cells expressing proCRH suggest that neither disruption of microtubules nor microfilaments affects the constitutive release of CRH_{1-41}. Furthermore, we observed that actin filaments play a role in mediating the release of CRH_{1-41} trough the regulated secretory pathway in transfected AtT20 cells (Perone and Castro, unpublished results). To date, nothing is known about the numerous associated microtubule-microfilament proteins which perhaps are the true motors in vesicular trafficking within eukarytic secretory cells (i.e., endocrine cells, neurons).

3. Cell-type specific targeting of prohormones/ proneuropeptides

From the previous section we can conclude that targeting and sorting domains sequences lack primary amino acid sequence homology. Therefore, other factors could determine protein sorting, such as tertiary structure and general physicochemical properties (i.e., hydrophobicity and hydrophilicity).

Work done in our laboratory and in others has recently shown that cellular factors must also play an important role in determining the intracellular targeting and final localisation of a given proprotein. Using pre-proCRH as a model proneuropeptide molecule, we demonstrated that the intact precursor is translocated to the cytoplasm (i.e., secretory pathway), and also to the nucleus within stably transfected CHO-K1 cells (Castro and Morrison, 1995; Castro et al., 1995a; Morrison et al., 1995) (Fig. 2). Nuclear proCRH is in close association with double stranded DNA, suggesting that it might play a role in the regulation of gene expression (Castro et al., 1995b). The intact pre-proCRH molecule expressed within COS-7 cells, in spite of being correctly targeted to the secretory pathway was not secreted into the extracellular medium. The lack of secretion is due to the formation of large precursor aggregates which did not exit the RER of these cells (Morrison et al., 1992; Castro and Morrison, 1997). When pre-proCRH was expressed within stably AtT20 cells, which possess a regulated secretory pathway and are capable of differentiating *in vitro* to achieve a neuronal phenotype (Tooze et al., 1989a), pre-proCRH was targeted to the regulated secretory pathway within these cells; the release of proCRH cleavage products, i.e. $proCRH_{125-151}$ and CRH_{1-41} (Fig. 3) was stimulated in response to extracellular stimulus indicating targeting to secretory vesicles (Castro et al., 1991). This was confirmed using immunofluorescence techniques and antibodies specific for CRH_{1-41}. Immunoreactive (IR)-CRH was seen as a bright punctuate pattern which accumulates at the tips of the cellular processess awaiting to be released upon stimulation (Fig. 1) (Castro et al., 1992; Castro and Morrison, 1997).

Böttger and Spruce (1995) working with another neuropeptide precursor, i.e., proenkephalin have demonstrated nuclear localisation of this precursor in rodent and human embryonic fibroblast cell lines (Swiss 3T3 and MRC-5 cells) and in rodent myoblast cells (C2C12 cells). These authors showed that when proenkephalin was expressed in COS cells, it was localised exclusively in the cytoplasm, while proenkephalin mutated at the first ATG codon or devoid of its signal peptide, was targeted to the nucleus as well as the cytoplasm (Böttger and Spruce, 1995). Results from our laboratory using CHO cells expressing proenkephalin (Lindberg et al., 1991) show only cytoplasmic localisation for immunoreactive-enkephalin as assessed using immuofluorescence techniques and confocal microscopy (Castro and Lindberg, unplublished data). The mechanism of nuclear translocation of prohormone/ proneuropeptides is at present unclear. Certain proteins need a nuclear localisation signal for nuclear import or to be associated with chaperone proteins carrying a nuclear localisation signal (Silver, 1991). Recently it has been reported a sugar dependent process for glycoprotein nuclear import (Duverger et al., 1995); this observation opens an interesting avenue to explore the role of carbohydrate in the mechanism of nuclear internalisation of prohormones/proneuropeptides. Nuclear translocation of prohormones/proneuropeptides could be due to transport of soluble cytoplasmic forms of these molecules which may arise through the usage of alternative initiation codons for translation. This might give rise to prohormones/proneuropeptides with truncated or extended signal peptide which prevent their translocation to the ER (Murray, 1996).

The results reviewed above point to the crucial role played by cellular factors in determining the intracellular localisation of prohormones/proneuropeptides. This draws the attention for the need of additional factors (molecular chaperones), besides the specific sorting domains which reside within the primary amino acid sequences of the prohormones/proneuropeptides

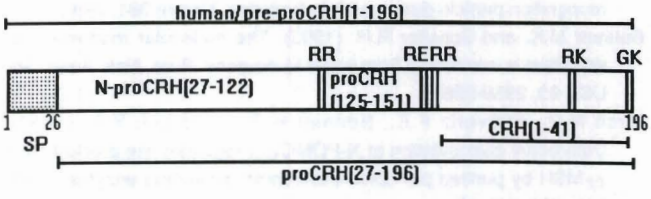

Fig. 3. Structure of the human pre-proCRH molecule. The various domains of the precursor molecule are noted as: SP, signal peptide spaning the first 26 amino acids of the precursor molecule; CRH_{1-41}, corticotrophin releasing hormone (aa: 1-41); N-proCRH27-122, H_2N-terminal procorticotrophin releasing hormone (aa: 27-122); $proCRH_{125-151}$, procorticotrophin releasing hormone (aa: 125-151). Putative sites of endoproteolytic cleavage are denoted by amino acids in the single letter code. R: arginine, K: lysine; G: glycine; S:serine; E: glutamic acid. Note that this figure is not drawn to scale.

themselves. These molecular chaperones could mediate folding, intracellular trafficking and sorting of neuropeptide/hormone precursors.

Future prospects

Although our knowledge on the molecular mechanisms underlying the intracellular sorting, trafficking and endoproteolytic processing of hormone/neuropeptide precursors has advanced rapidly over the last decade, there are still many questions which need to be answered. For example, the molecular mechanisms underlying the sorting of prohormones/proneuropeptides to the regulated secretory pathway; the accurate identification of the intracellular compartments where post-translational processing takes place; the intracellular localization and release of different products derived from a single prohormone/proneuro-peptide; and the molecular mechanisms underlying cell/tissue-specific sorting and processing. We hope that what we learn from these mechanisms will provide further insight into the molecular events which determine communication between cells of the endocrine and nervous systems.

Acknowledgements. This work is supported by the Biotechnology and Biological Sciences Research Council, Chemicals and Pharmaceuticals Directorate through a project grant to MGC. We also acknowledge the support which our laboratory receives from The Wellcome Trust, The Cancer Research Campaign, The Parkinson's Disease Society, The Royal Society, REMEDI, The Sir Halley Stewart Trust, Sandoz Foundation for Gerontological Research and The Faculty of Medicine Bequest Fund, University of Manchester. We thank Prof. A. M. Heagerty, Prof S. Tomilson and Dr. J. Brider for their support and encouragement.

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