Jens F. Rehfeld
Jens R. Bundgaard
Editors

Results and Problems in Cell Differentiation 50

Cellular Peptide Hormone Synthesis and Secretory Pathways

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Series Editors
Dietmar Richter, Henri Tiedge

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Cellular Peptide Hormone Synthesis and Secretory Pathways

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Preface

The concept of hormonal regulation using intercellular peptide messengers dates back to the discovery of secretin in 1902. The concept was simple: A peptide is released from specific hormone producing cells, endocrine cells, into circulation upon stimulation of the cells. The peptide hormone travels via blood to its target, the cells of which are equipped with specific receptors for high-affinity binding of the particular peptide hormone. Receptor binding subsequently elicits action of the target cells.

This concept has been seriously challenged by modern biochemistry and cell biology. Thus, it is now well established that the gene of a specific peptide hormone may be expressed in different types of endocrine cells, in neurons, and in some instances also in adipocytes, myocytes, osteoblasts, and immune cells. Today, only a few hormones – including the old master hormone insulin – represent the original endocrine paradigm. Instead, the widespread cellular synthesis now raises the question of how the body maintains the regulation of its functions by peptide hormones when a hormone may originate from a variety of cells.

Fortunately it has become apparent that several mechanisms act to ensure lack of interference. The first mechanism is simple. Peptide hormone gene expression in cells vary considerably during the ontogenetic development at all levels of the expression cascade. Secondly, biological barriers such as the blood–brain barrier ensure that the peptide hormones in peripheral circulation do not compete with the release of similar or identical peptides in the cerebral synapses. Thirdly, paracrine release of peptides between neighboring cells or autocrine secretion from self-stimulating cancer cells display a large concentration gradient in tissue so that locally released peptides do not interfere significantly with the systemic distribution of hormones in circulation. Finally – but not least – the cell-specific posttranslational processing helps to avoid interference by ensuring that different bioactive fragments of the prohormones are released from different cells. Thus, the fact that different cell types express the same hormone gene appears to be under control by counter-regulatory mechanisms in the normal organism. But the phenomenon is also of interest for the understanding of the dysregulation observed in several major diseases such as diabetes mellitus, cancer, and cardiovascular diseases.

The present volume of the well-established book series “Results and Problems in Cell Differentiation” is an attempt to illustrate the modern and universal concept

of peptide hormone biology with emphasis on the cellular hormone synthesis and secretory pathways. Originally a more comprehensive text was planned. Nevertheless, the actual volume illustrates well the key information in the up-to-date basic peptide biology that we wished to see unfolded and discussed in one book. We are grateful to the series editors, Dr Dietmar Richter and Dr Henri Tiedge, for the invitation to edit the volume. We also owe a large debt of gratitude to the authors for the excellent contributions and for their patience with the editorial process. Finally, we wish to thank Springer Verlag in general and Ms Ursula Gramm in particular for perfect collaboration.

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Jens R. Bundgaard

Contents

Maturation of Secretory Granules

Tanja Kögel and Hans-Hermann Gerdes

Abstract Exocrine, endocrine, and neuroendocrine cells store hormones and neuropeptides in secretory granules (SGs), which undergo regulated exocytosis in response to an appropriate stimulus. These cargo proteins are sorted at the *trans*-Golgi network into forming immature secretory granules (ISGs). ISGs undergo maturation while they are transported to and within the F-actin-rich cortex. This process includes homotypic fusion of ISGs, acidification of their lumen, processing, and aggregation of cargo proteins as well as removal of excess membrane and missorted cargo. The resulting mature secretory granules (MSGs) are stored in the F-actin-rich cell cortex, perhaps as segregated pools exhibiting specific responses to stimuli for regulated exocytosis. During the last decade our understanding of the maturation of ISGs advanced substantially. The use of biochemical approaches led to the identification of membrane molecules mechanistically involved in this process. Furthermore, live cell imaging in combination with fluorescently tagged marker proteins of SGs provided insights into the dynamics of maturing ISGs, and the functional implications of cytoskeletal elements and motor proteins.

1 Introduction

Secretory granules (SGs) are vesicular storage organelles of the secretory pathway of eukaryotic cells, which comprises the endoplasmic reticulum (ER), the Golgi apparatus, and the endosomal/lysosomal system, interconnected by tightly regulated vesicular transport steps. This extended endomembrane structure provides an uptake route for extracellular molecules through the endocytotic pathway and an outward route for secretory proteins by the so-called biosynthetic-secretory

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pathway. Exocrine, endocrine, and neuroendocrine cells have, in addition to the constitutive secretory pathway which is common to all cells, a regulated pathway of protein secretion (Kelly 1985). Proteins of both pathways are transported together from the ER to the Golgi apparatus, but their routes diverge at the most distal part of the Golgi – the *trans*-Golgi network (TGN). Constitutively secreted proteins such as extracellular matrix proteins, metalloproteases, or growth factors exit the TGN in constitutive vesicles (CVs). These vesicles fuse in a stimulus-independent manner with the plasma membrane (PM) to release their content. Regulated secretory proteins (RSPs) such as prohormones, proneuropeptides, and processing enzymes are packaged into SGs, which fuse with the PM in a stimulus-dependent manner. The biogenesis of SGs starts at the TGN, where RSPs are sorted into forming immature secretory granules (ISGs), which are converted to mature secretory granules (MSGs) in a complex maturation process. This process involves a number of protein sorting and membrane remodeling events including homotypic fusion of ISGs and budding of ISG-derived vesicles (IDVs). Furthermore, processing of RSPs to bioactive peptides is accomplished by prohormone convertases (PCs) during ISG maturation. Finally, SGs undergo priming and docking in the F-actin rich cortex. Exocytosis-competent SGs are stored in the cortical area of the cell and release their cargo in response to extracellular signals. Such signals are elicited by membrane depolarization upon opening of sodium channels, or by activation of specific surface receptors through ligand binding. Both events lead to an increased concentration of cytoplasmatic calcium through the opening of calcium channels in the PM and/or intracellular calcium stores (Meldolesi 2002).

The most prominent hallmark of SGs is the acidic dense core structure which is surrounded by a limiting membrane. This core consists of tightly packed aggregates of hormones and neuropeptides, as well as other signaling molecules, including catecholamines and monoamines. The respective composition of the aggregated secretory cargo varies with the cell type and is adapted to physiological demand. In addition to the storage of tissue-specific hormones and neuropeptides, SGs contain widespread markers present in many, if not all, different types of SGs. Such markers are represented e.g., by the granin family of acidic secretory proteins with its classical members chromogranin A (CgA), chromogranin B (CgB, also known as secretogranin-I), and secretogranin-II (Sg-II, also known as secretogranin C) (Huttner et al. 1991). Antibodies against the granins have been used as important tools for basic research on regulated protein secretion and as diagnostic tools for endocrine diseases. Another group of widely distributed marker proteins are the PCs, which include PC1/3, PC2 and carboxypeptidase E/H (Seidah et al. 1993; Steiner 1998). These enzymes are co-sorted with their substrates into ISGs and convert precursor proteins by cleavage at dibasic cleavage sites into bioactive peptides. The limiting membrane of SGs contains proteins required for SG acidification, uptake of non-proteinaceous molecules, transport, targeting, and regulated fusion with the PM.

SGs were first described morphologically in exocrine, zymogen-producing pancreatic acinar cells, and in endocrine, insulin-producing pancreatic β-cells (Palade 1956). Subsequent studies on the anterior pituitary gland employing high resolution electron microscopy (EM) autoradiography demonstrated the formation

of small ISGs at the TGN, which matured to MSG within 2 hours (Farquhar et al. 1978); for historical review see (Morvan and Tooze 2008). During the last two decades, the biogenesis of ISGs at the TGN and their subsequent maturation into mature, exocytosis-competent organelles was mainly studied in cell lines derived from anterior pituitary (AtT20 cells) (Cool and Loh 1994; Moore et al. 1983; Tooze and Tooze 1986) pheochromocytoma (PC12 cells) (Glombik et al. 1999; Tooze and Huttner 1990), endocrine pancreas (β, MIN6- and INS-1E-β-cells) (Ivarsson et al. 2005; Varadi et al. 2005; Wasmeier and Hutton 2001) or intestine (enterochromaffin cells) (Desnos et al. 2007). Moreover, systemic studies on knockout and transgenic mice provided further insights into the role of SG maturation under physiological conditions (Cool et al. 1997; Irminger et al. 1997; Methia et al. 1999; Thiele et al. 1997; Gondre-Lewis et al. 2006; Hendy et al. 2006; Kim et al. 2005; Rehfeld et al. 2008).

In this chapter, we will summarize current knowledge and concepts on SG biogenesis comprising the formation of ISGs at the TGN and their subsequent maturation. Although both steps involve different cellular compartments, they appear as a continuous biosynthetic process employing similar molecular mechanisms at both levels. This is best documented for the sorting and processing of regulated secretory cargo, which starts in the TGN and persists during SG maturation. Therefore, we will summarize the current knowledge about the molecules and mechanisms involved in the two biogenesis steps until the priming and segregation of exocytosis-competent SGs into different pools.

2 Formation of ISGs at the TGN

At the TGN, constitutive and regulated secretory proteins take different routes to the PM (Kelly 1985; Tooze and Huttner 1990). Whereas constitutively secreted proteins enter CVs, RSPs are sorted into ISGs. Two key mechanisms have been proposed for the sorting of RSPs from the TGN into forming ISGs: their selective aggregation and their targeting to specific membrane domains of the TGN. This led to the formulation of the "sorting for entry" hypothesis (Arvan and Castle 1998; Tooze 1998).

2.1 Aggregation

The aggregation of RSPs was first evidenced as electron-dense material in morphological studies on cells specialized for regulated protein secretion (Palade 1956). Subsequent investigations showed that such aggregates were not only detectable in ISGs and MSGs but also in the TGN, albeit of smaller size and less electron-dense (Smith and Farquhar 1966). This triggered the idea that aggregation could be involved in the targeting of RSPs to SGs and was further investigated in biochemical

experiments. Using in vitro assays, RSPs were shown to aggregate in the presence of millimolar calcium concentrations and a mildly acidic pH mimicking the milieu of the TGN Fig. 2. This was best demonstrated for the granins and also for a variety of other regulated proteins (Chanat and Huttner 1991; Colomer et al. 1996; Gerdes et al. 1989; Gorr et al. 1989; Rindler 1998; Shennan et al. 1994; Song and Fricker 1995; Vischer and Wagner 1994). Protons and divalent cations are thought to neutralize the negative charge of RSPs, thereby promoting their dense packaging. Further biochemical studies showed that constitutive secretory proteins were excluded from the formed aggregates (Chanat and Huttner 1991; Gerdes et al. 1989). This is in line with the observation that weak bases, neutralizing the pH in the TGN, caused missorting of RSPs into the constitutive pathway (Chanat and Huttner 1991; Gerdes et al. 1989; Moore et al. 1983; Tooze 1998), and highlighted the selective aggregation of RSPs as an important mechanism for the segregation of regulated from constitutive secretory proteins. Furthermore, abundantly expressed RSPs possibly function as sorting chaperones by providing aggregation seeds for those RSPs present at very low concentration and thus incompetent for sufficient self-aggregation. Such a chaperone function was shown for the sorting of a 23 kD product of proopiomelanocortin (POMC) by overexpressed CgB in AtT20 cells (Natori and Huttner 1996). Moreover, the proteolytic activity of PCs aids the aggregative sorting of RSPs. PCs are successively activated by the increasing acidification along the secretory pathway. This initiates precursor processing as early as in the TGN and thereby promotes the aggregative properties of bioactive products (Seidah et al. 2008). Finally, it was found that aggregation does not only segregate RSPs from constitutive secretory proteins but also different populations of RSPs from each other. This has been first demonstrated for the egg-laying hormone of *Aplysia californica*, which is processed in the TGN into two different peptides, which are then sorted to different types of SGs (Sossin et al. 1990). Together, these data support the concept that selective aggregation of RSPs plays a key role in sorting of cargo into SGs.

2.2 Sorting Motifs and Cargo–Membrane Interaction

During ISG formation, aggregates of RSPs are thought to interact with specific membrane domains in the TGN, which gives rise to the budding of ISGs. This raises two basic questions: First, do RSPs encode specific sorting signal(s) for this interaction and second, what is the nature of these membrane domains? Intensive studies over several decades in search of specific sorting signals that direct RSPs to SGs, have led to the identification of three types of targeting motifs comprising short amino acid sequences. One type consists of a disulfide-constrained loop structure located in the N-terminus of CgB, (Chap. 2) (Chanat et al. 1993; Glombik et al. 1999; Kromer et al. 1998). This loop structure, which is very homologous to the N-terminal loop structure in CgA (Benedum et al. 1987), was found to be essential and sufficient for directing secretory cargo to SGs (Glombik et al. 1999). Furthermore, it was shown

that the loop mediates calcium-independent membrane binding in the TGN of PC12 cells (Glombik et al. 1999). A related N-terminal loop structure of POMC was also necessary and sufficient for targeting a reporter protein to the regulated pathway of protein secretion (Cool and Loh 1994; Tam et al. 1993).

The second type of targeting motif in RSPs comprises paired basic amino acids (Dikeakos and Reudelhuber 2007). Such motifs have been found e.g., in proneurotensin (Feliciangeli et al. 2001), prorenin (Brechler et al. 1996), and progastrin (Bundgaard et al. 2004). The same paired basic amino acids appear to serve as cleavage sites for the membrane-bound endoproteases of the PCs, namely PC1/3 and PC2 (Dikeakos and Reudelhuber 2007). In support of a function in sorting, mutation of the PC2-specific dibasic cleavage site into a cleavage site for furin caused missorting of prorenin (Dikeakos et al. 2007).

Short α-helical domains comprise a third type of SG-specific targeting information. This motif was found in carboxypeptidase E (CPE) (Dikeakos et al. 2007), PC1/3, and PC2 (Dikeakos et al. 2007), prosomatostatin (Mouchantaf et al. 2001), CgA (Taupenot et al. 2002), and the endocrine peptide precursor VGF (Garcia et al. 2005). These, in most cases of C-terminal domains, were reported to direct regulated secretory proteins to SGs (Dikeakos et al. 2007). Furthermore, CPE, PC1/3 and PC2 were shown to interact with membranes through their short α-helical domains (Assadi et al. 2004; Dhanvantari et al. 2002; Lou et al. 2007). Important denominators of these shallow membrane interactions are hydrophobic patches and calcium binding (Dikeakos et al. 2009). Furthermore, evidence has been obtained that other RSPs may achieve SG-specific targeting by interaction with these membrane-associated RSPs. In this respect, proenkephalin, proinsulin, POMC (Cool and Loh 1998), and brain-derived neurotrophic factor (BDNF) have been proposed to interact with CPE (Lou et al. 2005). Furthermore, CgA, Sg-II and POMC-derived processing products were shown to bind to membrance-associated Sg-III (Han et al. 2008; Hosaka et al. 2004, 2005; Hotta et al. 2009).

The characterization of the lipid micro-domains to which SG-specific targeting domains bind, was guided by the recognition of cholesterol as an important factor in ISG formation (Wang et al. 2000). Subsequent studies provided evidence that PC2 (Blazquez et al. 2000), CPE (Dhanvantari and Loh 2000) PC3 (Arnaoutova et al. 2003) and Sg-III (Hosaka et al. 2004) were bound to cholesterol-rich membrane patches. The finding that Sg-III, in contrast to CPE, was not found in Triton X-100 insoluble rafts, suggests their association with distinct lipid micro-domains (Hosaka et al. 2004).

In summary, it emerges that RSPs are not targeted to SGs by a common sorting motif but rather through a variety of different motifs that are often combined in one protein. Furthermore, adaptor proteins, which bind to SG-specific cholesterol-rich membrane domains facilitate the sorting of soluble RSPs. Most likely, aggregates of RSPs interact with such adaptors. This results in multimerization of sorting motifs on the surface of these aggregates and thus increases the binding efficiency to membrane adaptors (Glombik et al. 1999). To date, such adaptor functions have been attributed to CPE (Cool et al. 1997) and Sg-III (Han et al. 2008; Hosaka et al.

2004), which possibly function as separate units. However, the quest for the initially proposed classical sorting receptor remains inconclusive. Such a transmembrane receptor is thought to initiate both coat protein binding and vesicle formation through its cytoplasmic domain. The claim that CPE is the sorting receptor for the regulated pathway of protein secretion remains most controversial (Cool et al. 1997; Irminger et al. 1997; Methia et al. 1999; Thiele et al. 1997).

3 Transport of Maturing ISGs

To gain information about the SG dynamics, live cell imaging has been frequently applied. However, the imaging of newly formed ISGs on their way to the cell cortex is challenging because regulated secretory cells contain thousands of MSGs with a $t_{1/2}$ of several days, while ISGs represent only a small percent of the total SG population with a short $t_{1/2}$ of e.g., ~45 min in neuroendocrine cells (Tooze et al. 1991). Therefore selective labeling of ISGs is necessary to follow their route. This was achieved by the application of a pulse/chase labeling protocol to PC12 cells (Kaether et al. 1997; Rudolf et al. 2001, 2003), where human CgB tagged with the temperature-sensitive mutant GFP (S65T) was expressed. In this way, pools of fluorescent SGs of a defined age (i.e., maturation status) were generated and followed through the cell. This showed that after biogenesis at the TGN, ISGs move in a microtubule-dependent manner within a few seconds to the F-actin-rich cell cortex (Rudolf et al. 2001) (Fig. 1, point 1). This, throughout uni-directional translocation, indicated the absence of dynein activity on the outward moving ISGs (Rudolf et al. 2001). In insulinoma cells fast, bidirectional movement of SGs was observed upon glucose stimulation. This transport was completely inhibitable by downregulation of kinesin-1 but only partially by downregulation of dynein by siRNA (Varadi et al. 2003). In the case of AtT20 cells recent biochemical investigations indicated that SGs interact via CPE and dynactin with a kinesin-2 (KIF3A) and a kinesin-3 (KIF1A), but not conventional kinesin-1 (KHC) (Park et al. 2008). Therefore, it remains to be investigated which kinesins are responsible for the outward transport of ISGs in other cell types. After the arrival of ISGs at the cell periphery, they remain restricted to the F-actin rich cortex, where they move actively in all directions along cortical F-actin (Fig. 1, point 2), mature (Fig. 1, point 3), and finally exocytose (Fig. 1, point 4) (Desnos et al. 2007; Rose et al. 2003; Rudolf et al. 2001, 2003; Varadi et al. 2005).

By applying the same pulse/chase protocol to PC12 cells coexpressing a dominant-negative myosin Va-tail, it was demonstrated that the cortical restriction of SGs and their F-actin dependent movement is dependent on myosin Va (Rudolf et al. 2003). Myosin Va is a processive motor (Tyska and Mooseker 2003) driven by ATP cycling (Trybus 2008). In control PC12 cells with functional myosin Va, the average velocity of ISGs in the cell cortex decreased during the first few hours resulting in ~60% immobile SGs after 180 min. From this fraction ~50% were tethered through F-actin (Rudolf et al. 2001). Confirming this notion, a similar

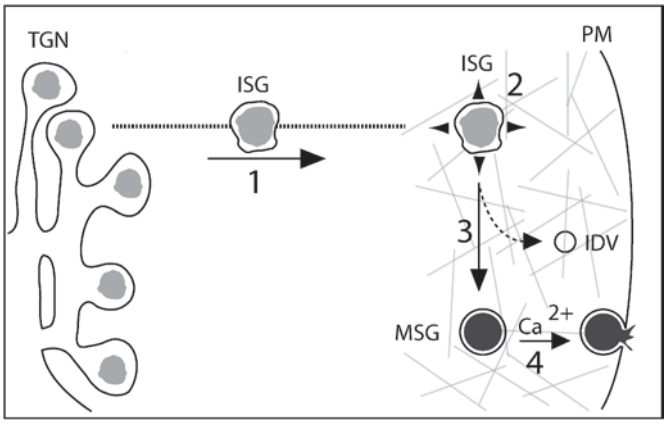

Fig. 1 *Scheme illustrating the dynamics of ISGs/MSGs in neuroendocrine cells*. Newly formed immature secretory granules (ISGs) are transported from the *trans*-Golgi network (TGN) along microtubules (1) to the cell periphery, where they are captured in the F-actin rich cell cortex. During their maturation, ISGs move F-actin-dependently in the cortex (2, 3) and give rise to ISG-derived vesicle (IDV) budding (*broken arrow*). After completion of maturation, the resulting mature secretory granules (MSGs) undergo docking near the PM (4) and exocytose in a stimulus-dependent manner. *Black lines* membrane; *broken black line* microtubule; *gray lines* F-actin; *gray/black spheres* aggregated secretory cargo; *black arrows/arrowheads* movement of SGs; *broken black arrow* budding of IDV

percentage of SGs was preserved in isolated PM sheets of PC12 cells, which contained the F-actin cortex (Avery et al. 2000; Martin and Kowalchyk 1997). Furthermore, myosin Va possibly plays a role in the tethering of ISGs, in addition to its motor function. This was evidenced by an in vitro assay, where the ATP-dependence of ISGs binding to F-actin declined with increasing age of the isolated ISGs, resulting in a loss of ATP-dependence after 180 min (our unpublished observations). In addition, it was found that myosin Va is implicated in the membrane remodeling of ISGs during their maturation (see below). These multiple roles of myosin Va demonstrated for ISGs of PC12 cells, raise the question whether the cortical transport of ISGs and their concurrent maturation (Rudolf et al. 2001) are coherent processes. So far, the pulse/chase-like system was only used to analyze ISG dynamics of PC12 cells and it remains to be investigated whether maturing ISGs of other cell types show similar characteristics.

4 Mechanistic Insights into ISG Maturation

ISG maturation is a tightly regulated process, which is composed of several discrete steps, and completes within a few hours.

4.1 Homotypic Fusion

Based on morphological studies of mammotrophs in the anterior pituitary gland of the lactating rat, Farquahr and colleagues proposed in 1966 that ISGs undergo homotypic fusion. The key observation for this proposition was that small (100–200 μm), Golgi-derived granules appeared to aggregate, resulting in polymorphous ISGs, which eventually rounded up to become the mature (600–900 μm) secretory granules (Smith and Farquhar 1966). Later, this concept was further validated using PC12 cell-derived in vitro reconstitution assays for ISG biogenesis (Tooze and Huttner 1990) and homotypic ISG fusion (Urbe et al. 1998) (Fig. 2, point 2). This led to the identification of molecules functionally involved in ISG–ISG fusion. Besides NSF (Urbe et al. 1998) and α-SNAP (Wendler et al. 2001), syntaxin 6 (Wendler et al. 2001) and synaptotagmin-IV (Ahras et al. 2006) were shown to be necessary for the ISG-specific membrane fusion. Syntaxin 6 was needed on both the donor and the acceptor vesicle in order to accomplish homotypic fusion (Ahras et al. 2006; Wendler et al. 2001). However, this fusion was independent of SNAP-25 and vesicle associated membrane protein-4 (VAMP4) (Ahras et al. 2006; Wendler et al. 2001). Thus, the requirements for the ISG–ISG fusion are different to those promoting the ISG–PM-specific fusion, namely syntaxin 1 and SNAP-25. It is of note that homotypic fusion was not only observed for ISGs during their maturation, but in some endocrine cell types also for MSGs upon stimulation of regulated exocytosis. In this case, homotypic fusion of MSGs precedes their subsequent fusion with the PM, resulting in bigger release quanta of cargo. Accordingly, this phenomenon has been referred to as quantum exocytosis (Cochilla et al. 2000; Orci and Malaisse 1980; Tooze et al. 1991).

4.2 Sorting by Retention

Maturation of ISGs includes, in addition to their homotypic fusion, refinement of the content and remodeling of the membrane. During this sorting process, proteins not destined for MSGs are removed together with excess membrane in clathrin-coated IDVs. This process has been termed “sorting by retention” (Fig. 2, point 3) (Arvan and Castle 1998; Tooze 1998). IDVs exocytose in an unstimulated manner, perhaps via the endosome, releasing their content in a process referred to as constitutive-like secretion (Arvan and Castle 1998). Several proteins have been found to exit from maturing ISGs. These include proteins from the homotypic fusion machinery, namely syntaxin 6 (Klumperman et al. 1998; Wendler et al. 2001), synaptotagmin-IV (Ahras et al. 2006; Eaton et al. 2000), and VAMP4 (Eaton et al. 2000). Synaptotagmin-IV removal was recognized as an important switch for ISGs to acquire responsiveness for regulated exocytosis (Eaton et al. 2000). In addition, the endoproteases carboxypeptidase D (Ahras et al. 2006; Dittie et al. 1996, 1997; Kakhlon et al. 2006; Klumperman et al. 1998; Varlamov et al. 1999; Wendler et al. 2001) and furin (Dittie et al. 1997), both predominantly located in the TGN

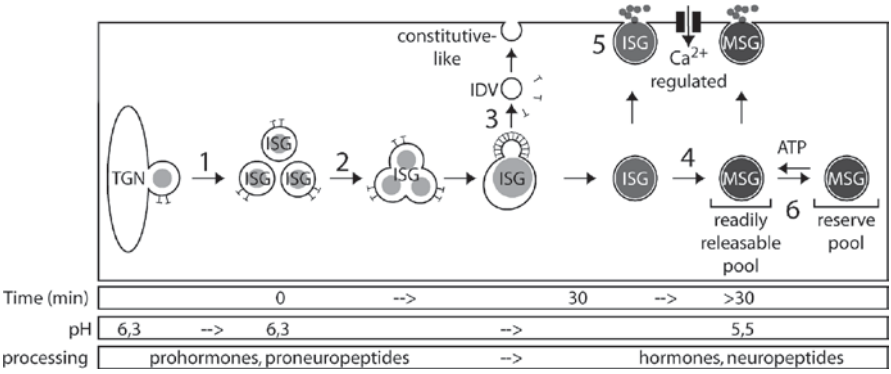

Fig. 2 *Scheme depicting the stepwise maturation of ISGs***.** (1) At the TGN, regulated secretory proteins are sorted into nascent ISGs (sorting by entry). (2) ISGs undergo homotypic fusion. (3) Proteins not destined for MSGs are removed together with excess membrane in clathrin-coated IDVs (sorting by retention), which exocytose stimulus-independent. (4) ISGs are converted into MSGs involving further acidification and condensation of the SG lumen, and processing of precursor proteins. (5) Both ISGs (after IDV-budding) and MSGs can be exocytosed in a regulated manner. A small fraction of MSGs forms the readily releasable pool. (6) The major fraction of MSGs is stored as reserve pool. Confinement to the reserve pool is reversible. *Upper panel*: *Black lines* membrane; *gray areas* aggregated regulated secretory proteins; *black arrows/arrowheads* steps of maturation. *Lower panels* indicate the age of the SGs, the pH of the SG lumen and the processing status of the cargo during the different maturation steps

but also present in ISGs, and M6PR (Klumperman et al. 1998), the sorting receptor for lysosomal proteins, are removed from maturing ISGs. Finally, poorly aggregating proteins are also thought to exit in IDVs, since their constitutive-like secretion was observed during the period of ISG maturation (Glombik and Gerdes 2000; Molinete et al. 2000). Recently, it was proposed that ISGs may contain presorted sets of protein: those which remain in ISGs during maturation and those which exit in IDVs (Katsumata et al. 2007). Evidence for this was obtained by the finding that the two removed proteins VAMP4 and syntaxin 6 were associated with the same lipid micro-domain but separated from VAMP2, a constituent destined for MSGs.

Several lines of evidence suggest that the transmembrane proteins M6PR, furin (Chap. 3), and VAMP4 fulfill a central role in the formation of IDVs. This role is executed through their cytoplasmatic tail, which binds to the clathrin adaptor protein complex-1 (AP-1) resulting in the recruitment of a clathrin coat (Dittie et al. 1997, 1999; Hinners et al. 2003). The binding of AP-1 was shown to be CKII-dependent (Dittie et al. 1999; Hinners et al. 2003) and stimulated by ADP-ribosylation factor-1 (ARF-1) (Dittie et al. 1996). For VAMP4, a phosphorylation-dependent recruitment of phosphofurin acidic cluster sorting protein (PACS-1) was shown to enhance AP-1 association to cargo (Hinners et al. 2003). Recently, it was demonstrated that the Golgi-associated γ-ear-containing ADP-ribosylation-factor binding proteins (GGAs) are also involved in the clathrin-mediated membrane remodeling of maturing ISGs

(Kakhlon et al. 2006). Collectively, these data emphasize that the budding of IDVs is an important step in ISG maturation.

The budding of IDVs is likely to take place during the early phase of maturation. This assumption is based on several observations. One is that in PC12 cells, the removal of furin from ISGs was detected between 12 and 30 min after their formation at the TGN (Rudolf 2001), (Fig. 2, point 3 and lower panel, top). Interestingly, this removal was monitored in the F-actin rich cortex and could be blocked by the expression of a dominant-negative mutant of myosin Va or knockdown of myosin Va expression by shRNA (our unpublished data). This suggests that the actin/myosin system may exert a mechanical force promoting membrane fission during IDV formation. In further support of a model that IDV budding takes place in the early phase of ISG maturation, the removal of VAMP4 from ISGs in AtT20 cells occurred ~45 min after ISG biogenesis (Eaton et al. 2000). In agreement with this finding ISGs of PC12 cells also gained competence for regulated exocytosis 30 min after their synthesis at the TGN (Tooze et al. 1991).

Recently two Rho GTP exchange factors (GEFs), kalirin and trio, were proposed to regulate the budding of IDVs from ISGs. These GEFs interact with the cytoplasmic tail of the SG-specific processing enzyme peptidylglycine α-amidating monooxygenase (PAM) (Ferraro et al. 2007). Overexpression of their N-terminal GEF domain in neuro-endocrine AtT20 cells enhanced constitutive POMC secretion from ISGs, thereby depleting cells of secretory cargo in the absence of secretagogue (Ferraro et al. 2007). On the other hand, selective pharmacological inhibition of endogenous GEF activity decreased the stimulus-independent release of hormone precursors and led to an accumulation of processed product in MSGs (Ferraro et al. 2007). This suggests that the activity of kalirin and trio modulates the extent of constitutive-like secretion during ISG maturation by controlling the budding of IDVs from ISGs. Together with the progression of endoproteolytic processing in maturing SGs over time, this modulation can determine the ratio of released unprocessed versus processed products. This leads to the intriguing speculation that secretory cells may fine-tune the set of released peptides by the regulation of IDV budding.

4.3 Acidification

During maturation the lumen of ISGs progressively acidifies along with further condensation of soluble cargo and dense core formation. This process is driven by the vacuolar proton ATPase of SGs, which transports protons into the lumen and due to the extremely low proton permeability of the SG membrane, leads to a decrease of the intragranular pH (Jefferies et al. 2008) from 6.3 (ISGs) to 5–5.5 (MSGs) (Urbe et al. 1997). For PC12 cells, it has been shown that the acidification is completed within a time frame of 90 min (Fig. 2), (Urbe et al. 1997). The resulting proton gradient drives the carrier-mediated uptake of monoamines from the cytosol (Johnson 1988) and activates PCs to process prohormones and

proneuropeptides into bioactive peptides (Seidah et al. 1993; Steiner 1998), (Fig. 2 Chap. 3).

The acidification of the SG matrix and processing of cargo was found to be independent of the removal of excess membrane via IDVs in some instances. This resulted from experiments with brefeldin A, which blocks ARF-1 recruitment to ISGs (Eaton et al. 2000), or from expression of the dominant-negative tail of myosin Va, which interfered with the removal of furin from ISGs (our unpublished results). In both cases the acidification-dependent processing of POMC (Fernandez et al. 1997) or Sg-II (our unpublished results), respectively, (Chaps. 3 and 5), was unchanged. Therefore, ARF-1 recruitment and myosin Va function seem to be essential for the removal step, but dispensable for acidification and processing. These results are contrasted by a study, where IDV budding was inhibited by interference with the function of the clathrin adaptor GGA3. This led to a retention of M6PR, syntaxin-6, and VAMP4 in MSGs and to a decrease in PC2 activity (Kakhlon et al. 2006). Thus, in contrast to the ARF-1- and myosin Va-dependent budding, GGA3-mediated budding of IDVs seems to be necessary for precursor processing and acidification of the ISG matrix. These data illustrate the complexity of ISG maturation as a tightly regulated multi-step process, where the functionally involved proteins control distinct steps. Further studies are necessary to unravel the underlying mechanisms.

5 Maturation into Vesicle Pools

5.1 Formation of SG Pools with Different Release Probabilities

Maturation of SGs is generally regarded as the part of SG biogenesis, which leads to dense-cored vesicles containing bioactive peptides. However, before these SGs can fuse with the PM, they have to go through additional modification steps leading to tethering, priming, and docking (Sorensen 2004). During this process SGs are segregated into different pools with specific release probabilities (Almers 1990; Burgoyne and Morgan 2003; Neher and Zucker 1993; Rose et al. 2003; Sorensen 2004). Therefore, in a broader sense, the formation and stabilization of distinct SGs pools can be regarded as part of their maturation.

In numerous studies two major pools of SGs have been described: a readily releasable pool undergoing immediate exocytosis under stimulation and a slower reacting reserve pool that facilitates long-lasting exocytosis under sustained stimulation (Almers 1990; Neher and Zucker 1993). For many cell types such as adrenal chromaffin cells (Bittner and Holz 1992; Neher and Zucker 1993; von Ruden and Neher 1993), pancreatic β-cells (Rorsman et al. 2000), and pituitary melanotrophs (Thomas et al. 1993) it was shown that most SGs belong to the reserve pool, whereas only a small portion of ~1% is initially

rapidly releasable (Burgoyne and Morgan 2003; Sorensen 2004). To enter the readily releasable pool, SGs undergo several priming steps at a basal intracellular calcium concentration of 0.1–2 μM, while the actual exocytosis requires a calcium concentration of several micromolar (Sorensen 2004). Key regulators for the transit of SGs from the reserve pool to the readily releasable pool (Fig. 2, point 6) are calcium, cortical F-actin and the SNARE complex at the PM. This process is delicately balanced by the action of many regulating factors. These include the F-actin severing proteins scinderin (Trifaro et al. 2000) and gelsolin (Miyamoto et al. 1993) as well as proteins promoting F-actin assembly, such as Rho GTPase Cdc42 and the guanine nucleotide exchange factor intersectin-1L (Gasman et al. 2004).

Upon stimulation of exocytosis, the F-actin in the cell cortex becomes partially depolymerized, while fine F-actin tracks leading toward exocytotic sites remain or become polymerized (Giner et al. 2005; Tchakarov et al. 1998; Vitale et al. 1995). This process was shown to be a prerequisite for exocytosis (Malacombe et al. 2006), and is thought to clear the way for the transport of SGs from the reserve pool. Compatible with this notion is the finding that cell depolarization induces the movement of SGs into formerly unvisited areas (Degtyar et al. 2007). This implies that, at least in some instances, the reserve pool and the readily releasable pool are spatially separated.

The transfer of SGs to the readily releasable pool (Fig. 2, point 6) was described as an ATP-dependent process (Burgoyne and Morgan 2003; Duncan et al. 2003). Consistently, only sustained but not immediate secretion, requires the presence of magnesium and ATP in bovine chromaffin cells (Bittner and Holz 1992; Parsons et al. 1995). This suggested the involvement of an F-actin-dependent motor protein, which was subsequently identified as myosin Va. In support of such a model, the inhibition of myosin Va by addition of a specific antibody against the head domain reduced the amount of regulated exocytosis only after sustained stimulation in chromaffin cells (Rose et al. 2003; Watanabe et al. 2005). Furthermore, the expression of the dominant-negative tail domain of myosin Va reduced the amount of exocytosis in neuroendocrine PC12 cells (our unpublished data) and endocrine cells (Desnos et al. 2007; Varadi et al. 2005).

Although fast and slow reacting SG pools are common for many different cell types, PC12 cells seem to lack an equivalent of the readily releasable pool. This is evidenced by the finding that even at high temporal resolution, PC12 cells exocytose at a very slow rate compared to chromaffin cells (Sorensen 2004). Consequently, the release of SGs from PC12 cells should always include priming (Sorensen 2004). This hypothesis is in agreement with the earlier finding that in differentiated growth cones of PC12 cells and in hippocampal neurons, mobile vesicles support exocytosis more efficiently than immobile vesicles (Han et al. 1999; Silverman et al. 2005).

Further evidence of a cell type-specific variation of regulated exocytosis is provided by the finding that knockdown of myosin Va by shRNA increased exocytosis of SG in neuroendocrine PC12 cells (our unpublished data), but led to a reduction in endocrine cells (Desnos et al. 2007; Ivarsson et al. 2005; Varadi et al. 2005). This

points to a regulatory role of myosin Va in exocytosis, which must depend on additional, cell type specific factors. In summary, these data support the model that myosin Va is essential for the maintenance, mobilization, and replenishment of the releasable pool of SGs, but not for the exocytotic event.

An additional regulatory level of exocytosis becomes evident when SGs are monitored on a longer time scale. This was demonstrated for chromaffin cells upon expression of atrial natriuretic factor fused to a fluorescent protein, which shifts its color from green to red within 16 hours (Duncan et al. 2003). With this system, newly synthesized SGs (green) were shown to immobilize below the PM and, upon nicotine stimulation, to exocytose in preference to the older SGs (red). Older SGs seemed to be retrieved from the cell cortex to more central areas of the cell (Duncan et al. 2003) which may indicate their turnover. The preferential release of newly formed SGs is in agreement with previous data showing that newly synthesized peptides are secreted foremost (Noel and Mains 1991). Assuming that the bioactive peptide cocktail of SGs is under the control of physiological signals, newly formed SGs are probably those which fit best to physiological demands.

5.2 Rabs, Rab Effectors and Higher Regulatory Networks

Rab proteins, a family of small GTPases with more than 60 members, are thought to play a major role in the segregation of SGs into different vesicle pools. In a systematic screen of all family members, only Rab3 and Rab27 isoforms were found to be implicated in the trafficking of SGs (Tsuboi and Fukuda 2006). Both exert their function through effectors, which are often long, synaptotagmin-like (Slp) proteins that recruit cell signaling molecules such as kinases or calcium, bind to cytoskeletal elements, the PM, parts of the exocytotic machinery and/or motor proteins. For Rab27, it was shown that it links myosin Va to SGs, and that it is a positive regulator of exocytosis (Desnos et al. 2003; Fukuda 2003b; Gomi et al. 2007; Tsuboi and Fukuda 2006; Zhao et al. 2002). Furthermore, Rab27a associates with SGs in both PC12 and chromaffin cells and with insulin-containing SGs in pancreatic MIN6 β-cells through effector proteins, Slp4/granulophilin (Yi et al. 2002) and synaptotagmin-like protein lacking C2 domains (Slac)2-c/MyRIP (Fukuda and Kuroda 2002), respectively. Both effectors were shown to reduce exocytosis of SGs and facilitate the tethering of SGs to F-actin (MyRIP) or to exocytotic sites (granulophilin) (Desnos et al. 2003; Fukuda 2003a; Fukuda and Kanno 2005; Fukuda et al. 2002; Fukuda and Kuroda 2002; Tsuboi and Fukuda 2006). The tethering of SGs to distinct structural elements suggests that synaptotagmin-like proteins in conjunction with Rab27 may play a role in segregating SGs into distinct vesicle pools.

The other Rab protein implicated in SG trafficking, Rab3, is expressed in four isoforms in mammals: Rab3A, Rab3B, Rab3C, and Rab3D. Whereas Rab3A–C are predominantly expressed in neurons, Rab3D prevails in nonneuronal tissues such as peptide secreting cell lines. In contrast to the positive regulation of exocytosis by Rab 27 in all investigated cases, both impairment of Rab3 isoform function as well as overexpression of Rab3 uniformly inhibited regulated exocytosis (Chung et al.

1999; Coppola et al. 1999; Holz et al. 1994; Iezzi et al. 1999; Johannes et al. 1994; Martelli et al. 2000; Schluter et al. 2002; Tabellini et al. 2001; Thiagarajan et al. 2004; Weber et al. 1996). Furthermore, two lines of evidence suggest that the Rab3D isoform may play a role in the maturation of ISGs: it was found that Noc2, a Slac, interacts with Rab3 (Shibasaki and Seino 2005) and Rab27 (Tsuboi and Fukuda 2005), and Noc2 knockout mice contain exocrine SGs of abnormally large size and irregular shape (Shibasaki and Seino 2005). This observation is reminiscent of studies on Rab3D-knockout mice, which contain twofold larger SGs in the exocrine pancreas and the parotid gland (Riedel et al. 2002). As SG size is likely to be determined during the process of maturation, these observations identify Noc2 together with Rab3D as prime candidates to function in SG maturation.

6 Summary

During the last decade biochemical approaches and live cell imaging have refined our understanding of ISG maturation. Significant progress has been made in identifying molecules and mechanisms underlying distinct maturation steps and in understanding how these events are organized in time and space. It appears that maturation of ISGs is more complex than anticipated and reflects more than just the condensation of the SG matrix and the processing of precursor proteins. In particular the findings that (a) the acquisition of responsiveness to exocytotic stimuli is tightly regulated, (b) the secreted bioactive peptide cocktail is adjusted by the regulation of IDV budding and (c) SGs are segregated into distinct vesicle pools with defined release probabilities, suggest that the maturation of ISGs is not only under cellular control but also integrated into higher regulatory networks. Therefore, future studies should address the impact of physiological signals on ISG maturation.

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Chromogranins A and B and Secretogranin II as Prohormones for Regulatory Peptides from the Diffuse Neuroendocrine System

Karen B. Helle

Abstract Chromogranin A (CgA), chromogranin B (CgB), and secretogranin II (SgII) belong to a family of uniquely acidic secretory proteins in elements of the diffuse neuroendocrine system. These "granins" are characterized by numerous pairs of basic amino acids as potential sites for intra- and extragranular processing. In response to adequate stimuli, the granins are coreleased with neurotransmitters and hormones and appear in the circulation as potential modulators of homeostatic processes. This review is directed towards functional aspects of the secreted CgA, CgB, and SgII and their biologically active sequences. Widely different effects and targets have been reported for granin-derived peptides. So far, the CgA peptides vasostatin-I, pancreastatin, and catestatin, the CgB peptides $CgB_{1–41}$ and secretolytin, and the SgII peptide secretoneurin are the most likely candidates for granin-derived regulatory peptides. Most of their effects fit into patterns of direct or indirect modulations of major functions, in particular associated with inflammatory conditions.

1 Introduction

Chromogranin A (CgA), chromogranin B (CgB), and secretogranin II (SgII) are well established as members of a family of uniquely acidic proteins that are ubiquitous in secretory cells of the nervous, endocrine, and immune system (Huttner et al. 1991; Winkler and Fischer-Colbrie 1992). Five, more selectively distributed, acid-soluble and heat-stable proteins of neuroendocrine origin are also included in this family. As reviewed elsewhere (Helle 2004), these are SgIII, SgIV (HISL-19 antigen), SgV (neuroendocrine secretory protein 7B2), SgVI (NESP55), and SgVII (the nerve growth factor inducible protein VGF). All granins, being products of distinct genes,

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are characterized by numerous pairs of basic amino acids for cleavage by the costored prohormone convertases PC1/3 and PC2 and extracellular proteases such as plasmin (Parmer et al. 2000). The exocytotic corelease of CgA, CgB, and SgII with their costored amines and peptide hormones is by now a well-established concept (Feldman and Eiden 2003; Montesinos et al. 2008). This review is directed towards the extracellular effects of the granin cargo in relation to their postulated role as modulators of major functions, aiming at a coherent picture of CgA, CgB, and SgII and their derived peptides (Fig. 1a-c) in normal and pathophysiological conditions.

2 Granins and Granin-Derived Peptides

Immunoreactive CgA-, CgB-, and SgII-like proteins are widespread among mammals and occur in lower vertebrates (Montero-Hadjadje et al. 2008). Within the CgA protein the vasostatin-I (VS-I) sequence is highly conserved across vertebrates from fish to man, while the sequences for pancreastatin (PST) and catestatin (CAT) are either lacking or poorly conserved in submammalian vertebrates (Montero-Hadjadje et al. 2008). Although CgA and CgB are products of different genes, analyzes of their primary structure and gene organization have revealed a closer relationship between these two than between either protein with SgII and other members of the granin family.

On the other hand, CgB, containing the highest number of potential cleavage sites, seems more extensively degraded in the bovine chromaffin granule extracts than CgA (Fischer-Colbrie et al.1985; Metz-Boutigue et al. 1993) while only three processed products of SgII have so far been reported (Montero-Hadjadje et al. 2008).

2.1 The Prohormone Concept

The first reported peptide originating from a granin was the CgA-derived pancreastatin (PST), acting as an inhibitor of glucose-stimulated insulin secretion in the porcine pancreas (Tatemoto et al. 1986). This discovery formed the basis for the prohormone concept (Eiden 1987), implying that granins may serve as precursors of smaller peptides that, once released into the extracellular space, might serve some autocrine, paracrine, and/or endocrine function. The experimental support for this concept is steadily growing. There are numerous reports now on biological effects of granin-derived peptides, notably from CgA and SgII. These peptides have been postulated to participate in a wide range of processes such as innate immunity, inflammatory reactions, cardiovascular modulations, and several homeostatic regulations (Metz-Boutigue et al. 1998; Koeslag et al. 1999; Helle and Aunis 2000; Helle 2004; Fischer-Colbrie et al. 2005; Helle et al. 2007). Notably CgA and SgII appear to be involved in mechanisms of disease, such as hypertension, heart failure, and inflammatory syndromes (Taupenot et al. 2003; Ceconi et al. 2002; Ferrero et al. 2004; Di Comite et

al. 2009; Zhang et al. 2008; 2009a) Although a coherent picture of the physiological impact of these granin-derived peptides is yet to be drawn, the available information lends substantial support for significant contributions of peptides derived from CgA, CgB, and SgII as modulators of normal and pathophysiological functions.

2.2 CgA

CgA was the first granin to be isolated and characterized as a uniquely acidic protein costored and coreleased with the catecholamine hormones from the bovine adrenal medulla (Helle 2004). Contrary to earlier assumptions, CgA is not only a product of neuronal and glandular elements of the neuroendocrine system but appear also as a product of cardiocytes and polymorphonuclear neutrophils (PMNs). Accordingly, vertebrate hearts have proved fruitful as models for functional effects of not only VS-I (Corti et al. 2002, 2004; Tota et al. 2003; Imbrogno et al. 2004; Cerra et al. 2006, 2008; Cappello et al. 2007; Gallo et al. 2007) but also of CAT (Mazza et al. 2008; Angelone et al. 2008).

The N-terminal peptides $CgA_{1–76}$ and $CgA_{1–113}$ obtained from the retrogradely stimulated bovine adrenal medulla (Helle et al. 1993) were named vasostatins, VS-I and VS-II, respectively (Fig. 1a), as a reflection of their suppressive effects in precontracted isolated human conduit vessels (Aardal and Helle 1992; Aardal et al. 1993). VS-I is a natural cleavage product of CgA in man and larger mammals (Stridsberg et al. 2000) but not in the rat (Glattard et al. 2006) due to the absence of a pair of dibasic amino acids in the position 77–78, giving rise to a glutamine-rich, longer peptide, betagranin (rat $CgA_{1–128}$, Hutton et al. 1988). As illustrated in Fig. 1a, prochromacin is the largest VS-I free CgA peptide in bovine chromaffin granules (Metz-Boutigue et al. 1993; 1998) and occurs also as the main CgA productin the urine of carcinoid patients (Gadroy et al. 1998).

Prochromacin encompasses five other well-conserved domains of CgA, i.e. PST, WE-14, parastatin, catestatin (CAT), and GE-25 (Fig. 1a). In human plasma, PST occurs as a slightly elongated form and a substantially larger intermediate (Curry et al. 1990). In the pancreatic islet cells, PST is colocalized with insulin, glucagon and somatostatin (Schmidt and Creutzfeldt 1991), and histamine in the enterochromaffin cells of antrum of the stomach (Håkanson et al. 1995).

Parastatin was first isolated as a 74 residues long fragment from the porcine parathyroid CgA and the name reflects its inhibitory effect on secretion of both parathormone (PTH) and CgA from the porcine parathyroid cells (Fasciotto et al. 1993). As illustrated in Fig. 1a, parastatin comprises not only the highly conserved CAT domain but also GE-25 (Kirchmair et al. 1994). Processing of CgA to CAT occurs by intra- and extracellular processing (Parmer et al. 2000; Biswas et al. 2008). Biological activity has been assigned to CAT in a number of tissues such as bovine chromaffin cells (Mahata et al. 1997), the human baroreceptor centre of the nucleus tractus solitarius (Mahapatra 2008), porcine parathyroid cells (Fasciotto et al. 2000), rat mast cells (Krüger et al. 2003) in frog (Mazza et al. 2008) and rat heart (Angelone et al. 2008), and in Gram-positive and negative bacteria (Briolat et al. 2005; Radek et al. 2008).

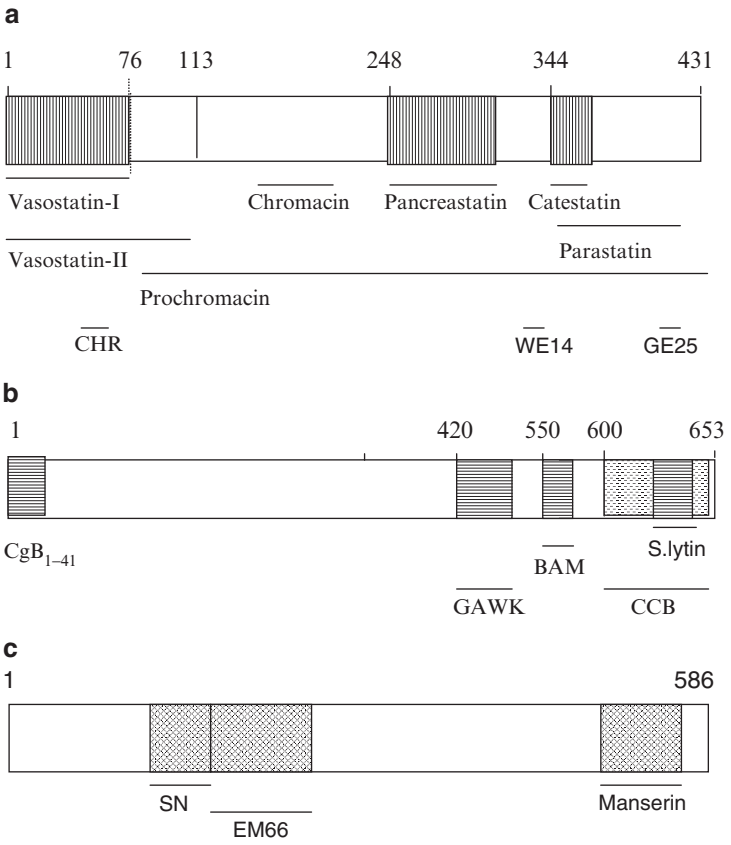

Fig. 1 Schematic illustration of peptides derived from (**a**) chromogranin A: vasostatin-I ($bCgA_{1–76}$), chromofungin (CHR, $CgA_{47–66}$), prochromacin ($bCgA_{79–431}$), chromacin ($bCgA_{173–194}$), pancreastatin ($bgA_{248–296}$), parastatin ($bCgA_{348–420}$), catestatin ($bCgA_{344–366}$), WE14 ($CgA_{316–330}$), GE25 ($_{367–391}$); (**b**) chromogranin B; $bCgB_{1–41}$, GAWK ($bCgB_{420–493}$), BAM ($bCgB_{547–560}$), CCB ($bCgB_{597–653}$), secretolytin (S. lytin, $bCgB_{614–626}$); and (**c**) secretogranin II: secretoneurin (SN, rat $SgII_{154–186}$), EM66 (rat $SgII_{189–256}$), manserin (rat $SgII_{529–568}$)

2.3 CgB

CgB is the largest and the least acidic of the granins, yet sharing with CgA not only the similar sized and structured disulfide-bridged loop at the N-terminus, but also the calcium binding capacity and aggregating properties (Huttner et al. 1991). Analogous to CgA, CgB is widespread in neuroendocrine cells of mammals, being expressed in species- and tissue-specific ratios relative to CgA and costored hormones (Rosa et al. 1985; Fischer-Colbrie et al. 1985). However, CgB, postulated to be released in constant ratio to insulin, has recently been shown to be largely segregated from CgA in the secretory granules, revealing that only 27% contained both CgA and CgB (Giordano et al. 2008).

As illustrated in Fig. 1b, cleavage at position 42–43 (Strub et al. 1995) results in the biologically active peptide $CgB_{1–41}$ (Russell et al. 1994), while cleavage at the C-terminus results in the antimicrobial peptide, secretolytin (Strub et al. 1995). So far, no biological activity has been assigned to other CgB peptides such as GAWK and CCB, being abundant in human pituitary gland extracts (Benjannet et al. 1987) and BAM-1745 and particularly in the arcuate nucleus of the hypothalamus of the human brain (Marksteiner et al. 1999).

2.4 SgII

SgII was initially identified as a sulfated secretory protein from the bovine anterior pituitary and the rat PC12 cell line, and the primary structures of bovine, rat, and human SgII were deduced from the respective cDNA sequences (Huttner et al. 1991).

Three peptides have so far been identified in SgII, i.e. secretoneurin (SN), Fischer-Colbrie et al. 1995), EM66 (Anouar et al. 1998), and the 40 amino acid residues long peptide, manserin (Yajima et al. 2004), as illustrated in Fig. 1c. SN is the most highly conserved region in SgII (Montero-Hadjadje et al. 2008) and immunoreactive SN is widely distributed (Kirchmair et al. 1993, 1994), overlapping partly but not completely established neurotransmitter and neuropeptide systems (Marksteiner et al. 1993). The order of free SN is (by concentration): intestine > brain > anterior pituitary > pancreas, and adrenal (Wiedermann 2000). Moreover, the N- and C-terminal domains of SN have been immunodetected in all insulin-positive cells, most of the glucagon cells, and some of the pancreatic poloypeptide cells while no SgII peptide could be detected in the somatostatin cells (Stridsberg et al. 2008). A wide range of biological activities has been assigned to SN (Vaudry and Conlon 1991; Kirchmair et al. 1993; Kähler and Fischer-Colbrie 2000) and there are indications of a functional relevance for EM66 in the control of food intake and/or the stress associated with fasting (Boutahricht et al. 2005).

3 Functional Aspects

3.1 Compensatory Upregulation of CgB and SgII in CgA Null Mice

Knockout technology has provided novel insight into granin functions. For instance, a compensatory increase in CgB has been demonstrated in the secretory granules of the adrenal medulla in CgA null mice, excreting elevated levels of catecholamines (Mahapatra et al. 2005; Hendy et al. 2006) despite reduced capacity for storage and exocytosis of catecholamines (Montesinos et al. 2008) and differences in developmental abnormalities in adrenomedullary morphology (Hendy et al. 2006). Moreover, a two- to threefold upregulated expression of CgB and other

members of the granin family (SgII–SgVI) appeared to compensate for the CgA deficiency in the CgA null mice (Hendy et al. 2006). Also, pancreatic CgB and SgII epitopes were expressed in the CgA null mice, although in lower levels than in the wild type (Portela-Gomes et al. 2008; Stridsberg et al. 2008) and plasma insulin was decreased although plasma glucose and glucagon levels were normal, consistent with increased glucagon cell function in the absence of CgA (Portela-Gomes et al. 2008). Although essential hypertension is associated with high plasma CgA, an elevated blood pressure is also evident in the ChgA null mice characterized by a higher than normal catecholamine secretion (Mahapatra et al. 2005). Intriguingly, an alleviation of hypertension could be obtained by genetic humanization of the Chga null mice or by venous infusion of exogenous CAT (Mahapatra et al. 2005), suggesting a hypotensive effect of CgA via CAT. However, it remains to be clarified whether the hypotensive effect of CAT is secondary to a CAT-evoked histamine release from mast cells in mice, as is the case in the rat (Kennedy et al. 1998), or to a modulation of the baroreceptor centre of the nucleus tractus solitarius, as suggested for the human CAT variant (Gly364-Ser) (Mahapatra 2008).

3.2 Circulating Granins

There is a relatively constant background of granins in the peripheral circulation and in the cerebrospinal fluid (CSF). Normal human serum contains low nanomolar concentrations not only of CgA (O'Connor et al. 1993) but also of CgB (Stridsberg et al. 1995; Aardal et al. 1996) and SgII (Kirchmair et al. 1994; Ischia et al. 2000). Taking into account the fact that all three granins occur in the brain and are released from the respective regions upon adequate stimuli, it is noteworthy that CgA, CgB, and SgII are represented in CSF by acidic domains largely devoid of biological activities (Stark et al. 2001; Helle 2004). The possibility that the basic and/or less acidic, biologically active peptides that may remain bound to their target tissues, might account for the unexpected and selective CSF patterns.

A vast number of reports on pathologically high plasma CgA have accumulated since the first documentation of increased levels in patients with neuroendocrine tumors (O'Connor and Bernstein 1984). Plasma CgA is by now a commonly used diagnostic and prognostic marker for tumors of neuroendocrine origin, using antibodies raised to a range of epitopes along the CgA molecule (Stridsberg et al. 2004; Greenwood et al. 2006; Børglum et al. 2007). Plasma CgA is also elevated in patients with a range of systemic diseases including renal and hepatic failure, cardiac arrest, and essential hypertension (Taupenot et al. 2003) as well as in inflammatory conditions such as heart failure (Corti et al. 2000; Ceconi et al. 2002), acute coronary syndromes (Jansson et al. 2009), rheumatoid arthritis (Di Comite et al. 2006, 2009), systemic lupus erythematosis (Di Comite et al. 2006) and acute systemic inflammatory response syndrome (Zhang et al. 2009a). It seems well established that increased plasma CgA is predictive of shorter survival, not only in patients with metastatic neuroendocrine tumors (Arnold et al. 2008; Nikou et al. 2008), but also

in chronic heart failure (Ceconi et al. 2002) and in the critically ill, nonsurgical patients (Zhang et al. 2008; 2009a). Intriguingly, lower than normal serum CgA has been reported for patients suffering from self-reported food hypersensitivity in association with symptomatic carbohydrate malabsorption (Valeur et al. 2008). This points to circulating CgA being implicated in functional gastrointestinal disorders yet to be elucidated.

Whether plasma CgA, CgB, and SgII serve solely as passive markers of the secretory state of the various elements of the diffuse endocrine system or, in addition, as active and functional contributors to homeostatic regulations of normal and clinical conditions, would depend on the ability of the prohormone and/or its derived peptides to activate or modulate relevant cellular functions.

3.3 Sources and Effects of Granin Peptides

3.3.1 Neurons and Chromaffin Cells

CgA coreleased with the catecholamine cargo from the sympathoadrenal components appears to be the major source for the autocrine, negative feedback control of the adrenomedullary release exerted by the CAT domain (Mahata et al. 1997). The mechanism for this specific, noncompetitive inhibition involves the neuronal nicotinic acetylcholine receptors (nAChRs), suggesting the open state of the channel as the target (Herrero et al. 2002). In addition, CAT, like substance P (SP) also inhibited the nicotine-induced desensitization of the receptor (Mahata et al. 1999).

SN occurs in high concentrations in several regions of the brain, the endocrine cells of the gastrointestinal tract, and in peripheral sympathetic and sensory nerves (Fischer-Colbrie et al. 1995). In the terminals of sensory nerves, SN is colocalized with SP and calcitonin gene-related peptide (CGRP) (Klimaschewski et al. 1995). In response to mechanical or immunological injury, the release of these sensory peptides results in neurogenic inflammation characterized by chemotaxis of leucocytes and their transendothelial passage to the sites of injury (Kähler and Fischer-Colbrie 2000).

3.3.2 Extraneuronal Sources

The heart, the gastrointestinal tract, and immune cells such as the polymorphonuclear neutrophils (PMNs), have recently attracted attention as extraneuronal sources of CgA in the rat and frog atrial myocytes (Steiner et al. 1989; Glattard et al. 2006) and the hypertrophied human ventricular myocardium (Pieroni et al. 2007). CgA is normally costored with ANP in classical secretory granules in the atrial myocardium while, in the hypertrophied human ventricular myocardium. CgA is expressed, colocalized and constitutively released together with BNP upon increased wall stress (Pieroni et al. 2007). Enterochromafin-like and enterochromaffin cells of the gastrointestinal tract also contain an abundance of CgA, costored and cosecreted with histamine (Håkanson

et al. 1995) or serotonin (Cubeddu et al. 1995) upon adequate stimuli. A range of CgA-derived fragments immunoreactive containing VS-I and CAT are produced and secreted by human PMNs when stimulated by the leukocidin Panton-Valentin (Lugardon et al. 2000; Briolat et al. 2005). Wherever PMNs accumulate in response to invading microorganisms, tissue inflammation, and sites of mechanical injury, this source of CgA peptides may affect a wide range of cells involved in inflammatory responses, e.g. endothelial, endocardial and epithelial cells, other leucocytes, fibroblasts, cardiomyocytes, and vascular and intestinal smooth muscle.

4 Granin Peptides and Targets

The first reported targets for granin peptides were the pancreatic β cells for the CgA-derived peptide PST (Tatemoto et al. 1986), the bovine parathyroid cells for $CgB_{1–41}$ (Russell et al. 1994) and the rat striatum for SN (Saria et al. 1993). During the last decade, the spectrum of targets has increased exponentially, notably for the CgA-derived peptides and for SN. There is also accumulating support for the vascular endothelium as a pivotal target not only for the CgA peptide VS-I but also for SN. In the following, the target systems will be discussed in relation to functions modulated by one or more of the granin peptides.

4.1 Antimicrobial Potencies and Innate Immunity

Among the different mechanisms integrated in the innate immunity, i.e. the inborn system of first defense against microorganisms, a range of natural cationic peptides have been isolated from insect lymph, skin of frogs, mammalian neutrophil granules, and plants as reviewed elsewhere (Helle et al. 2007). These peptides boost the innate immune responses by selectively modulating pathogen-induced inflammatory responses. During the last decade a range of natural antimicrobial peptides has been derived from the processing of granins, i.e. the CgA-derived VS-I, prochromacin, chromacin and CAT, and the CgB-derived secrelytin (Strub et al. 1995; Fig. 1a, b), implicating the adrenal medulla as a potential contributor to the innate immunity (Metz-Boutigue et al. 1998). Chromofungin (CHR, $CgA_{47–66}$) is the most active of the antifungal VS-I-derived peptides (Lugardon et al. 2001; Zhang et al. 2009b), revealing an amphipathic helical conformation related to a destabilization of the plasma membrane, allowing the peptide to penetrate by pore formation into fungi and yeast cells. Subsequently, the internalized CHR has been assumed to interfere with intracellular targets such as calcium-dependent calmodulin (CaM) dependent systems including the phosphatase activity of calcineurin (Lugardon et al. 2001).

Antimicrobial activity has also been assigned to the CgA-derived CAT and to the CgB-derived secretolytin. Consistent with an abundance of cationic charge, the active core of CAT, i.e. cateslytin ($CgA_{344–358}$), inhibits growth of Gram-positive and Gram-negative bacteria, a variety of filamentous fungi, and several

forms of yeasts (Briolat et al. 2005). Consistent with a role for CgA and CAT in immunoprotection, CAT penetration through human epidermis and inhibitory potencies against skin pathogens has more recently been demonstrated (Radek et al. 2008). Importantly, CgA was detected in keratinocytes and processed into CAT in human skin, while the expression of CAT in murine skin was increased in response to skin injury and infection.

Secretolytin (Fig. 1b) displays potent antibacterial activity against Gram-positive species and reveals sequence homology with the lytic domain of the insect cecropins and the pigmyeloid antibacterial peptide (Strub et al. 1995, 1996). No antimicrobial activity has so far been assigned to the negatively charged SN or to other domains of SgII.

Hence, evidence in favor of antimicrobial peptides derived from CgA and CgB is accumulating, seemingly providing protection against a wide variety of infections. These host defense peptides, notably VS-I, CAT, and secretolytin, have emerged as potential effectors for the innate immune system, suggesting roles in management of infections as antimicrobial peptides in their own right.

4.2 Inflammatory Conditions

Neurogenic apoptosis, inflammatory pain, and neuronal inflammation appear as potential conditions involving granin peptides. In particular, the endothelial barrier between the circulation and the underlying tissues has emerged as a conspicuous target for the granin peptides VS-I and SN revealing however, striking, counteracting effects on endothelial (EC) permeability. Moreover, granular immunocytes such as mast cells and PMNs have to be included as targets for CAT.

4.2.1 VS-I and Nitrergic Neurons in Gatrointestinal Pain

Inflammatory, somatovisceral pain may be induced experimentally by peritoneal application of acetic acid in vivo, abolishing the spontaneous contractile activity and decreasing the excitatory component of the tonic response to transmural nerve and reducing motility in human and rat colonic segments (Ghia et al. 2004a, b, 2005). Although without intrinsic activity, the very N-terminal domain of VS-I ($CgA_{1–16}$) exerted a nociceptive effect similar to CGRP, and capsaicin but not SP. Moreover, $CgA_{4–16}$ counteracted the acetic acid sensitive L-type of Ca^{2+} channels on both the colonic smooth muscle and the afferent nerve terminals. When intraluminal pressure was applied as the stimulus to rat proximal colon in vitro, low nanomolar concentrations of VS-1 and $CgA_{7–57}$ produced a concentration-dependent, progressive decrease in the mean amplitude of the spontaneous contractions in the circular layer of smooth muscle without affecting the resting tone (Amato et al. 2005). Taken together these studies support the concept of suppressive effects of the entire VS-1 molecule on elements of the gastrointestinal tract, presumably via activation of primary inhibitory nitrergic afferents, in addition to a direct inhibition of smooth muscle contractility.

4.2.2 VS-I, Microglia, and Neurogenic Apoptosis

As resident macrophages in the nervous system, microglial cells support neuronal survival and differentiation. By their release of neurotrophins, secretion, and responses to cytokines and by their stimulation of astrocytes, the microglial cells play a major role in the immune response (Ciesielski-Treska and Aunis 2000). CgA and VS-1 have been shown to activate cultured rat microglia in a manner analogous to but not identical to microbial toxins, triggering secretion of heat-stable, diffusible neurotoxins and accumulation of NO and TNFα (Taupenot et al. 1996; Ciesielski-Treska et al. 1998). The CgA induced reactive phenotype resulted in microglial apoptosis and death (Ciesielski-Treska et al. 2001). Moreover, a series of characteristic features, which forego neuronal apoptosis, were apparent in the CgA and VS-I stimulated cocultures of microglia and cortical neurons (Ciesielski-Treska et al. 2001). While the acute microglial activation by CgA and VS-I may be beneficial to the host, prolonged microbial activation cascades have been implicated in the inflammatory processes associated with degenerative disorders like Alzheimer's, Pick's, and Parkinson's diseases (Kingham and Pocock 2000; Hooper and Pocock 2007).

4.2.3 CAT, VS-I-Derived CHR, and Activation of PMNs

CAT has recently been reported to stimulate chemotaxis in human PMNs in a concentration-dependent manner with maximal potency at 1 nM, similar to that of the formylated chemoattradctant Met-Leu-Phe (fMLP; Egger et al. 2008). Intriguingly, the naturally occurring human variants of CAT varied in potencies, being highest for Pro370Leu and lowest for Gly364Ser. Moreover, CAT stimulated Akt- and extracellular signal related kinase (ERK) phosphorylation, and the effect was blocked by antagonists to a wide range of signaling pathways, indicating involvement of tyrosine kinase receptor-, G-protein-, and sphingosine-1-phosphate signaling. The authors conclude on a role for CAT as an inflammatory cytokine, of possible implications for the extensive microglial activation and neuronal damage in relation to the CgA-containing Alzheimer plaques.

Moreover, CAT and the cationic and amphipathic CHR domain of VS-I (Fig. 1a) have most recently been shown to activate unstimulated human PMNs by provoking a transient influx of Ca^{2+} and leading to exocytosis of a series of relevant immunoregulating processes (Zhang et al. 2009b). The mechanism for this activation by CAT and CHR involves CaM binding and subsequent activation of Ca^{2+}-independent phospholipase A_2. Thus, CgA released from bacteriotoxin-stimulated PMNs might provide paracrine stimuli for unstimulated PMNs, thereby propagating their immunoregulatory contributions.

4.2.4 CAT and Histamine Release from Mast Cells

As granular immunocytes, mast cells reside in the barrier tissues where they orchestrate inflammatory responses. In rat mast cells the N-terminal, biologically active domain

of CAT, i.e. cateslytin ($CgA_{344–358}$), is a potent activator of histamine release (Krüger et al. 2003), accounting for the reduced pressor response to an intravenous injection of CAT in rats sensitive only to the H1 type of histamine blockers (Kennedy et al. 1998). Moreover, the potency and efficacy of cateslytin were higher than of the cationic wasp venom mastoparan and the sensory neuropeptide SP (Krüger et al. 2003). A pertussis-toxin (PTX)-sensitive, peptidergic, and receptor-independent pathway has already been established for mastoparan, SP, and other amphiphilic cationic neuropeptides on histamine release from rat mast cells (Jones and Howl 2006). Hence, it seems likely that the cateslytin domain of CAT may stimulate mast cell release by a similar PTX-sensitive pathway (Helle 2009), in marked contrast to the inhibitory, autocrine effect of CAT on adrenomedullary catecholamine release (Mahata et al. 1997). By comparison, neither the VS-I derived peptides nor WE-14 were able to elicit histamine release from the rat mast cells (unpublished observations).

4.2.5 VS-I, SN, and EC Integrity

The vascular (ECs) and endocardial endothelia (EECs) form barriers against transvascular exchange of fluids, proteins, and blood cells. ECs and EECs may themselves be targets for granin-derived peptides, whether released locally or delivered via the circulation, affecting secretion, contractile properties, and transport of other cells and substances through gaps in the otherwise confluent monolayer. Notably, VS-I (Ferrero et al. 2004) and SN (Kähler et al. 2002b) may modulate transendothelial transport of leukocytes as part of the inflammatory response, however in opposite directions.

In vivo and in vitro experiments strongly suggest that CgA via VS-I at pathophysiological concentrations at and above 7 nM may prevent the TNFα-induced extravasation of macromolecules by targeting to EC in mouse liver in vivo and in cultured monolayers (HUVEC) in vitro (Ferrero et al. 2004). Analogously, VS-I also inhibited TNFα-induced formation of gaps in cultured arterial EC (Blois et al. 2006). In addition, VS-1 also partially inhibited thrombin- and vascular endothelial growth factor (VEGF)-induced permeability through confluent monolayers of HUVECs (Ferrero et al. 2004). Taken together, these findings suggest a role for VS-I in the protection of the EC barrier against the gap-forming, permeabilizing activity of TNFα by a mechanism involving cytoskeletal reorganization and downregulation of the transmembrane protein inter-cellular VE-cadherin responsible for for cell–cell adhesion (Ferrero et al. 2004). A pivotal role for VS-I as an inhibitor of the PTX- and TNFα activated p38MAP kinase phosphorylation was demonstrated in pulmonary arterial EC (Blois et al. 2006), implicating VS-1 in the protection of a Gαi-coupled tonic inhibition of the p38MAPK activity in the PTX-sensitive pulmonary EC (Garcia et al. 2002; Helle 2009).

In HUVECs, the protective effect of VS-1 on EC integrity is not limited to inhibition of gap formation induced by proinflammatory agents, but also appears to inhibit motility and basal ERK phosphorylation, leading to a more quiescent stage without apoptotic or necrotic effects (Belloni et al. 2007). Importantly, VS-1 also inhibited the VEGF-induced ERK phosphorylation, cell migration, proliferation, morphogenesis, and invasion of collagen gels in various in vitro assays (Belloni et al. 2007).

Contrary to VS-I, SN has been reported to impair the integrity of the EC barrier by reducing the expression of Zona occuludens-1 and occludin and activating JNK and ERK1/2, but not p38MAPK in human coronary arterial EC (Yan et al. 2006).

Of note, SN was almost as effective as TNFα in stimulating transmigration of PMNs via an EC pathway involving PTX, CTX, and staurosporine-sensitive signaling (Kähler et al. 2002a). In addition, SN may recruit immunocompetent monocytes and PMNs to the sites of injury. A selective SN-induced chemotaxis of human monocytes in vitro and in vivo (Reinich et al. 1993) and their adhesion to arterial and venous EC (Kähler et al. 2002a) appear to precede their transendothelial migration (Kähler et al. 1999; Kähler and Fischer-Colbrie 2000). Hence, with respect to of EC permeability, VS-I and SN appear to have striking, opposite effects.

4.3 Other Cardiovascular Functions

A range of inhibitory effects by VS-I has been reported for blood vessels and elements of the heart. The first experimental models were isolated segments of human intrathoracic arteries and saphenous veins (Aardal and Helle 1992; Aardal et al. 1993; Angeletti et al. 1994), revealing suppressive effects on precontracted vessel segments. Most recently, several models of the vertebrate heart have been introduced (Corti et al. 2002; Imbrogno et al. 2004; Cerra et al. 2006). Common to the vertebrate hearts is a negative myocardial inotropy elicited not only by the highly conserved VS-I domain in CgA but also by CAT.

4.3.1 VS-I and Vasodilatation

In human vessel segments, the natural bovine VS-I + VS-II and the synthetic $CgA_{1–40}$ suppressed the ET-1 contractions independent of EC and extracellular calcium, affecting the maximal sustained tension response but not the potency for ET-1 (Aardal and Helle 1992; Aardal et al. 1993). Inhibitory effects of VS-I and $CgA_{1–40}$ were also evident in isolated and pressurized bovine coronary resistance arteries (Brekke et al. 2002). Here, $CgA_{1–40}$ evoked dilatation independent of other constrictors over a functional range of transmural pressures. Moreover, the intrinsic and concentration-dependent dilator effects persisted at moderately elevated extracellular K^+ (Brekke et al. 2002), but was prevented by PTX and by antagonists to several subtypes of K^+ channels, suggesting vasodilatation by a $CgA_{1–40}$ and VS-I induced hyperpolarization via opening of K^+ channels in the smooth muscle.

4.3.2 VS-I and CAT on Myocardial Inotropy

The vertebrate heart, consisting of the epicardium, the myocardium, EEC, and the coronary blood supply, is a complex system to analyze for tissue-specific effects

of any given substance. As a simplistic first model, the avascular myocardium of the frog (*Rana esculenta*) was chosen for the assessment of myocardial effects of CgA peptides (Corti et al. 2002). A calcium-dependent negative inotropism was observed in response to the recombinant human $STACgA_{1-78}$ (VS-1) This effect in the frog was independent of EEC, adrenergic, and muscarinic receptors and was completely antagonized by Ba^{2+}, suggesting involvement of K^+ channels and hyperpolarization in the cardiomyocytes (Corti et al. 2004). Moreover, VS-1 also counteracted the characteristic inotropism exerted by the β-adrenoceptor agonist isoprenaline (ISO). The natural loop structure of frog and bovine VS-I was essential for both the negative inotropism and the counteraction of the β adrenoceptor activation (Tota et al. 2003).

In contrast, in the eel (*Anguilla anguilla*), VS-I derived peptides induced a negative basal and ISO stimulated myocardial inotropy that was dependent on EEC functions, notably the NO-cGMP-PKG pathway (Imbrogno et al. 2004). Analogously, in the Langendorff preparation of the nonworking rat heart, a perfusion with VS-1 caused a negative inotropic effect including inhibition of the inotropic response to ISO via EC-dependent NO production (Cerra et al. 2006, 2008), suggesting that, **w**hatever the subcellular signaling route, VS-1 may exert negative inotropic effects on vertebrate hearts. Intriguingly, VS-I was ineffective on the basal contractility on rat papillary muscle while partially reducing the effect of ISO stimulation via EC-derived NO production (Gallo et al. 2007). Moreover, removal of EC and inhibition of NO synthesis and PI3K activity abolished the antiadrenergic effect of VS-1, indicating that the antiadrenergic effect in the rat heart is also due to a PI3K-dependent NO release from EC rather than to a direct action on the cardiomyocytes. Moreover, two different pathways appear to mediate the protective activity of VS-1 against ischemic insults in the rat heart, one via A1 receptors and the other by NO release, both converging on PKC (Cappellio et al. 2007). Enhancing NO production, either through a direct control of eNOS or through modulation of Gαi/o proteins, is one alternative, another being PKG controlling intracellular calcium homeostasis and utilization. PKG may also exert a feedback regulation of Gαi/o proteins, thereby generating a circuit of interactions converging to depress contractility. Taken together, the findings support the concept of a cardiosuppressive function of VS-I in vertebrates, which apart from the frog, appears to be mediated by EC-dependent NO production. Of note, not only VS-1 but also CAT has been shown to exert negative myocardial inotropy and to noncompetitively inhibit the β-adrenoceptor on the cardiomyocyte, presumably mediated by the relaxing effect of the EC-derived NO release mediated by Akt/PKB signaling to eNOS (Angelone et al. 2008). In addition, a noncompetitive inhibition of the ET-1 receptor in the rat cardiomyocyte has also been assigned to CAT (Angelone et al. 2008). However, in contrast to VS-I, CAT also increased heart rate and coronary pressure, suggesting significant peptide specific differences in coupling to some tissue responses. Moreover, the EC-dependent, PTX-sensitive negative inotropic responses to both VS-1 and CAT in the rat heart raise the question whether these two distinctly different CgA sequences may act competitively or synergistically, targeting to identical or different PTX-sensitive Gαi/o subunits in the EC membrane.

4.3.3 VS-I and SN on Cell Motility

Cell-adhesive effects of the intact human CgA and VS-1 have been observed in human and mouse fibroblasts and in human coronary artery smooth muscle cells, but not in neuroblastoma cells (Gasparri et al. 1997; Ratti et al. 2000). Importantly, the antiadhesive effect of the intact prohormone could be changed into a proadhesive effect upon limited tryptic treatment (Corti et al. 2004b). An indirect mechanism, probably dependent on stimulated synthesis of other cell surface proteins, was suggested from 3 to 4 h lag time for these antiadhesive effects.

In contrast, SN actively stimulated cell motility in human skin fibroblasts but failed to induce proliferation (Kähler et al. 1997a). SN also induced a directed, selective migration of cultured rat aortic smooth muscle cells and stimulated cell proliferation and DNA synthesis (Kähler et al. 1997a, b; Kähler and Fischer-Colbrie 2000).

4.3.4 VS-I and SN in Angiogenesis and Vasculogenesis

Angiogenesis is defined as the generation of new vessels by sprouting from the already existing vasculature, stimulated by VEGF and the basic fibroblast growth factor in vivo. Vasculogenesis implies, on the other hand, de novo formation of vessels from circulating endothelial progenitor cells in the embryo, from bone-marrow derived endothelial progenitor cells or from circulating precursor cells in postnatal neovasculogenization (Kirchmair et al. 2004a, b). There is to date only one report implicating a role for VS-1 in angiogenesis (Belloni et al. 2007). Here, an inhibitory effect of VS-1 on the formation of capillary-like structures could be demonstrated in a matrigel assay in a rat model. In addition, VS-1 inhibited the VEGF-induced migration, proliferation, morphogenesis, and invasion of collagen gels in HUVECs in vitro. Analogous to VS-1, SN inhibited the proliferation of HUVEC when stimulated by fibroblast growth factor (Kähler et al. 1997a).

However, more recent reports have demonstrated that SN may act as an angiogenic cytokine comparable in potency to VEGF when assayed in a mouse cornea neovascularization model in vivo, stimulating a dose-dependent and specific capillary tube formation in a matrigel assay in vitro (Kirchmair et al. 2004a). Here, SN also stimulated proliferation and exerted antiapoptotic effects on EC. In a separate study with the same model, systemic injections of SN led to an increase in circulating stem cells and endothelial progenitor cells to sites of vasculogenesis in vivo, confirming stimulatory effects on proliferation and antiapoptotic effects (Kirchmair et al. 2004b).

VEGF is an angiogenic cytokine that is enhanced by hypoxia like a range of other angiogenic factors. It has also been shown that SN is upregulated by hypoxia, however in a tissue-specific manner, being present in muscle cells but not in EC, vascular smooth muscle cells, or pituitary tumor cells (Egger et al. 2007). Hence, SN may play a role in hypoxia-driven induction of neovascularization in ischemic diseases like peripheral or coronary artery disease, diabetes, retinopathy, central ischemia, or in solid tumors (Fischer-Colbrie et al. 2005; Egger et al. 2007).

4.4 CgA Peptides as Modulators of Calcium and Carbohydrate Homeostasis

The parathyroid hormone (PTH), being costored and coreleased with CgA (Cohn et al. 1982), is a primary homeostatic regulator of plasma Ca^{2+}. While the release of PTH is stimulated at low plasma Ca^{2+}, the hormone release is inhibited not only by elevated plasma Ca^{2+} via hyperpolarization (Välimäki et al. 2003) but also by the CgA peptide VS-I at low plasma Ca^{2+}as effectively as by the physiologically high concentrations of Ca^{2+} (Angeletti et al. 2000).

PST inhibition of the first phase of the glucose-stimulated secretion of insulin (Tatemoto et al. 1986) was sensational, implicating a novel role for CgA in regulation of carbohydrate metabolism. By now, it is well established that the islet cells of the endocrine pancreas together with the liver and adipose tissue represent essential targets for PST in the homeostatic regulation of plasma glucose.

4.4.1 VS-I, PST and Parastatin/CAT on PTH Secretion

Both the natural and synthetic VS-I and the N-terminal $CgA_{1–40}$ actively inhibit PTH release in the bovine parathyroid cells (Russell et al. 1994; Angeletti et al. 1996). Also $CgB_{1–41}$, has been shown to be an active inhibitor of PTH release (Russell et al. 1994), suggesting that the N-terminal loop domains of CgA and CgB may perform the same function in this tissue, whether serving as autocrine (CgA) or endocrine (CgB) inhibitors of PTH secretion (Angeletti et al. 2000). A partial inhibition of PTH secretion by PST was regarded as physiologically irrelevant due to the low degree of CgA processing into PST in this tissue (Drees and Hamilton 1992). The CAT-containing parastatin (Fig. 1a) was also found to inhibit the cosecretion of PTH and CgA in the porcine parathyroid (Fasciotto et al. 1993, 2002), but with markedly lower potency than with VS-I in the bovine parathyroid cells. Hence, these findings suggest that three domains of CgA may contribute to modulation of PTH secretion and that VS-I via its N-terminal domain $CgA_{1–40}$ appears as the most likely autocrine inhibitor of PTH release at low plasma Ca^{2+} in the bovine parathyroid cells.

4.4.2 PST on Carbohydrate Homeostasis

CgA processing in the human gastrointestinal tract reveals cell and region-specific patterns (Portela-Gomes and Stridsberg 2001, 2002a, b; Portela-Gomes et al. 2008). Although the physiological relevance of PST in humans has been questioned due to the low degree of processing (Schmidt and Creutzfeldt 1991), a later study indicated that human PST (hP-16) corresponding to the amidated C-terminus of $hCgA_{286–301}$, might be involved in reduction of elevated blood glucose and insulin levels after oral glucose load in nondiabetic humans (Siegel et al. 1998). The endocrine role of PST in humans has also been approached by a different experimental design

(O'Connor et al. 2005). PST infusion into the brachial artery at a supranormal concentration was without intrinsic effects, yet it reduced the A-V glucose difference, and inhibited uptake of glucose and free fatty acids without affecting blood flow. The regulatory effects of PST on liver and adipose tissues are to date best documented in vitro, as extensively reviewed elsewhere (Sanchez-Margalet et al. 2000). In the rat, hepatocytes and adipocyte PST inhibit insulin-mediated glucose transport, glucose utilization, and lipid synthesis. A lipolytic effect has also been demonstrated in addition to a PTX stimulated basal and insulin-stimulated protein synthesis (Gonzalez-Yanes and Sanchez-Margalet 2002). These in vivo and in vitro results support the concept of hepatocytes and adipocytes as well pancreatic β cells as likely targets for PST in the rat. However, the postulated inhibitory role of PST on the first phase of glucose-stimulated insulin release from the human pancreas (Tatemoto et al. 1986) still awaits experimental support.

5 Towards a Unifying Concept for Extracellular Functions of CgA, CgB, and SgII

The release of CgA, CgB, and SgII with costored amines and peptide hormones from elements of the diffuse neuroendocrine system upon adequate stimuli from the external environment and internal milieu is well-established. Although a coherent picture of the functional implications of CgA, CgB, and SgII and their derived peptides is still not complete, it is evident from the accumulated evidence that a wide range of processes associated with homeostasis appear to be modulated by one or several of the peptides derived from these granins.

As illustrated in Table 1, CgA emerges notably as a multifunctional prohormone, giving rise to at least three peptides, modulating not only calcium and carbohydrate

Table 1 Reported actions of granin-derived peptides with functions involved in homeostatic regulations

	CgA	CgA	CgA	CgA	CgB	CgB	SgII
	VS-I	PST	CAT	Parastatin	$CgB_{1–41}$	Secretolytin	SN
Calcium metabolism	Inhib	Inhib	–	Inhib	Inhib	–	–
Carbohydrate metabolism	–	Inhib	–	–	–	–	Disrupt
EC integrity	Prot	–	–	–	–	–	–
Heart, blood vessels	Inhib	–	Inhib	–	–	–	–
Innate immunity	Anti	–	Anti	–	–	Anti	–
Microglia, mast cell	Act	–	Act	–	–	–	–
GI pain	Inhib	–	–	–	–	–	Act
Cell motility etc.	Inhib	–	Act	–	–	–	
Angiogenesis	Inhib	-	-	-	-	-	Act
Vasculogenesis	-	-	-	-	-	-	Act

Inhib Inhibition, *Act* activation, *Prot* protection, *Disrupt* disruption, *Anti* antimicrobial, not demonstrated

metabolism but also EC integrity, myocardial inotropy, microbial control, innate immunity gastrointestinal pain, cell adhesion, migration, and proliferation. Intriguingly, the N-terminal VS-I stands out as the most versatile among the CgA peptides, affecting all sectors but carbohydrate metabolism. While three CgA domains and the N-terminal CgB peptide may inhibit PTH release from the parathyroid, PST appears as the only granin peptide with modulating potentials on carbohydrate metabolism.

Another aspect of considerable interest is the apparent convergence of the two structurally different CgA peptides, VS-I and CAT, on the heart, both inhibiting myocardial contractility via activation of PTX-sensitive EC production of NO in the rat heart.

Nevertheless, the most striking aspect of the granin peptides is their association with inflammatory conditions. It seems likely that concerted effects of VS-I, CAT, and secretolytin may be relevant for the first-line host-defense against invading microorganisms. Moreover, several immunocompetent cells also respond to CgA peptides. For example, the rat microglia becomes activated by VS-I to cause neuronal apoptosis, while not only the rat mast cells but also human PMNs may respond to CAT, to evoke markedly different responses, i.e. histamine release and cellular migration and secretion, respectively. Furthermore, activation by VS-I of primary inhibitory nitrergic afferents in elements of the gastrointestinal tract points to a contribution to pain reduction during inflammatory conditions.

Although being devoid of antibacterial potencies, one may regard SN as an indirect contributor to innate immunity in view of its activation of chemotaxis, transendothelial extravasation, and migration of leukocytes. In this context the oppositely directed effects of VS-I and SN on EC permeability are particularly important. Where SN appears to induce EC permeability for transendothelial transport of immunocompetent leucocytes, VS-I seemingly protects the integrity of the EC barrier against the disruptive effects of proinflammatory agents. It is by no means clear to what extent these opposite effects occur within the same frames of time and space. Rather than a direct competition between VS-I and SN on regulation of EC permeability, it is tempting to speculate that there may be a timelag between the release of SgII derived SN from sensory nerves in response to a mechanical or inflammatory injury and the release of CgA and VS-I from activated PMNs at site of inflammation. If SN initially triggers transendothelial passage of leukocytes including PMNs, a subsequent release of CgA-derived VS-I and CAT from activated PMNs might serve to combat the microbial invasion. In addition, the SN-induced EC leakage might be counteracted by VS-I to protect EC against further barrier disruption and transendothelial leakage of cells and solutes. In the case of CgA releasing tumors, VS-I may protect the host against transendothelial transport of tumor-derived products.

Intriguingly, VS-I and SN also appear to exert opposite effects on new formation of blood vessels. While VS-I appears to inhibit VEGF-induced cell migration, proliferation, morphogenesis, and invasion of collagen gels inherent in angiogenesis, SN has, in contrast, been shown to activate EC chemotaxis, proliferation, angiogenesis, and vascularization while inhibiting EC apoptosis, suggesting a significant role for SN also in tissue repair.

Two aspects remain presently unanswered, namely the question of receptors and concentrations needed to obtain the reported effects. For VS-I or SN, there is to date no reported extracellular receptor, while for CAT the nicotinic acethylcholin receptor in the sympatoadrenal system mediates only the autocrine inhibitory effect on the adrenal medulla. For VS-I and PST, there are reports on peptide-binding membrane proteins of the order of 70–80 kDa coupled to G-proteins. It has been postulated that hydrophobic and amphipathic properties of VS-I and CAT might allow for their receptor-independent penetration into and activation of cells (Helle 2009). However, a similar mechanism seems rather unlikely for the highly acidic SN.

With respect to effective concentrations, many of the reported responses to the CgA peptides may come into play under pathophysiological conditions, e.g. during hypertension, cardiac heart failure, and inflammatory conditions in various organs. On the other side, the local concentrations of SN at sensory nerve terminals near a blood vessel may be high enough to induce EC permeability for tissue repair during mechanical and inflammatory injuries.

To conclude, although the physiological impact of these granin-derived peptides is yet to be fully understood, the accumulated evidence on significant contributions of peptides derived from CgA, CgB, and SgII lend substantial support to the hypothesis that these costored and coreleased granins serve as prohormones for regulatory peptides with impact on a wide range of normal and pathophysiological functions.

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Cell-Specific Precursor Processing

Jens F. Rehfeld and Jens R. Bundgaard

Abstract The singular gene for a peptide hormone is expressed not only in a specific endocrine cell type but also in other endocrine cells as well as in entirely different cells such as neurons, adipocytes, myocytes, immune cells, and cells of the sex-glands. The cellular expression pattern for each gene varies with development, time and species. Endocrine regulation is, however, based on the release of a given hormone from an endocrine cell to the general circulation from whose cappilaries the hormone reaches the specific target cell elsewhere in the body. The widespread expression of hormone genes in different cells and tissues therefore requires control of biogenesis and secretion in order to avoid interference with the function of a specific hormonal peptide from a particular endocrine cell. Several mechanisms are involved in such control, one of them being cell-specific processing of prohormones. The following pages present four examples of such cell-specific processing and the implications of the phenomenon for the use of peptide hormones as markers of diseases. Notably, sick cells – not least the neoplastic cells – often process prohormones in a manner different from that of the normal endocrine cells.

1 Introduction

The fundamental concept of endocrinology originates back to the discovery of the first hormone, secretin, by Bayliss and Starling (1902). The concept is simple: A hormone is released from specific endocrine cells into circulation upon appropriate stimulation of the cells. Via blood the hormone reaches its more or less distant target cells which are equipped with receptors for high-affinity binding of the particular hormone. Receptor binding subsequently elicits the intended action of

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the target cell. For example, gastric acid in the duodenum is neutralized by the bicarbonate secreted from exocrine pancreatic cells to the duodenal lumen after the release of secretin from the duodenal S-cells to blood and subsequent secretin binding to the pancreatic ductular cells (Bayliss and Starling 1902; Leiter et al. 1994). And glucose homeostasis in the body is achieved by glucose and incretin induced secretion from the pancreatic beta-cells from where insulin reaches its target cells in the liver and muscles. This monolithic concept still prevails in the descriptions of endocrine regulation in textbooks of physiology and medicine.

Modern biochemistry and molecular biology have, however, challenged and changed not only general cell biology but also basic endocrinology. Thus, it is now well established that the gene of a given peptide hormone is expressed in different types of endocrine cells, neurons and in some instances also in cells such as adipocytes, myocytes, osteoblasts, and immune cells (for review, see Rehfeld 1998b). Today, only a few hormones – including the old master hormone insulin – represent the original endocrine paradigm. Instead, the understanding of widespread cellular expression now raises the question of how the body maintains a fine-tuned regulation of its functions by individual peptide hormones, when a given hormone may originate from a broad variety of cells. Fortunately, it has become apparent that several mechanisms act to ensure lack of interference. The first mechanism is simple and quantitative. Thus, peptide hormone expression in cells and organs vary considerably during development –at the transcriptional, posttranscriptional, translational and posttranslational levels. For instance, the fetal endocrine pancreas in mammals expresses large amounts of bioactive gastrin peptides which, however, disappear soon after birth so that antroduodenal G-cells take over more than 95% of the normal gastrin production for the rest of life (Larsson et al. 1976; Brand et al. 1984a, b). Secondly, biological barriers such as the blood–brain barrier ensure that the peptide hormones in peripheral circulation do not compete with neuronal release of similar or identical neuropeptides in the cerebral synapses. Thirdly, peripheral paracrine release of peptides between neighboring cells or autocrine secretion from self-stimulating cells display a large concentration gradient in tissue, so that locally released peptides do not interfere significantly with the systemic distribution of hormones in circulation. Finally, the cell-specific processing of prohormones contributes to avoid interference of the same hormone by ensuring that different bioactive fragments of the prohormones are released from different cells. Thus, functional implications of the fact that different cell types express the same hormone gene are under control by counter-regulatory physiological mechanisms in the normal organism.

Apart from a theoretical and general interest in cell-specific precursor processing, the phenomenon has direct interest for several diseases; for instance, for various forms of tumors and cancer. Hence, not only do rare neuroendocrine tumors produce hormonal peptides of various kinds; but also major gastrointestinal, genital, pancreatic, and pulmonary cancers release peptide hormones that may accelerate the growth of malignant cells and thus play a significant carcinogenetic role (for reviews, see Rehfeld and van Solinge 1994; Watson et al. 2006). Moreover, both for specific neuroendocrine tumors and common cancers the peptide hormones

and their precursors may serve as useful biomarkers. For this purpose processing-independent analysis (PIA) may be of use because PIA bypasses analytical problems derived from cell-specific processing (Bardram and Rehfeld 1988; Goetze et al. 2002; Rehfeld and Goetze 2003). Finally, cell-specific products of hormone biogenesis may prove useful molecular targets in modern cancer therapy (for review, see Watson et al. 2006).

In the following, four examples of cell-specific precursor processing are first presented and some clinical implications are described. Subsequently, the principles and practice of PIA are reviewed.

2 Examples of Cell-Specific Prohormone Processing

2.1 Progastrin

In adult vertebrates, G-cells in the antral part of the stomach express by far the most progastrin, but G-cells are present also in the duodenum, although to a lesser extent (Larsson et al. 1974; Larsson and Rehfeld 1979a). G-cells are equipped with an extensive machinery of processing enzymes located along the secretory pathway (Bundgaard et al. 1995, 2004; Bundgaard and Rehfeld 2008). Matured by the enzymes, the original progastrin molecule ends up in secretory granules almost completely processed to bioactive alfa-amidated gastrins, of which nearly 90% are gastrin-17 and 5–10% are gastrin-34 (Håkanson et al. 1982; Hilsted and Rehfeld 1987). Half of both gastrin-17 and gastrin-34 are *O*-sulfated (Gregory and Tracy 1964; Gregory et al. 1964; Brand et al. 1984b; Bundgaard et al. 1995, 1997). Beyond these major gastrins, a few additional alfa-amidated gastrins are also synthetized (gastrin-71, gastrin-14 and gastrin-6), but in low, almost negligible, amounts in normal G-cells (Hilsted and Rehfeld 1987; Rehfeld and Johnsen 1994; Rehfeld et al. 1995). Also inactive complementary flanking fragments of progastrin as well as some processing intermediates are present in the secretory G-cell granules (Rehfeld and Johnsen 1994; Goetze et al. 2006; Smith et al. 2006). In the normal organism almost all bioactive gastrin in circulation originates from the antrum and duodenum in the forms of gastrin-17 and gastrin-34.

The gastrin gene is expressed in cell types other than antroduodenal G-cells. Quantitatively, the other cells contribute only little to the circulating gastrins in plasma in adults. This is because the level of expression in adults is low, and because the biosynthetic processing is cell-specific. So far, we have encountered expression of the gastrin gene outside the antroduodenal mucosa in the distal small intestine originating from the so-called TG-cells (Larsson and Rehfeld 1979a); in unidentified cells in the colon (van Solinge et al. 1993a; Lüttichau et al. 1993); in endocrine cells in the fetal and neonatal pancreas (Larsson et al. 1976); in pituitary corticotrophs and melantrophs (Rehfeld 1978a; Rehfeld and Larsson 1981; Larsson and Rehfeld 1981); in oxytocinergic hypothalamo-pituitary neurons (Rehfeld 1978a, b; Rehfeld et al. 1984); in a few cerebellar and vagal neurons (Rehfeld 1991;

Uvnäs-Wallensten et al. 1977); in the adrenal medulla (Bardram et al. 1989); in the bronchial mucosa (Rehfeld et al. 1989); in the postmenopausal ovaria (van Solinge et al. 1993b); and in the spermatogenic cells (Schalling et al. 1990).

The function of gastrin synthesized outside the antroduodenal G-cells is unknown, but suggestions can be offered. The first possibility is paracrine regulation of growth. Secondly, it is possible that the low concentration of peptides is without significant function in the adult, but is a relic of a more comprehensive fetal synthesis. A third possibility is that the low cellular concentration reflects constitutive rather than regulated secretion where the peptides are stored in secretory granules.

Although the extra-antral synthesis of gastrin may be without functional significance in the normal adult organism, the phenomenon is interesting from a cell-specific, biosynthetic and carcinogenetic point of view. For instance, no gastrin-17 is synthesized in the pituitary corticotrophs due to the lack of prohormone convertase 2 (PC2) (Rehfeld 1978a; Rehfeld and Larsson 1981; Rehfeld et al. 2002a, 2008a). Moreover, the gastrin-34 produced in the corticotrophs is not sulfated (Rehfeld and Larsson 1981). Vice versa, the amidated gastrin synthesized in neonatal, pancreatic islets is completely sulfated gastrin-17 (Brand et al. 1984a, b; Cantor et al. 1986), suggesting that PC2 and tyrosyl-protein sulfotransferases are highly active in islet cells. The main features of the cell-specific progastrin processing in antral G-cells, pancreatic islet cells, and pituitary corticotrophs are illustrated in Fig. 1.

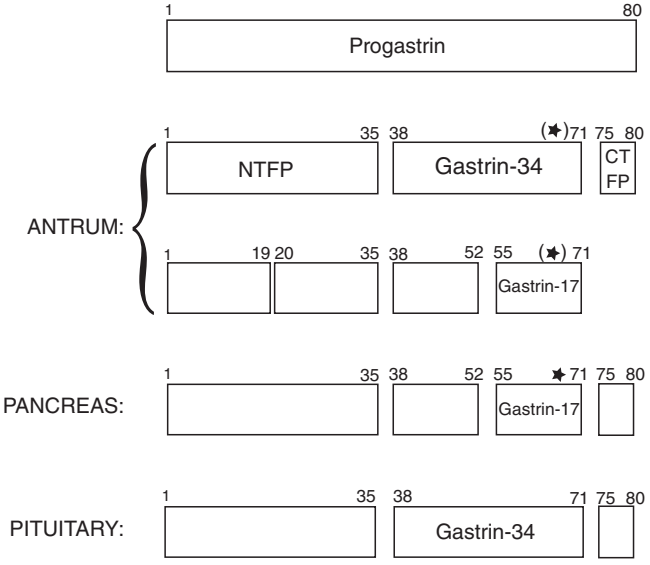

Fig. 1 Simplified scheme of the cell-specific processing of progastrin in the antrum, the pancreas and the pituitary gland. The numbers show amino acid positions in the 80 amino acid residue progastrin sequence. The *asterixes* show the position fo the *O*-sulfated tyrosol residue in the bioactive gastrin-34 nad gastrin-17 sequences. NTFP and CTFP are acronyms for "N- and C-terminal flanking peptides," respectively

2.2 Procholecystokinin

Although mammals synthesize the most cholecystokinin (CCK) in cerebral neurons, an essential portion is also synthesized in the endocrine I-cells of the small intestine (Larsson 1980). Hence, almost all CCK in plasma originates from the I-cells (Rehfeld et al. 2001). The I-cells, however, are so disseminated in the intestinal mucosa, and the intensity of the biosynthesis in per gram of intestine is so small that dynamic biosynthesis studies have so far been impossible to perform. However, the dynamics of cerebral CCK synthesis have been studied in detail (Goltermann et al. 1980; Stengaard-Pedersen et al. 1984). In addition, a variety of procholecystokinin (proCCK)-derived peptides of different lengths have been identified from extracts of both the small intestine and the brain (Blanke et al. 1993; Eberlein et al. 1992; Eng et al. 1984; Rehfeld and Hansen 1986). Combination of these results with those of knockout of prohormone convertase genes in mice and general knowledge about peptide hormone synthesis has given a picture of the cell-specific proCCK processing, both in intestinal I-cells and cerebral CCK neurons (Rehfeld et al. 2008b).

After translation of CCK mRNA and removal of the N-terminal signal peptide, proCCK is transported from the rough endoplasmatic reticulum (RER) to the Golgi apparatus. A few endoproteolytic cleavages by prohormone convertase 1 (PC1) and, for instance, serine phosphorylation may occur late in RER. But otherwise, as for other peptide hormones, the first posttranslational modifications occur in the Golgi where proCCK is almost completely tyrosyl *O*-sulfated. Partial endoproteolytic cleavages at di- and monobasic sites also begin in the trans-Golgi apparatus and continue towards the basal parts of the I-cells where the processing continues in the secretory granules, ending up with the main products, carboxyamidated and tyrosyl *O*-sulfated CCK-58, CCK-33, CCK-22, and CCK-8 (Mutt and Jorpes 1971; Rehfeld 1978b; Reeve et al. 1986; Eng et al. 1984).

PC1 is responsible for the endoproteolytic maturation of proCCK in the intestinal I-cell. Thus, the synthesis of hormonally active CCK is almost entirely blocked in the small intestine of PC1 knockout mice (Rehfeld et al. 2008b). In contrast, the intestinal synthesis of bioactive CCK peptides is intact in PC2 knockout mice (Rehfeld et al. 2002b). As for the gastrins, the last and decisive processing step in the synthesis of bioactive CCK peptides occurs during maturation in the secretory granules. The secretory granules in the endocrine I-cells also contain the enzyme(s) necessary for amidation which removes glyoxylate from the glycine-extended CCK to complete the synthesis of bioactive CCK peptides (Eipper et al. 1992).

As a result of the elaborate biosynthetic pathway, the intestinal I-cells release a mixture of proCCK products from the secretory granules. A few per cent are nonamidated precursors and the longest possible bioactive product of proCCK, CCK-83 (Eberlein et al. 1992). However, the most bioactive CCKs from the gut are the medium-sized peptides CCK-58, CCK-33 and CCK-22 (Eberlein et al. 1988; Mutt and Jorpes 1971; Rehfeld 1978b; Eng et al. 1984), although small amounts of

the short CCK-8 and CCK-5 are also present (Dockray et al. 1978; Shively et al. 1987; Rehfeld et al. 2001). The distribution and release patterns vary among species.

The CCK gene is expressed in several cells other than the small intestinal I-cells. Entirely predominant are CCK neurons which are abundantly distributed in all regions of the central nervous system (Rehfeld 1978b; Crawley 1985; Dockray et al. 1978; Larsson and Rehfeld 1979b), though the cerebellar neurons produce CCK in significant amounts only in the fetal state (Mogensen et al. 1990; Rehfeld et al. 1992). The highest expression occurs in neocortical regions, which explains why CCK is the most abundant peptide system in the human brain (Rehfeld 1978a, b; Crawley 1985). CCK peptides are also widely expressed in peripheral neurons, primarily in the intestinal tract and also in the genitourinary tract and elsewhere (Larsson and Rehfeld 1979a, b; Rehfeld and Lundberg 1983). Low-level expression has been found in pituitary corticotrophs (Rehfeld 1986, 1987), in thyroid C-cells (Rehfeld et al. 1990), in the adrenal medulla (Bardram et al. 1989), in the bronchial mucosa (Rehfeld et al. 1989), and in the spermatogenic cells of certain mammals (Persson et al. 1989).

The cerebral processing of proCCK is only marginally affected in PC1 knockout mice (Rehfeld et al. 2008b), whereas lack of PC2 profoundly disturbs the neuronal processing (Rehfeld et al. 2002b). PC5/6 is expressed also in CCK neurons and can cleave proCCK (Cain et al. 2003). Together, the data suggest that the cerebral maturation of proCCK requires mainly PC2 but, to a smaller extent, PC5/6 also, whereas PC1 has minimal effect on neuronal proCCK processing.

Presumably, due to the cell-specific expression of the processing enzymes, the difference in proCCK processing between intestinal I-cells and cerebral CCK neurons becomes striking: I-cells accumulate and, as mentioned, secrete mainly the medium-sized CCK-58, -33 and -22 to circulation, whereas cerebral neurons release the small CCK-8 and -5 to the synaptic clefts. This difference is highly expedient. Circulating CCK from the gut has to travel a considerable distance in blood before it reaches its target. Circulating peptides, therefore, need slow clearance from blood. The medium-sized CCKs meet this requirement. In contrast, transmitter peptides reach their synaptic target immediately after release and then need rapid clearing. The short CCK-8 and -5 peptides are, accordingly, cleared very quickly. Hence, cell-specific proCCK processing is indeed desirable from a functional point of view, which is further illustrated by the blood–brain barrier's complete separation of the circulating from the synaptic CCK peptides. Figure 2 summarizes the main features of the cell-specific proCCK processing in intestinal I-cells, cerebral neurons, and pituitary corticotrophs.

2.3 Proglucagon

The different processing of proglucagon (PG) in the pancreatic islets and the gut is a third example of decisive and clear-cut cell-specific precursor processing. In pancreatic alfa-cells glucagon, the CRPP, a fragment corresponding to the PG 64-69, and the

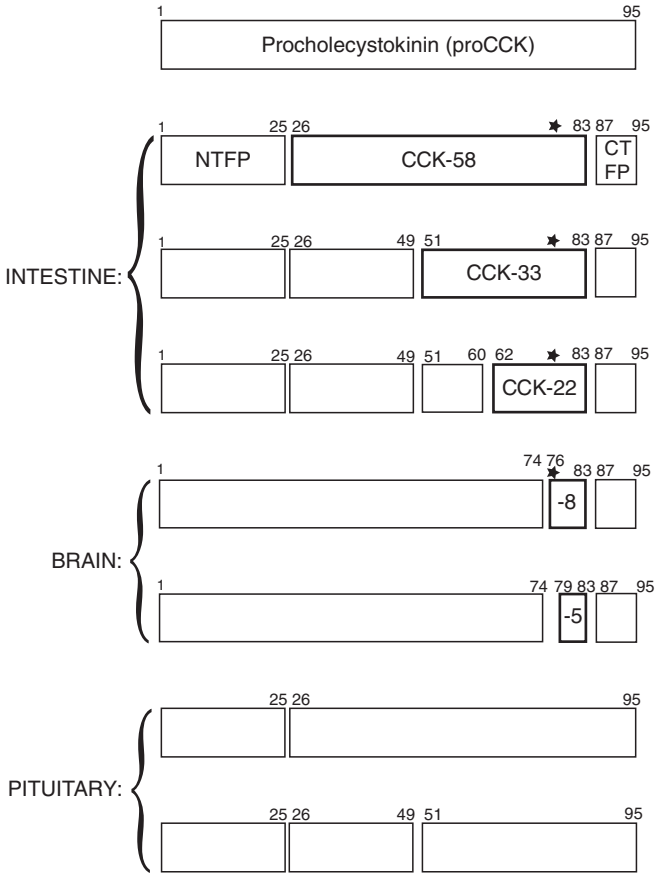

Fig. 2 Simplified scheme of the cell-specific processing of procholesystokinin (proCCK) in the small intestine, the brain, and the pituitary gland. The numbers show amino acid positions in the 95 amino acid residue proCCK sequence. The *asterixes* show the position of the *O*-sulfated tyrosyl residue in the bioactive CCK-58, CCK-33, CCK-22, and CCK-8 sequences. NTFP and CTFP are acronyms for "N- and C-terminal flanking peptides," respectively

major PG fragment corresponding to PG 72-60 (Holst et al. 1994) are synthesized, and they are all secreted in parallel upon stimulation. The processing in the alfa-cells is ensured by PC2, which has been demonstrated to be both necessary and sufficient for cleaving pancreatic PG as described (Rouille et al. 1994). Consequently, PC2 knockout mice cannot process PG in the alfa-cells (Furuta et al. 1997). Therefore, these mice have lower blood glucose concentrations and improved tolerance to glucose than the wild-type littermates. They also develop alfa-cell hyperplasia in a way seen in mice with deletion of the glucagon receptor gene (Gelling et al. 2003).

The small intestinal L-cell processing results in the synthesis of glicentin, corresponding to PG residues 1–69, part of which may be cleaved further to

oxyntomodulin which corresponds to PG 33-69 (Baldissera and Holst 1984; Orskov et al. 1986). The PG sequence that corresponds to the major PG fragment contains pairs of basic amino residues, cleavage sites for the prohormone convertases, flanking both of the glucagon-like peptide sequences in the cDNA. It was, therefore, predicted that the prohormone might be cleaved at these sites (Orskov et al. 1986), and that the sequences of the intestinal glucagon-like peptides, GLP-1 and GLP-2, would correspond to PG 72–108 and 126–158, 126–159 or 126–160. However, identification of the naturally occurring peptides from the human small intestine revealed that the structure of native GLP-1 corresponds to PG 78–107 (Holst et al. 1987). This turned out to be important since the truncated peptide was found to be a potent stimulator of insulin secretion, whereas full-length GLP-1 was inactive (Holst et al. 1987; Mojsov et al. 1987). The designation GLP-1 now covers only the truncated peptide. It was also found that the glycyl residue in position 108 of PG serves as a substrate for amidation of the C-terminal arginyl (Orskov et al. 1989). However, in contrast to other alfa-amidated peptide hormones (for instance, CCK and gastrin) the biological consequences of the amidation of GLP-1 are unclear. The amidated and the glycine-extended forms have similar bioactivities and overall metabolism (Orskov et al. 1993), although the amidated peptide may show higher stability towards plasma enzymes (Wettergren et al. 1998). In humans almost all of the GLP-1 secreted from the gut is amidated (Orskov et al. 1994), whereas in many animals (rodents, pigs) part of the secreted peptide is GLP-1-(7-37) (Hansen et al. 2000; Mojsov et al. 1990). This poses special problems with respect to the measurement of GLP-1 secretion. The sequence of naturally occurring GLP-2 corresponds to PG 126-158 (Buhl et al. 1988; Hartmann et al. 2000). A comparison of GLP sequences among species shows that the GLP-1 sequence is fully preserved in mammals, and the sequence homology is pronounced across other classes of animals (Kieffer and Habener 1999). GLP-2 shows more variation.

The endoproteolytic processing of PG in the intestinal L-cells is due to PC1 (Ugleholdt et al. 2004; Zhu et al. 2002). This is a striking parallel to the above mentioned proCCK processing in the intestinal I-cells. Here, PC1 is also responsible for the entire endoproteolytic processing, whereas the extra-intestinal proCCK processing in cerebral neurons is due mainly to PC2. Interestingly, the N-terminal cleavage site of GLP-1 is not a classical pair of basic amino acids but a single arginyl residue. However, coexpression of PC1 and PG has demonstrated efficient cleavage at this site (Rouille et al. 1997). Accordingly, mutations in PC1 lead to abnormalities in GLP-1 processing and secretion, associated with multiple endocrinopathies (Jackson et al. 2003), and mice with a deletion of the PC1 gene are unable to process PG to GLP-2 and GLP-1 (Ugleholdt et al. 2004; Zhu et al. 2002). Interestingly, it was recently demonstrated that virus-mediated expression of PC1 in the pancreatic alfa-cells increases islet GLP-1 secretion, resulting in improved insulin secretion, increased survival after cytokine treatment, as well as enhanced performance after transplantation to mouse models of type 1 diabetes (Wideman et al. 2006).

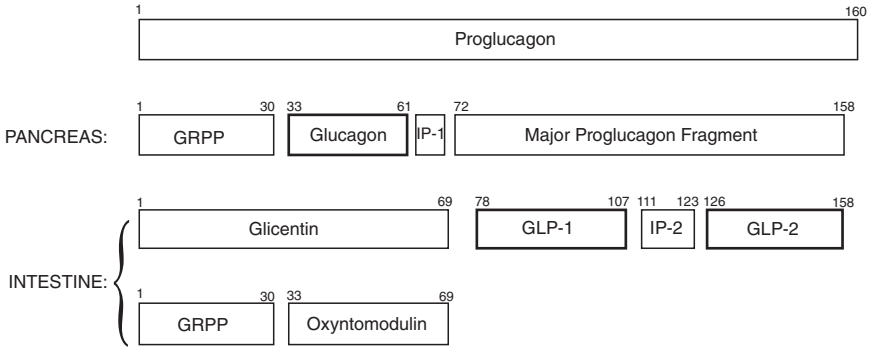

Fig. 3 Simplified scheme of the cell-specific processing of proglucagon in the pancreas and in the small intestine. The numbers indicate amino acid positions in the 160 amino acid proglucacon sequence. GPPP is an acronym for GRPP *glicentin-related pancreatic polypeptide*; IP-1 *intervening peptide*-1; IP-2 *intervening peptide*-2

A scheme of the cell-specific processing of PG in pancreatic alfa-cells versus intestinal L-cells is presented in Fig. 3.

2.4 Proneurotensin

A fourth example of cell-specific prohormone processing is proneurotensin. The neurotensin (NT) gene is expressed predominantly in the hypothalamus and the neurons of some other brain areas and nuclei, in the adrenal glands, and in endocrine, small intestinal N-cells. The whole biosynthesis and processing story of proneurotensin is detailed later by Kitabgi (2009) in this volume (see also Kitabgi 2006). In the present context it suffices to state that proneurotensin contains two bioactive sequences in its C-terminal part: the tridecapeptide NT and the hexapeptide neuromedin N (NN). Both peptides are bioactive, either in their native, short forms or when they are extended only at the N-terminus due to lack of N-terminal endoproteolysis (large NT and large NN). As illustrated in Fig. 4, proneurotensin in cerebral neurons is processed quite efficiently to release both NT and NN in their short forms, which is expedient for neurotransmitter peptides, as discussed for the similar short CCK peptides in the brain (*vide supra*). In the small intestinal N-cells, however, the processing differs to release mainly large NT and large NN (Fig. 4). The large forms are subsequently released to blood as hormones. As discussed for proCCK, it is an advantage for hormonal peptides which are diluted into the entire vascular bed that they are cleared relatively slowly from blood, in order to maintain an adequate concentration when they reach their more or less distant target. Consequently, the cell-specific processing of proneurotensin is also highly appropriate.

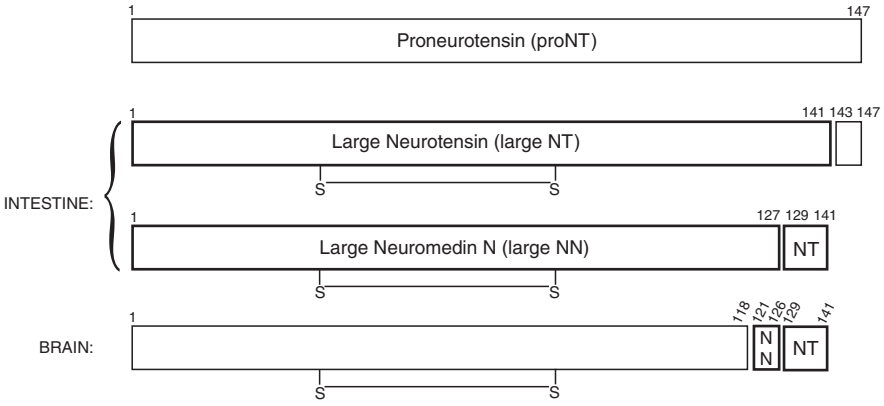

Fig. 4 Simplified scheme of cell-specific processing of proneurotensin/neuromedin N. Rat pre-pro-NT/NN is 169 residues long and starts with a 22 residues signal peptide. The NN and NT sequences are located near the C-terminus of the precursor. The disulfide bridge between Cys^{61} nad Cys^{110} is also shown. This organization is conserved in all the species from which the precursor has been cloned. Below the precursor are represented the biologically active products, i.e., NT, NN, large NT, and large NN that are differentially generated following tissue-specific pro-NT/NN processing

3 Significance of the Cell-Specific Processing in Disease

Diseases of peptide producing cells cause several sorts of malfunctioning in the biogenesis and secretion of the peptides. Also the last biogenetic phase, the posttranslational processing, may be affected in a way that has heavy clinical consequences. The cellular processing of precursors is normally a precisely balanced sequence of overlapping enzymatical modifications where, for instance, amino acid derivations may influence the efficiency of subsequent endoproteolytic cleavages at basic amino acid residues as well as the N- or C-terminal trimmings (Bundgaard et al. 1995) – and vice versa. Generally, the posttranslational modifications are never complete, leaving varying amounts of intact precursors and partly processed intermediates along the secretory pathway. Some of the prohormones and processing intermediates are subsequently released by constitutive or constitutive-like secretion, but portions of the precursors may also be secreted in a regulated manner together with the bioactive endproducts from secretory granules (Bundgaard and Rehfeld 2008; Rehfeld et al. 2004).

Diseases may diminish the production of bioactive peptides, either as a primary cause, as seen by the autoimmune destruction specifically of the insulin cells in type-1 diabetes mellitus, or as secondary consequences of cell and tissue destructions in inflammatory diseases, as seen in atrophic gastritis that affects the endocrine cells in the stomach. The endocrine symptoms then reflect shortage in secretion of the specific hormone. Diseases may also increase the cellular expression of peptide hormone genes with subsequent translational increase of the synthesis of secretory proteins.

To which extent the increased expression of prohormones per se leads to endocrine symptoms then depends on the efficiency of the posttranslational processing (Jensen et al. 1989; Jackson et al. 2003). Enhanced prohormone expression often occurs by malignant transformation of cells. Clear-cut examples are the relatively rare neuroendocrine tumors, of which most are slow-growing cancers (Rehfeld 1998a). But also frequent and common cancer forms express peptide hormone genes. These cancers include, for instance, all the major gastrointestinal carcinomas (colorectal, gastric, esophageal, pancreatic) as well as all forms of lung cancer and ovarial cancer (for review, see Rehfeld and van Solinge 1994). Even sarcomas express genes of peptide hormones at the proprotein level (Reubi et al. 2004; Friedman et al. 1992).

It is well known that neuroendocrine tumors originate from neuroendocrine cells, sometimes from cells that were active mainly in the fetal state of development (oncofetal carcinogenesis). But also the common cancers seem to originate from tissue that in the normal, adult state contain cells that express the peptide hormone genes. Generally, the level of expression is often low in nonmalignant cells, but it increases after the malignant transformation. Hence, peptide synthesis in tumors is probably not ectopic but reflect the phenomenon of widespread expression of bioactive peptide systems in several different cell types and organs, also in the normal organism (Rehfeld and van Solinge 1994).

The processing of propeptides in diseased cells often deviates from that of the corresponding normal cells. There are two types of deviations. First, with increased expression and subsequent translation, the cells produce more propeptide than the posttranslational machinery can process. Therefore, only a smaller fraction of the precursors mature to bioactive products, and the sick cells consequently release mainly unprocessed and partly processed precursors (Jensen et al. 1989). In malignant cells the capacity for maturation is sometimes so reduced that only inactive prohormones and processing intermediates are released from the cells (Rehfeld et al. 1989; van Solinge et al. 1993a, b; Bardram 1990; Reubi et al. 2004). The second type of deviation is simply due to lack of expression of appropriate processing enzymes, so that the cells produce only the intact prohormone, as seen in, for instance, Ewing's sarcoma which releases only proCCK to circulation (Reubi et al. 2004).

The widespread expression of peptide systems in tumor cells has clinical significance in three respects: First, since many bioactive peptides are also growth factors, the increased production and secretion may stimulate tumor growth by autocrine, endocrine or paracrine routes. The increased peptide synthesis may, in other words, have pathogenetic significance, which leads to the second role where the peptides or enzymes in the biosynthetic machinery may be targets for new forms of therapy, such as vaccination, immunotherapy, or specific enzyme blockade (Watson et al. 2006; Rehfeld and Goetze 2006). The last clinical role for the peptides is in diagnosis (Rehfeld 1998a; Rehfeld and Goetze 2003). As indicated, however, proper diagnostic measurements of peptide concentrations in plasma or elsewhere requires the understanding of the posttranslational processing machinery and the deviations from the normal processing seen in diseased cells. In this situation, PIA may be of help.

4 Processing-Independent Analysis

cDNA for most peptide hormones and neuropeptides has been cloned, sequenced, and the proprotein structures deduced. Today, we also know the specific substrate structure for many processing enzymes, both for endoproteolytic cleavage and for amino acid derivatization (Steiner 1998; Müller and Lindberg 2000). It is, consequently, possible to select sequences of prohormones and proneuropeptides which are neither processed nor too short to be immunogenic. The selected sequence must neighbor a proteolytic cleavage site.

The PIA is then developed as follows: The selected prohormone sequence (approximately ten amino acids) is synthesized with either a C-terminal or a N-terminal residue permitting a directional coupling to a suitable carrier, and antibodies specific for the selected sequence are produced. In this way, a specific radioimmunoassay for either the N- or the C-terminus of the processing-independent sequence is developed. The sample to be assayed is then preincubated with a suitable endoprotease, often trypsin, which mimics cleavage at basic residues somewhat resembling the cleavage of the specific prohormone convertases. In addition to exposing the epitope, the endoproteolytic cleavage ensures that fragments of equal size are released so that prohormone, processing intermediates, and mature products are quantitated with equimolar potency. The analytical principle is outlined in Fig. 5.

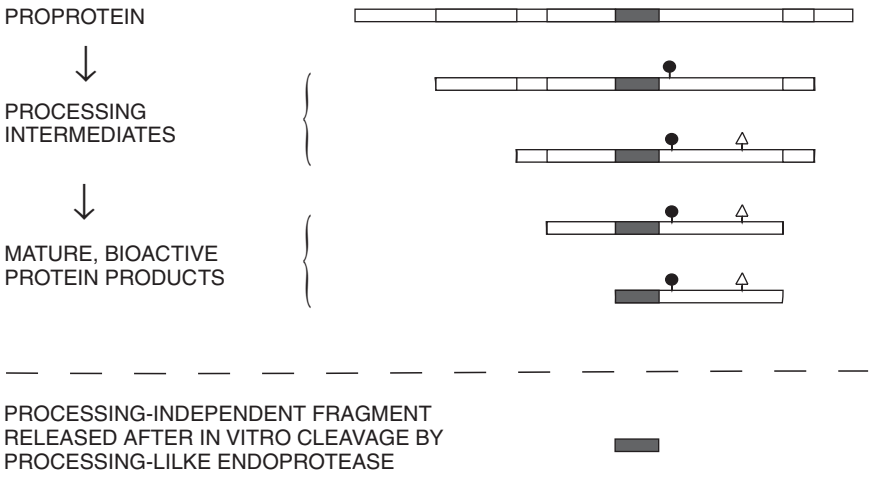

Fig. 5 General scheme of the posttranslational maturation of secretory proteins and peptides. The scheme also shows the principle of the PIA. *Shaded bar*, illustration of a peptide sequence which does not undergo posttranslational modifications but is present in precursors, processing intermediates, and bioactive mature products. *Dots, arrowheads*, amino acid derivatizations. The "processing-independent" sequence must be localized between suitable cleavage sites. Thus, by in vitro treatment with appropriate endoproteases one "processing-independent" peptide fragment is released per molecule translated proprotein, irrespective of the degree of processing. After inactivation of the endoprotease added, in vitro quantitation of the "processing-independent" peptide can be performed by a suitable assay

Table 1 Plasma concentrations of conventionally measured gastrin (alfa-amidated gastrin) versus the PIA measured total progastrin product in a gastrinoma patient (pmol l^{-1}; see Bardram 1990)

	Alfa-amidated gastrin	Total progastrin product
Gastrinoma suspected	27	276
After 3 years: no tumor found	60	642
After 4 years: after tumor resection	10	75
After 6 years: hepatic metastases	1,500	7,200

In accordance with the general design, we have developed specific radioimmunoassays for fragments of human progastrin, chromogranin A, proCCK, proBNP, and proCNP (Bardram and Rehfeld 1988; Paloheimo and Rehfeld 1994; Goetze et al. 2002; Nielsen et al. 2005; Børglum et al. 2007). These sequences follow trypsin-sensitive arginyl or lysyl residues and contain a tyrosyl residue at the C-terminus. The specificity of the assays has been evaluated in detail. To give an example, we measured the concentration of the total progastrin product in plasma from patients with gastrinomas, duodenal ulcer disease, and control subjects. The total progastrin product comprises the sum of unprocessed progastrins, processing intermediates, and mature bioactive gastrins (Bardram and Rehfeld 1988). The results were compared with those of conventional measurements of bioactive gastrin. They showed that almost all progastrin in G-cells of normal subjects mature to bioctive, alfa-amidated gastrins, and that metastatic gastrin-producing carcinomas mature only 23% of the precursors in contrast to the 54% in the nonmetastatic gastrinomas. Finally, analysis of plasma sampled over a period of several years from a gastrinoma patient showed that the concentration of the total progastrinproduct was grossly enhanced 3 years before the tumor was diagnosed. Hence, proper diagnosis could have been made years earlier with the PIA type of analysis (Table 1) (Bardram 1990).

A prospective evaluation of the use of PIA in routine diagnostics has shown that PIA contributes essentially to ensuring correct diagnosis for small, early gastrinomas with uncharacteristic clinical symptoms, i.e., borderline gastrinomas. In contrast, PIA did not improve the diagnostic sensitivity significantly for gastrinoma patients with consistently enhanced concentrations of carboxyamidated gastrin in plasma (Jørgensen et al. 1998). For both proCCK progastrin and chromogranin A we could, however, show that PIA provides a significantly more accurate measure of the tumor burden for each individual patient than conventional RIA measurements (Bardram 1990; Børglum et al. 2007; Reubi et al. 2004).

5 Summary

Cell-specific precursor processing is a phenomenon that has been known for more than three decades. For the precursors of peptide hormones it has turned out to be much more widespread and general than first anticipated. Thus, expression of peptide hormone genes in a multitude of tissues and cells in the same organism appears to

be a rule for which there are only few exceptions, if any. The widespread cellular synthesis, however, has to be under tight steering in order to maintain the necessary and specific hormonal control of the bodily functions. One of the steering mechanisms is cell-specific processing, i.e., that different cell types produce and release different active fragments of the same prohormone. This review discusses different examples of normally occurring cell-specific prohormone processing. The review also points out the clinical and diagnostic impact on cell-specific processing that occurs not least in cancer cells and other neoplastic cells. Thus, the use of peptides as markers of disease requires not only knowledge about the general biology of cell-specific processing, but also insight into the diagnostic limits of conventional peptide assays. One way of dealing with this problem is to use PIA.

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ACTH: Cellular Peptide Hormone Synthesis and Secretory Pathways

Adam Stevens and Anne White

Abstract Adrenocorticotrophic hormone (ACTH) is derived from the prohormone, pro-opiomelanocortin (POMC). This precursor undergoes proteolytic cleavage to yield a number of different peptides which vary depending on the tissue. In the anterior pituitary, POMC is processed to ACTH by the prohormone convertase, PC1 and packaged in secretory granules ready for stimulated secretion. In response to stress, corticotrophin releasing hormone (CRH), stimulates release of ACTH from the pituitary cell which in turn causes release of glucocorticoids from the adrenal gland. In tissues, such as the hypothalamus and skin, ACTH is further processed intracellularly to alpha melanocyte stimulating hormone (αMSH) which has distinct roles in these tissues. The prohormone, POMC, is itself released from cells and found in the human circulation at concentrations greater than ACTH. While much is known about the tightly regulated synthesis of POMC, there is still a lot to learn about the mechanisms for differentiating secretion of POMC, and the POMC-derived peptides. Understanding what happens to the POMC released from cells will provide new insights into its function.

1 Introduction

POMC is a prohormone which undergoes proteolytic cleavage to produce a number of bioactive peptides, including ACTH and the melanocyte stimulating hormones (αMSH, βMSH and γMSH) which act via a number of tissue specific melanocortin receptors (MCRs) (Cone 1999). POMC is produced in a range of tissues, but most is known about its synthesis and processing in the anterior pituitary, where ACTH is produced as part of the HPA axis (Bicknell 2008; Oliver et al. 2003; Raffin-Sanson et al. 2003) (Fig. 1a).

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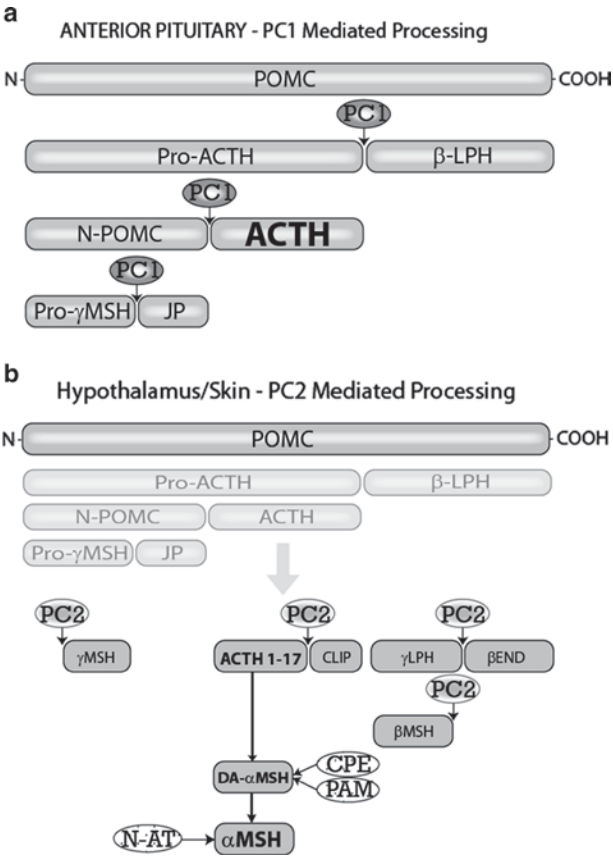

Fig. 1 The processing of pro-opiomelanocortin (POMC) to form peptide fragments. (**a**) POMC processing in the anterior pituitary, driven by the prohormone convertase 1 (PC1) enzyme produces ACTH as the main biologically active POMC fragment. *POMC* pro-opiomelanocortic, *ACTH* Adrenocorticotropic hormone, *LPH* Lipotropin, *MSH* Melanocyte-stimulating hormone, *JP* Joining peptide, *PC* Prohormone convertase.(**b**) POMC processing in the hypothamalus, skin and intermediate lobe of the pituitary utilises PC1-derived POMC peptide fragments as substrates for PC2 mediate processing, producing αMSH as the main biologically active POMC fragment. *CLIP* Corticotropin-like intermediate peptide, *END* Endorphin, *CPE* Carboxypeptidase E, *PAM* Peptidyl amidating monoxygenase, N-*AT N*-acetyl transferase

The main role of ACTH is to stimulate glucocorticoid production by the adrenal glands. ACTH modulates both the diurnal secretion of glucocorticoids and the acute release of glucocorticoids as part of the stress response. Glucocorticoids are critical for survival and can affect the transcription of up to 20% of the expressed human

genome (Donn et al. 2007) by "fast" nongenomic mechanisms, "slow" genomic and long-term epigenetic mechanisms. Glucocorticoids affect both developmental and physiological pathways and, therefore, the regulation of glucocorticoid release is essential for maintenance of life.

The POMC gene is also expressed in extra-pituitary sites, notably the hypothalamus and the skin, where further processing of ACTH can result in αMSH (Fig. 1b). POMC and its derived peptides are important in regulation of energy balance and skin pigmentation (Pritchard et al. 2002; Challis et al. 2002; O'Rahilly et al. 2003; Pritchard et al. 2003). This is evidenced by genetic deletions in the human POMC gene where patients have obesity, red hair and pale skin.

The nature and degree of processing varies in different tissues and in different physiological states. However, it is quite difficult to monitor the degree of processing in vivo and therefore the physiological consequences of alterations in the rate and extent of POMC processing are not well-defined.

2 POMC Synthesis and Its Regulation

The POMC gene is well conserved across species, highlighting the functional significance of POMC and its peptide products. The regulation of POMC synthesis, particularly in the pituitary, occurs primarily at the level of transcription, regulated by a promoter region 5′ to the gene. There is basal synthesis of POMC independent of stimulation (Lugo and Pintar 1996); however, promoter elements are present in the POMC gene region that are responsive to stimulation by corticotropin-releasing hormone (CRH), glucocorticoids and arginine vasopressin (AVP) along with elements that control the developmental expression of POMC and contribute to differential tissue expression (White 2005).

POMC gene expression is under the main positive regulatory control of CRH, and negatively regulated by glucocorticoids in the pituitary – the classical example of a negative feedback loop. The orphan receptor NGFI-B is central to the control of pituitary POMC transcription. CRH, mediated in-part by cAMP response element binding protein (CREB), upregulates NGFI-B expression resulting in increased POMC transcription and glucocorticoids mediate their inhibitory effect on POMC transcription via interaction of the glucocorticoid receptor (GR) with DNA-bound NGFI-B (Lavoie et al. 2008; Martens et al. 2005; White 2005).

There are some tissue-dependent differences in the control of POMC expression. The POMC promoter elements described above function to some extent in all tissues studied; but in the hypothalamus other distant regulatory elements have been discovered which modulate hypothalamic regulation of POMC transcription (de Souza et al. 2005). This may explain why there are certain differences between pituitary and hypothalamic expression such as the stimulatory action of glucocorticoids in the hypothalamus.

3 POMC Processing: The Peptides Produced and the Cleavage Pathway

POMC is processed in a compartmentally regulated manner by the action of multiple enzymes to form a panel of peptides. The initial processing of POMC by the prohormone convertase 1 enzyme (PC1) produces proACTH and βLPH. ProACTH is then further cleaved by PC1 to ACTH, N-POC and joining peptide and βLPH is cleaved by PC1 to γLPH and β-endorphin (Fig. 1a). Depending on cell type, the peptides derived from PC1 action on POMC are then cleaved by prohormone convertase 2 (PC2) to generate peptides which are further post-translationally modified to αMSH, βMSH and γMSH (Fig. 1b)

3.1 Processing of POMC to ACTH

Initial processing of POMC occurs primarily by the action of PC1 which is a subtilisin-like serine endoprotease (Bergeron et al. 2000). PC1 processes POMC in the trans-Golgi network and regulates trafficking to the secretory pathway, in which it subsequently cleaves POMC to ACTH. The initial processing involves cleavage by PC1 at dibasic amino acids. The surrounding amino acids influence the kinetics of processing and the initial cleavage by PC1 is between proACTH and βLPH, followed by the release of ACTH (Mains and Eipper 1990) (Fig. 1a). The importance of PC1 is evidenced by PC1 knockout mice which have a severe pituitary defect in the processing of ACTH from POMC (Zhu et al. 2002).

PC1 is produced as an inactive zymogen that is autocatalytically processed in the endoplasmic reticulum to form a heterodimer of the active enzyme along with its inhibitory prosegment. PC1 enzyme activity is established by a second autocatalytic event to cleave the inhibitory prosegment and the enzyme is sorted to secretory granules where reducing pH (6.0–5.5) establishes the fully active enzyme capable of cleaving POMC (Seidah et al. 2008; Zhou and Mains 1994). Overexpressed PC1 in a cellular system leads to more extensive cleavage of POMC to smaller products (Zhou and Mains 1994).

PC1 can exist as a membrane associated form where it is anchored to lipid rafts in secretory granule membranes. There is evidence that a transmembrane domain targets PC1 to secretory granules (Arnaoutova et al. 2003), although this is controversial (Stettler et al. 2005) and it has been shown that mutations of an RRGDL sequence within PC1 prevented its entry to secretory granules and hence the formation of the 66 kDa PC1 (Lusson et al. 1997).

PC1 has a cofactor (proSAAS) which is a potential endogenous inhibitor, although how it affects cleavage of POMC is unclear at present. ProSAAS is expressed primarily in neuroendocrine tissues (Fricker et al. 2000) and it is the processed C-terminal peptide that is associated with the inhibition of PC1 as evidenced by overexpression experiments (Che et al. 2004). However overexpression does not affect processed peptide products (Fortenberry et al. 2002) and there is

evidence that the inhibitory actions of proSAAS C-terminal peptides may be dependent on cell type (Lee et al. 2004).

Other protease enzymes may contribute to POMC processing as demonstrated by recent observations from the cathepsin L knockout mice which have much lower levels of ACTH, β-endorphin, and αMSH in the pituitary. In addition, in POMC expressing cells the overexpression of cathepsin L has been shown to increase the amount of ACTH and β-endorphin in the regulated secretory pathway. Therefore these data suggest that cathepsin L may be involved in the processing of POMC peptides in secretory vesicles along with PC1 (Funkelstein et al. 2008).

3.2 Processing of ACTH by PC2

ACTH can be cleaved to ACTH1-17 and ACTH18-39 by PC2 acting at a specific arginine dibasic amino acid sequence (Bergeron et al. 2000) (Fig. 1b). PC2 cleaves at dibasic amino acids but has a different selectivity to PC1 (Benjannet et al. 1991). PC2 mediates a slower processing of POMC than PC1 which is totally blocked at 20 °C and requires an acidic intracellular compartment (Paquet et al. 1996). Therefore processing by PC2 only occurs in the secretory granules and not in the trans-Golgi network.

The biosynthesis of PC2 is tightly linked with the protein 7B2 which is thought to bind to the prosegment of PC2 at residues 242–248 (Apletalina et al. 2000). 7B2 is required for efficient transport and activation of the enzyme (Bergeron et al. 2000) although the carboxy-terminal of 7B2 inhibits PC2 (Fortenberry et al. 1999).

The interaction of 7B2 with PC2 differs from that of proSAAS with PC1, despite both potently inhibiting PC enzymes via a C-terminal peptide. 7B2 expression affects the rate of conversion of pro-PC2 to PC2 in the late secretory pathway (Zhu and Lindberg 1995) compared to proSAAS which has no apparent effect on the extremely rapid conversion of pro-PC1 to the 87-kDa active form in the endoplasmic reticulum (Fortenberry et al. 2002). After 7B2 precursor processing, the C-terminal fragment of 7B2 remains associated with proPC2, thereby preventing autocatalytic conversion of the proenzyme until the appropriate site for activation in the secretory pathway is reached (Braks et al. 1996).

3.3 Post-Translational Processing to Produce αMSH

The ACTH 1-17 fragment formed by PC2 action requires further post-translational processing to generate functional αMSH (Fig. 1b).

3.3.1 Carboxypeptidase E (CPE)

CPE acts as an exopeptidase in secretory vesicles by inducing the removal of basic C-terminal amino acids from ACTH 1-17 to form ACTH 1-13 (Fig. 1b). ACTH

1-13 is the precursor of active αMSH. There may be a functional association of CPE with PC2 as the addition of CPE results in more efficient processing by PC2 in vitro (Day et al. 1998).

There is evidence to suggest that CPE can also act as a sorting receptor that directs POMC to the regulated secretory pathway. The C-terminal of CPE has an amphiphilic α-helix that can associate with membranes at low pH thus allowing the binding of CPE to secretory granule membranes, and facilitating the sorting of processed peptides (Dhanvantari et al. 2002).

3.3.2 Peptidyl Amidating Monoxygenase (PAM)

PAM amidates a glycine residue at the C-terminal of ACTH 1-13 after the COOH group has been removed by CPE. This results in des-acetyl αMSH (Fig. 1b). Interestingly, overexpression of PAM in the pituitary cell line AtT-20 can result in decreased POMC processing, probably due to altered cytoskeletal organisation (Ciccotosto et al. 1999).

3.3.3 N-acetyl Transferase (N-AT)

This acetyl transferase catalyses the addition of an acetyl group on the N-terminal of des-acetyl αMSH (Fig. 1b) (Wilkinson 2006). The reports on acetylation are contradictory with some suggesting that αMSH is more active than des-acetyl αMSH (Suli-Vargha et al. 1992) while others suggest it is a mechanism for inactivating peptides as in β-endorphin (Lindberg et al. 1979) This is discussed in greater detail in the section on processing in the hypothalamus (Sect. 2.44).

4 The Secretion of Processed POMC Peptides

4.1 The Cellular Secretory Pathway

POMC moves from the endoplasmic reticulum to the trans-Golgi network where it is sorted for delivery to secretory granules (Fig. 2a). The precursors, along with other regulated secretory pathway proteins, are sorted and packaged into immature secretory granules (ISGs) that bud off from the trans-Golgi network (TGN). Maturation of the ISGs involves many steps before ultimately forming dense-core secretory granules (DCSGs)- the key organelles for secretion of hormones in endocrine cells in response to stimulation. Immediately after budding of ISGs from the TGN, acidification within these organelles occurs. It has been reported that the TGN, ISGs and DCSGs have increasingly acidic pH; this state is maintained in

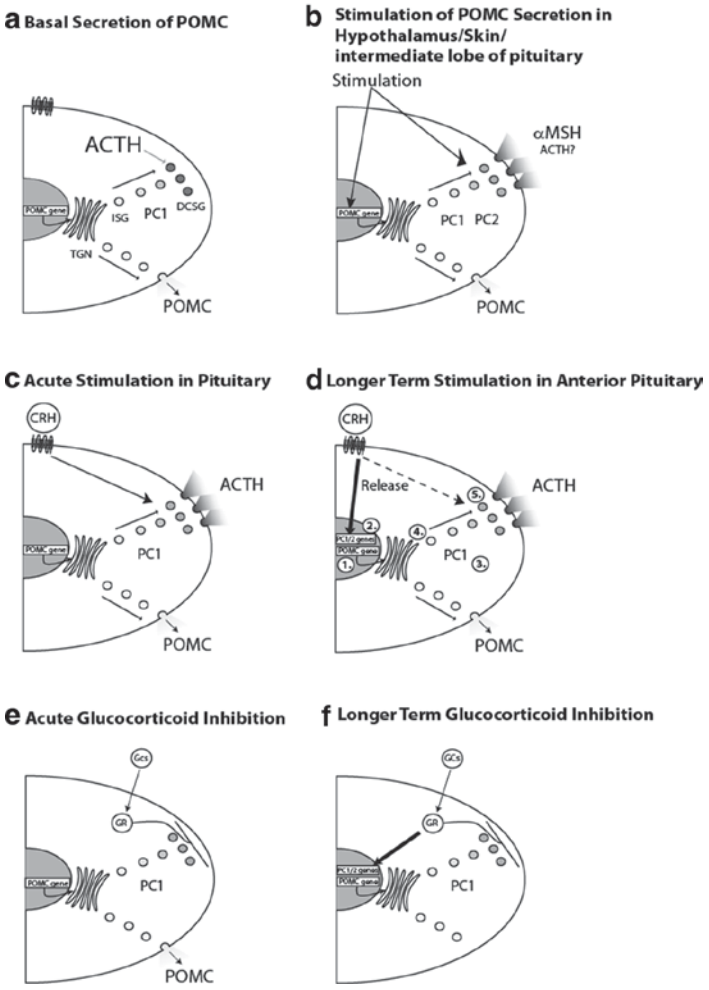

Fig. 2 The secretion of POMC and POMC processed peptides. (**a**) POMC is secreted basally by pituitary corticotroph cells and other POMC peptide secreting cells. ACTH, processed from POMC by the action of PC1, is stored into dense core secretory granules (DCSG). TGN = trans-Golgi network; ISG = immature secretory granules. (**b**) In the hypothalamus/skin/intermediate lobe of the pituitary, αMSH, possibly along with ACTH, are released upon stimulation. POMC is also secreted possibly via a constitutive-like pathway. (**c**) In the anterior pituitary ACTH is released upon acute stimulation with CRH. POMC is also secreted but not subject to stimulation. (**d**) In the anterior pituitary, long term chronic stimulation with CRH leads to an upregulation of the POMC gene. ACTH is released by the stimulation with CRH. (**e**) In the anterior pituitary, glucocorticoids can inhibit ACTH secretion in an acute, nongenomic, manner. GR = glucocorticoid receptor, Gcs = glucocorticoids. (**f**) In the anterior pituitary, chronic exposure to glucocorticoids inhibits ACTH secretion by genomic mechanisms including the inhibition of PC1 and POMC transcription

DCSGs by the expression of membrane channels such as aquaporin-1 (Arnaoutova et al. 2008). Sulphation of POMC, unlike some secretory proteins, is not required for the intracellular transport, sorting and proteolytic processing of its regulated secretory proteins (van Kuppeveld et al. 1997).

Onset of processing and ISG release are distinct biochemical processes with different kinetics and separate cytosolic requirements. It has been suggested that a two-step mechanism is involved, with onset of processing followed by ISG release (Andresen and Moore 2001). Currently, a "sorting-for-entry" model for POMC is supported by the majority of studies where targeting to secretory granules is determined in the TGN (Dumermuth and Moore 1998) and may involve interactions with proteins such as chromogranin B (Natori and Huttner 1996).

There is increasing evidence that POMC is sorted to the DCSGs via an N-terminal three-dimensional sorting motif that can bind to membrane CPE as a sorting receptor (Cool et al. 1995). The sorting signal consists of four conserved residues in the N-terminal of POMC that form a loop structure utilising two disulfide bridges formed between Cys28/Cys50 and Cys34/Cys46. Mutation of POMC close to the N-terminal at Cys28 did not have any effect on sorting (Cool et al. 1995). However, when the sorting signal in POMC is mutated there is an accumulation of the mutant in the Golgi, as well as high basal secretion, indicating that the mutant form of POMC is inefficiently sorted to the regulated secretory pathway (Loh et al. 2004). The recent identification of a patient with a mutation in the N-terminal of POMC, at Cys28, which affects processing, highlights the importance of this sequence in efficient POMC peptide production (Creemers et al. 2008).

The sorting of POMC also involves oligermerisation, a step that is independent of the N-terminal (Cawley et al. 2000), and there may be cell specific effects of different POMC domains (Gorr and Darling 1995).

Integrity of the trans-Golgi network and sorting of POMC into secretory granules are important for POMC processing (Tanaka et al. 1997). PC1 mediates rapid processing of POMC and PC1 dependent action is relatively insensitive to 20 °C temperature blockade (which arrests secretory pathway transport at the trans-Golgi network).

After processing, ACTH is packed and stored in DCSGs and is released when exocytosis is stimulated by secretagogues such as CRH, possibly acting via effects on calcium channels. The exocytosis of ACTH is inhibited via mechanisms grouped under the term "fast effects" (2–60 min) which are mainly mediated by the nongenomic action of glucocorticoids (Spiga et al. 2008).

4.2 POMC Processing in the Anterior Pituitary

Corticotroph cells, expressing the POMC gene and secreting ACTH, comprise 5–20% of the adult pituitary cell population (Levy 2002). The main POMC-derived peptides found in the circulation in humans are ACTH, N-pro-opiocortin (N-POC), joining peptide (JP) and β-lipotrophin (βLPH) - all products of PC1 processing

(Gibson et al. 1994) (Fig. 1a). Corticotroph cells do not express PC2 and consequently do not process POMC to αMSH, γMSH and βMSH (Fig. 1b).

Regulation of the secretion of ACTH is under acuteshort-term control (e.g. acute stress responses) (Fig. 2b), but it is also subject to long-term stimulation by stressors such as chronic inflammation (Fig. 2d, e). The immediate, stress-activated release of ACTH is mediated by hypothalamic CRH and augmented by AVP. The stress-initiated secretion of ACTH is then subject to feedback inhibition by glucocorticoids (Fig. 3).

This process can be modulated by locally produced intrapituitary factors such as leukemia inhibitory factor (LIF), which is secreted by folliculo-stellate (FS) cells and by corticotroph cells. LIF acts to enhance glucocorticoid repression (Auernhammer et al. 1998). There are many other factors which have been shown to affect ACTH secretion in particular experimental paradigms, e.g. PACAP, catecholamines, DOPA, serotonin, GABA, directly or via effects on CRH (White 2005). However there are not many studies showing how they are involved in ACTH physiological or pathological processes (Fig. 3).

ACTH secretion from the pituitary is pulsatile with high frequency/low amplitude pulses that are calcium-dependent and independent of hypothalamic stimulation, suggesting the existence of an intrinsic intrapituitary pulse-generating mechanism (Gambacciani et al. 1987) which is under the command of the suprachiasmatic

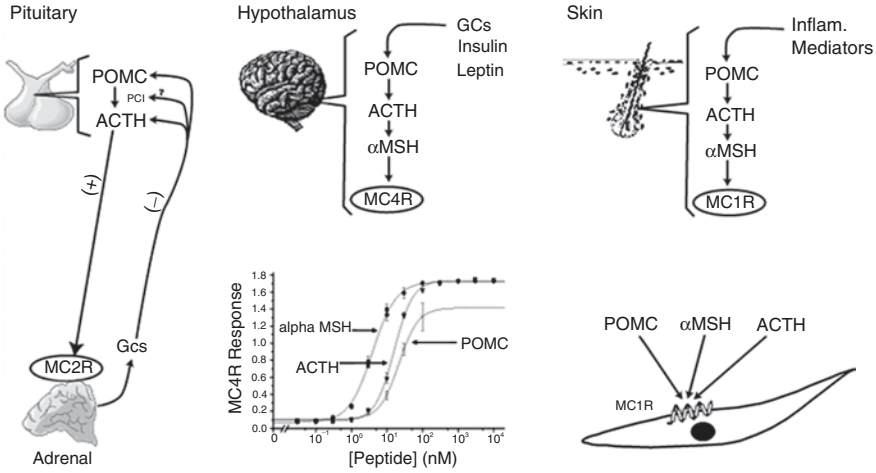

Fig. 3 The tissue specific processing of POMC. POMC processing in the anterior pituitary produces ACTH which stimulates the MC2 receptor (MC2-R) on the adrenal gland to produce glucocorticoids (Gcs) Glucocorticoids in turn downregulate ACTH production by inhibition at the POMC gene, the processing enzyme (PC1) and ACTH secretion. In the hypothamalus POMC is processed via PC1 and PC2 to ultimately form αMSH. Hypothalamic processing is regulated by glucocorticoids, insulin and leptin. The hypothalamic MSH receptor (MC4R) can also be activated by ACTH and full-length POMC (Pritchard et al 2003).In the skin POMC is processed by PC1 and PC2 to form αMSH under the regulation of inflammatory mediators. The MSH receptor in the skin is MC1-R which can also be activated by other POMC peptide fragments (Rousseau et al 2007)

nucleus (SCN) in the hypothalamus.There is also a circadian rhythm to the HPA axis and ACTH secretion in particular.

Glucocorticoids can inhibit ACTH release from the pituitary quite rapidly (Fig. 2c). The "fast-effect" of glucocorticoids on ACTH secretion may be due to a glucocorticoid-dependent reduction in CRH stimulation mechanisms. This could occur via the channels involved in stimulatory release of secretory granules. Since CRH acts through calcium signalling,it is thought that glucocorticoids exert their "fast-effects" by blocking this mechanism (Antoni 1996) (Fig. 2c and 2e).

Another "fast effect" of glucocorticoids on ACTH secretion may be due to mechanisms involving Annexin 1 (ANXA1). ANXA1 is a glucocorticoid inducible gene that was identified as an anti-inflammatory protein. ANXA1 has also been shown to affect neuroendocrine secretion as demonstrated by the observation that intracerebroventricular injection of ANXA1 generated inhibitory effects similar to glucocorticoids on ACTH release. ANXA1 is expressed in the FS cells which maintain contact with endocrine secretory cells in the pituitary. Glucocorticoids have been shown to augment expression of ANXA1 in FS cells and also enhance the translocation of ANXA1 from the cytoplasm to the plasma membrane outer surface via an exocytosis independent mechanism. ANXA1 is then able to exert inhibitory effects on CRH stimulated ACTH secretion (Buckingham 2006). This is mediated via cell surface receptors, possibly involving members of the formyl peptide receptor (FPR) family acting on SNARE protein interactions that are critical for exocytosis (John et al. 2008).

4.3 POMC Processing in the Intermediate Pituitary

In the melanotrope cells of the intermediate lobe of the pituitary, POMC is processed by PC1 to ACTH, N-POC and βLPH. However because of the presence of PC2 in these cells further processing occurs to form αMSH, γMSH and βMSH respectively (Fig. 1b).

Rodents have a well developed intermediate lobe and therefore αMSH is released into the blood stream. There is usually more POMC expressed in the intermediate lobe than in the anterior lobe (Day et al. 1992) and this therefore will impact on the types of peptides in the circulation. However in humans, the intermediate lobe is only present in the fetus as a distinct area and in adults reduced to a thin layer of cells between the anterior and posterior pituitary. POMC expression is inhibited by dopamine in the intermediate lobe, which therefore reduces αMSH secretion.

PC2 in the intermediate lobe is not regulated by glucocorticoids as demonstrated by adrenalectomy (Dong and Day 2002). However the expression of both PC1 and PC2 are inhibited in the intermediate lobe by dopamine (Day et al. 1992) along with CPE and PAM (Oyarce et al. 1996). Evidence from PC2 and 7B2 knockout mice showed an increased number of secretory granules in melanotrophs and, in the case of PC2, enhanced accumulation of POMC , suggesting that PC2 and 7B2 may have roles in secretion as well as processing (Laurent et al. 2004).

Another factor potentially involved in the melanotroph processing of POMC is plasmin. A deficiency of plasminogen reduces processing of β-endorphin and αMSH, and interferes with normal brain function (Wang et al. 2004).

4.4 POMC Processing in the Hypothalamus

POMC processing in the hypothalamus is very similar to that found in melanotroph cells of the pituitary intermediate lobe resulting in the production of MSH peptides (Fig. 1b), although with some differences in regulation. There is considerable evidence implicating hypothalamic POMC and αMSH in the regulation of food intake. Leptin stimulates the release of hypothalamic POMC peptides and they are in turn mediated by the melanocortin-4 receptor (MC4-R). Children with mutations and mice with deletions in POMC, MC4-R and leptin are all grossly obese ((Pritchard et al. 2002) (Fig. 3).

Defects in POMC processing are implicated in human obesity. The importance of PC1 in energy homeostasis is evidenced by a child with inactivating mutations in PC1 who displays an obese phenotype. The loss of PC1 also resulted in defects in processing of a number of other hormones; so it is difficult to implicate individual hormones and neuropeptides (Jackson et al. 1997). Also, CPE deficient mice are obese and hyperglycemic (Naggert et al. 1995) due to missorting of prohormones. Serum POMC peptides are negligible in these mice, whereas some abnormal larger POMC peptides are present (Cool et al. 1997).

There is relatively little information on the processing of POMC and on the levels of precursors and MSH peptides in the hypothalamus. Much of the evidence implicating αMSH comes from knock-out studies and from studies giving synthetic POMC derived peptides (Pritchard and White 2007). POMC itself is secreted by rat hypothalamic cells in culture (Liotta et al. 1980). In the hypothalamus, both POMC and ACTH are secreted from neurons in the arcuate nucleus and in humans they are easily detected in cerebro-spinal fluid (CSF) with POMC approximately 40-fold higher than ACTH (Tsigos et al. 1993). Moreover, comparison of the ratio of POMC to ACTH in lean and obese Zucker rats indicates that central POMC processing is impaired in obesity with higher POMC and less ACTH (Pritchard and White 2007). This is consistent with changes in the processing enzymes observed in obesity (Pritchard et al. 2003). Interestingly, αMSH was not detected in CSF or hypothalamic extracts when POMC and ACTH were easily detectable (Pritchard and White 2007).

POMC-derived peptides have been identified in the brain after HPLC analysis of hypothalamic extracts. The peptides measured were N-POMC (1-49), joining peptide, αMSH, des acetyl-αMSH, βMSH and β-endorphin (Castro and Morrison 1997) although there was no comment as to whether POMC itself was present.

In rat hypothalami extracts, des acetyl-αMSH appears to be more predominant than αMSH, therefore demonstrating tissue specificity of *N*-acetyltransferase action as there is much more αMSH than des acetyl-αMSH in the intermediate lobe of the pituitary (Emeson and Eipper 1986). Des acetyl-αMSH injected

intracerebroventricularly has no effect on food intake whereas αMSH inhibits food intake (Tsujii and Bray 1989). This suggests that regulation of MSH acetylation may be central in the control of behaviour. There is also some intriguing data to suggest that des-acetyl αMSH is more sensitive to degradation than acetylated αMSH (Guo et al. 2004; Wilkinson 2006). Therefore differences in biological activity of des-acetyl αMSH and αMSH and in their sensitivity to protease degradation may explain observed functional differences between the two forms of αMSH.

Leptin not only regulates the biosynthesis and processing of POMC, but also some of the PC1 and PC2 expression. Leptin has been shown to increase PC1 and PC2 promoter activities and food deprivation in rats, leading to low serum leptin levels, resulting in a decrease in PC1 and PC2 gene and protein expression in the paraventricular and arcuate nucleus of the hypothalamus (Nillni 2007). Leptin also activates *N*-acetyltransferase in POMC neurons leading to increased hypothalamic levels of αMSH (Guo et al. 2004). (Fig. 2b).

In the hypothalamus, peptides derived from POMC function through the MC4 receptor (MC4-R). The natural agonist ligand of MC4R is presumed to be αMSH antagonised by agouti-related protein (AGRP). However, it has been demonstrated that βMSH, des acetyl-αMSH and ACTH all have similar potencies to αMSH at MC4R (Pritchard et al. 2004) (Fig. 3). Mutations in βMSH in a child with obesity suggest that it may well be involved in regulation of food intake (Challis et al. 2002; Lee et al. 2006). In addition, even POMC itself has some activity at the MC4-R. These observations imply that many POMC-derived peptides could have a role in appetite regulation (Pritchard et al. 2004) (Fig. 2b).

4.5 POMC Processing in the Skin

The skin and hair follicle have all the components of the HPA axis (Paus et al. 2006; Slominski et al. 2007). In epidermal melanocytes and keratinocytes, POMC, ACTH and αMSH (Luger et al. 2000; Rousseau et al. 2007) (together with convertases PC1 and PC2 (Rousseau et al. 2007) (Deacon et al. 2000)) have been detected. The coordinated production of the cutaneous HPA axis components could enable the skin to regulate local responses to stress (Slominski et al. 2005) (Fig. 3).

Recent data indicates that POMC itself is the main peptide released from human hair follicle melanocytes, epidermal melanocytes and human skin keratinocytes. Moreover, CRH not only increases the release of this precursor but also activates its intracellular processing. (Rousseau et al. 2007)

There is accumulating evidence that there is a key role for the cutaneous HPA axis components in the efficacy of tissue repair, although many important questions remain to be addressed. This is supported by the multiple functions which αMSH exerts on skin importantly as a potent immunosuppressant (Bohm et al. 2005; Rouzaud et al. 2006; Scholzen et al. 2000).

4.6 Secretion of ACTH Precursors by Extra-Pituitary Tumors

In most cases of pituitary corticotroph adenomas the processing of POMC to ACTH appears to be normal (Stewart et al. 1994). However there can be some disruption of ACTH processing in corticotroph macroadenomas (Gibson et al. 1996) where decreased PC1 expression may result in an increase of the secretion of the precursors POMC rather than ACTH (Tateno et al. 2007).

In most extrapituitary tumors that express POMC, the processing is inefficient, resulting in increased secretion of unprocessed POMC. Small cell lung carcinoma (SCLC) is the most common example of these extrapituitary tumors and very frequently there are elevated levels of POMC in plasma and a high ratio of POMC to ACTH (Oliver et al. 2003; Stewart et al. 1994). In one patient with a phaeochromocytoma, the levels of POMC in the patient's plasma correlated with increased skin pigmentation (White et al. 2000).

Glucocorticoid resistance in POMC expressing extrapituitary tumors is usually due to mutations in the GR (Gaitan et al. 1995; Ray et al. 1996) or altered GR pathway function (Waters et al. 2004). Recently it has been shown that expression of wild-type GR can induce apoptosis in SCLC cell lines (Sommer et al. 2007), suggesting that there is a survival advantage conferred on glucocorticoid resistant cell lines and implying that POMC expression and ACTH secretion may be biomarkers of this malignancy.

4.7 Other Sites of POMC Processing

POMC is also expressed, but at a much lower level, in other tissues such as the testis, ovary, placenta, duodenum, liver, kidney, adrenal medulla, lung, thymus, heart and lymphocytes (Buzzetti et al. 1989; Chen et al. 1986; DeBold et al. 1988; Grigorakis et al. 2000; Jingami et al. 1984; Lacaze-Masmonteil et al. 1987; Millington et al. 1999; Slominski et al. 2007). Little work has been done to look at POMC processing in these tissues; however, processing of POMC in the placenta results in significant amounts of POMC secretion along with ACTH, β-LPH, α-MSH and β-endorphin (Grigorakis et al. 2000).

Immune cells express the machinery required for processing POMC into active peptides and are able to release these peptides from secretory granules. (Mousa et al 2004). Early papers predicted that immune cells secreted significant amounts of these peptides but given the number of these cells in the body, the concentration of ACTH in the human circulation would be sufficient to cause Cushing's Syndrome. One explanation is that the peptides are degraded soon after release in the extracellular environment and do not reach the circulation.

5 Incomplete Processing of POMC: When, How and Why?

It is difficult to resolve how POMC processing at the cellular level results in the specific peptides released from the tissues. It does not seem to be, as some text books would suggest, that the presence of PC1 enables ACTH to be produced, whereas the presence of PC1 and PC2 allows predominantly αMSH. The concentration of specific peptides in blood or in the paracellular environment (Fig. 2d) could depend upon (1) the regulation of the POMC gene, (2) the synthesis of the convertases, (3) the activity of these enzymes and their cofactors, (4) the sorting of the peptides into specific secretory vesicles and/or (5) the factors which regulate the release of the vesicles. In addition it may well depend upon the different half lives of the various peptides as discussed for αMSH and des acetyl-αMSH in the hypothalamus.

Thus the human pituitary produces ACTH, N-POC, βLPH but very little β-endorphin (Gibson et al. 1994). In addition, highly specific two-site immunometric assays for POMC and ACTH have been used to compare ratios of POMC:ACTH in different tissues and body fluids in order to understand the degree of processing. Based on these assays, POMC is detected in the plasma of normal human subjects at concentrations of 5–40 pmol l^{-1} which are higher than the ACTH levels (<0.9–11pmol l^{-1}). This indicates that processing in the normal pituitary is incomplete and POMC is released into the circulation at a 5:1 molar ratio to ACTH (Gibson et al. 1994).

It has been shown in man, in vivo, that CRH stimulation of the pituitary for periods as little as 3 min regulates acute secretion of ACTH but not of POMC, suggesting that POMC and ACTH are in different secretory granules, with POMC exiting the pituitary cell by a constitutive-like pathway.

Evidence described above indicates that the intermediate lobe of the pituitary, the hypothalamus and the skin produce primarily αMSH but when comparisons are made on a molar basis it appears more complex and both ACTH and POMC can be detected at higher concentrations than αMSH.

There are two potential explanations for the presence of unprocessed POMC, secreted from POMC expressing cells. Either POMC processing in the regulated secretory pathway is incomplete and secretory granules contain high levels of unprocessed material, or alternatively, significant quantities of POMC are secreted in a constitutive-like manner. The dynamics of release of POMC and ACTH have been examined in AtT20 mouse pituitary adenoma cells. Under basal conditions there is an excess of intracellular POMC compared to ACTH (200 pmol l^{-1} compared to 33 pmol l^{-1}) (Fig. 4). After 2 h stimulation with CRH there is a 1.3-fold increase in intracellular ACTH and a 2-fold increase in secreted ACTH with no change in secreted POMC. After stimulation with CRH for 72 h there is no increase in POMC or ACTH intracellularly, but there is a 1.2-fold increase in POMC and a 1.3-fold increase in ACTH secreted into the cell medium. These data imply that CRH stimulates release of ACTH from the cells in a nongenomic manner but POMC is secreted constitutively and can only be regulated genomically (Fig. 4).

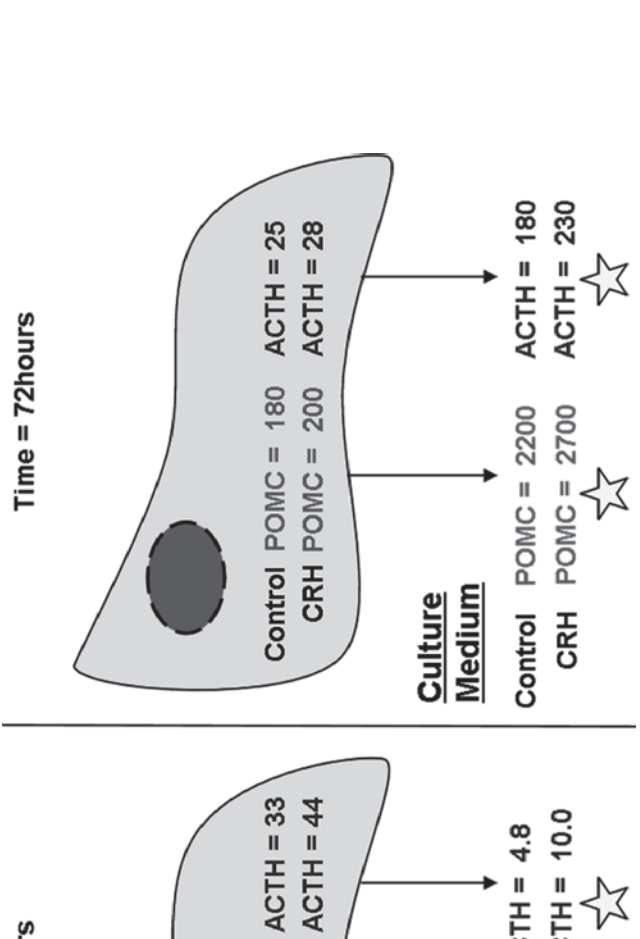

Fig. 4 The unregulated secretion of POMC. The relative levels of POMC peptides inside and outside the cell when treated with CRH.Actively dividing AtT20 cells were grown in culture for either 2 or 72 h. The concentration of POMC and ACTH (pmol l^{-1}) was assayed in the cell-culture medium and in cell extracts (White 2008 unpublished data)

5.1 Extracellular Modification of POMC

The extracellular proteolytic processing of POMC peptides has received little attention despite a potentially important role in controlling the local activation of MCRs. Recently studies in the skin have shown that specific peptidases such as neprilysin (neutral endopeptidase; NEP) or angiotensin-converting enzyme (ACE) can act on ACTH-like peptides (Konig et al. 2006). Nardilysin (Seidah and Prat 2002), neprilysin and ACE (Konig et al. 2006) function in the extracellular environment. It is also possible for extracellular tissue kallikreins to digest ACTH and potentially POMC (Fig. 5). Therefore extracellular processing of POMC could well generate ACTH-like peptides. However it is unlikely to generate α-MSH, as the latter requires intracellular post-translational processing.

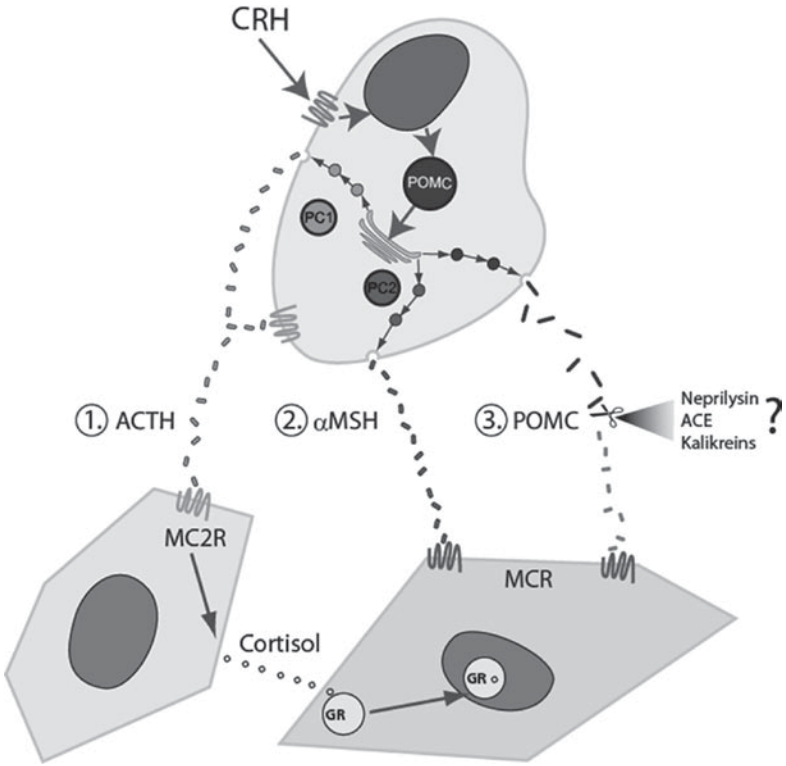

Fig. 5 The extracellular modification of POMC. POMC producing cells can secrete (1) ACTH via PC1 processing (2) αMSH via PC2 processing (3) POMC constitutively. ACTH stimulates local/systemic glucocorticoid release by interaction with MC2-R. αMSH can generate effects on cells via MC1-R (skin) or MC4-R (in the hypothalamus). Possible extracellular digestion of POMC by protease enzymes such as Kalikrein, neprilysin and ACE may result in fragments of POMC that are physiologically active

There is a precedent for extracellular processing in that adrenal secretory serine protease (AsP) has been shown to be involved in the processing of N-POMC-derived peptides (Hansen et al. 2004). The fragment produced [N-POMC (1-52)] acts as an adrenal mitogen (Bicknell 2008).

6 Conclusion

The roles of POMC peptides are most clearly evidenced by two patients who carry inactivating mutations in the POMC gene. These patients have adrenal insufficiency, severe obesity, pale skin and red hair, highlighting the importance of POMC derived peptides at the adrenal gland, in the hypothalamus and in the skin respectively (Coll et al. 2004b; Krude et al. 1998). It is anticipated that the effects of ACTH at the adrenal gland predominate and treatment to restore glycaemic control is important for survival.

However inherited abnormalities in POMC synthesis and processing of POMC-derived peptides in both humans and mice have also helped shape our current understanding of the importance of the melanocortin system in human energy balance (Coll et al. 2004a). Mutations in the POMC gene which impair the synthesis or structure of POMC-derived peptides do predispose to human obesity (Creemers et al. 2008).

Regulation of the synthesis and secretion of POMC peptides is clearly critical and there are still areas that are not easily understood. There is clearly a need for acute release of ACTH from pituitary cells, in response to stress, to activate rapid release of glucocorticoids. Over a longer time-frame, regulation of POMC synthesis will adjust the supply of precursor peptide available to the cell, and altered POMC processing will influence the ratio of precursors to ACTH secreted by the cell. ACTH and POMC precursor peptides are both secreted but are subject to different regulation, and partitioning of POMC into ISGs is likely to account for its constitutive-like secretion. Release of POMC itself from pituitary cells could simply relate to inefficient processing in the secretory granules, but it is also possible that the ratios of POMC and ACTH differ with different regulatory factors which would not occur if these factors just targeted release of the vesicles.

Regulated secretion of the POMC precursor peptide may provide a mechanism to allow selective processing of the prohormone at target organs as has been demonstrated with N-POC (White and Gibson 1998). Release of POMC could be an "overflow pathway" reflecting the need for subtle mechanisms (acute and chronic, repetitive and sustained) to regulate ACTH release. Thus it would allow for differential regulation of the POMC gene and the processed peptides. However it is tempting to speculate that there is regulation of processing to produce peptides with varying potency, and even to release the prohormone for extracellular processing at its site of action.

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Neurotensin and Neuromedin N Are Differentially Processed from a Common Precursor by Prohormone Convertases in Tissues and Cell Lines

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Abstract Neurotensin (NT) is synthesized as part of a larger precursor that also contains neuromedin N (NN), a six amino acid NT-like peptide. NT and NN are located in the C-terminal region of the precursor (pro-NT/NN) where they are flanked and separated by three Lys–Arg sequences. A fourth dibasic sequence is present in the middle of the precursor. Dibasics are the consensus sites recognized and cleaved by specialized endoproteases that belong to the family of proprotein convertases (PCs). In tissues that express pro-NT/NN, the three C-terminal Lys–Arg sites are differentially processed, whereas the middle dibasic is poorly cleaved. Processing gives rise mainly to NT and NN in the brain, NT and a large peptide with a C-terminal NN moiety (large NN) in the gut, and NT, large NN, and a large peptide with a C-terminal NT moiety (large NT) in the adrenals. Recent evidence indicates that PC1, PC2, and PC5-A are the prohormone convertases responsible for the processing patterns observed in the gut, brain, and adrenals, respectively. As NT, NN, large NT, and large NN are all endowed with biological activity, the evidence reviewed here supports the idea that posttranslational processing of pro-NT/NN in tissues may generate biological diversity of pathophysiological relevance.

1 Introduction

Neurotensin (NT) is a tridecapeptide (Fig. 1) first isolated from bovine hypothalamus (Carraway and Leeman 1973) and shortly after, from bovine small intestine (Kitabgi et al. 1976). In the brain, NT is predominantly expressed in neurons within discrete brain area and nuclei. The effects of centrally administered NT include the well documented interaction of the peptide with dopaminergic systems (Geisler et al. 2006; Caceda et al. 2006), the ability to induce opioid-independent analgesia (Dobner 2006),

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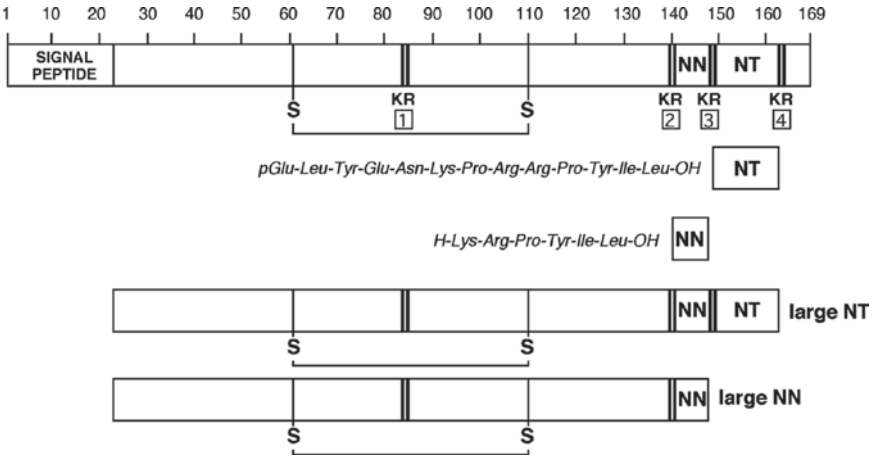

Fig. 1 *Representation of the rat neurotensin (NT)/neuromedin N (NN) precursor and of precursor-derived processing products*. Rat prepro-NT/NN is 169 residues long and starts with a 22 residues signal peptide. The NN and NT sequences are located in tandem near the C-terminus of the precursor. The four Lys–Arg (KR) dibasic processing sites are represented by *thick double bars* and are numbered 1–4 from N- to C-terminus. The disulfide bridge between Cys^{61} and Cys^{110} is also represented. This organization is conserved in all the species from which the precursor has been cloned. The biologically active products, i.e., NT, NN, large NT, and large NN, that are differentially generated following tissue-specific pro-NT/NN processing, are represented below the precursor. The amino acid sequences of NT and NN are shown in *italic*

and inhibition of food intake (Beck 2000). In the periphery, NT is primarily produced in endocrine N cells scattered throughout the jejuno-ileal mucosa. The peptide is released upon food ingestion and regulates a number of digestive processes including gastrointestinal motility and pancreatic and biliary secretion (Zhao and Pothoulakis 2006). In addition, NT exerts growth-promoting effects on normal gastrointestinal tissues and on cancer cells derived from a variety of organs (Evers 2006; Carraway and Plona 2006). Besides the brain and gut, NT is found in other tissues such as the adrenals where its functions remain poorly understood.

Peptide hormones and neuropeptides like NT are synthesized and stored within neuroendocrine cells and released upon appropriate stimuli. They share a number of common features that characterize their biosynthesis (Mains et al. 1990), as follows: (1) Almost all neuropeptides and peptide hormones are synthesized as part of larger inactive polypeptide precursors. Often, a precursor may contain in its sequence more than one potentially active peptide. (2) Neuropeptide sequences in the precursor are flanked by clusters of basic residues, most often dibasics, which serve as cleavage sites for releasing the peptides in active form from the precursor. (3) Cleavage at basic residues is effected by a limited set of proteolytic enzymes that belong to a larger family of proprotein convertases (PCs). (4) In the case of a multipeptide precursor, PC-mediated processing often generates different combinations of active peptides in the tissues that express the precursor.

NT biosynthesis in tissues follows these rules as will be reviewed here.

2 The Neurotensin Precursor Also Contains Neuromedin N

Ten years after the discovery of NT, the purification of an NT-related hexapeptide named LANT6 from chicken intestine was reported (Carraway and Ferris 1983). One year later its mammalian hexapeptide counterpart, neuromedin N (NN), was isolated from porcine spinal cord (Minamino et al. 1984). NT and NN share a common C-terminal tetrapeptide (Fig. 1) and exhibit similar pharmacological activity profiles. In 1987, the NT precursor cDNA was cloned from canine intestine and bovine brain (Dobner et al. 1987) and in 1988 the sequence of the rat precursor gene was reported (Kislauskis et al. 1988). These studies revealed that NT and NN were products of the same precursor and were encoded by the same exon in the precursor gene. Furthermore, in all three species, the precursor molecule consisted of a highly conserved polypeptide of 169 (rat)–170 amino acids starting with a N-terminal signal peptide and containing one copy each of NT and NN in its C-terminal region. Figure 1 depicts the organization of the rat NT/NN precursor. The signal peptide was shown to be 22 residues long (Bidard et al. 1993; Carraway and Mitra 1991). NN precedes NT and is separated from it by a Lys–Arg sequence. Two other Lys–Arg sequences flank the N-terminus of NN and the C-terminus of NT. A fourth Lys–Arg pair (Arg–Arg in the canine and bovine precursor) occurs in the middle of the precursor. These four dibasic sites - denoted 1, 2, 3, and 4 from N- to C-terminus (Fig. 1)- represent consensus cleavage sites for the PCs, thus suggesting that pro-NT/NN may be processed to generate various sets of peptides in addition to NT and NN. Noticeably, it was found that site 1 is enclosed within a loop delimited by a disulfide bridge that links the only two cysteine residues (conserved in all species) present in the proprotein (Feliciangeli et al. 2001).

3 Pro-NT/NN Is Differentially Processed in Tissues and Cell Lines

With the use of region-specific antibodies raised against various portions of the pro-NT/NN sequence combined with protease treatment of tissue and cell extracts and HPLC analysis, it was possible to analyze the composition of precursor-derived products in the tissues and cell lines that endogenously express pro-NT/NN. Protease treatment served to unmask antigenic sequences within unprocessed or partially processed precursor fragments. Essentially two types of treatment were applied: pepsin digestion (Carraway et al. 1992) and Arg-directed trypsin cleavage following reversible protection of Lys residues (Bidard et al. 1993). Both methods quantitatively excised immunoreactive sequences from pro-NT/NN and large pro-NT/NN fragments, thus allowing their subsequent measurement. This in turn permitted to calculate the extent to which each pro-NT/NN dibasic site was endogenously processed. These studies revealed that site 1 in the middle of the disulfide bond-delimited loop was poorly, if at all, processed in all systems examined, possibly because of its

conformational environment. In contrast, processing of sites 2 to 4 was observed in most systems, albeit to different extent. This led to the production of different combinations of four biologically active products, i.e., NT, NN, large NT, and large NN, the latter two starting right after the signal peptide and ending with the NT and NN sequence, respectively (Fig. 1).

3.1 Differential Processing in the Brain, Gut and Adrenals

In the brain, cleavage at sites 2, 3, and 4 was nearly complete (Carraway et al. 1992, 1993; de Nadai et al. 1994; Woulfe et al. 1994), giving rise to high amounts of NT and NN and small quantities of large NN and large NT (Table 1). A minor cleavage at site 1 was also observed in some brain regions (de Nadai et al. 1994). In contrast, in the gut, cleavage at site 2 was largely incomplete whereas cleavages at sites 3 and 4 were substantial (Carraway and Mitra 1990; Shaw et al. 1990), resulting in high amounts of NT and large NN and small amounts of NN (Table 1). Yet another processing pattern was observed in the adrenal medulla (Carraway et al. 1993) where cleavage at site 4 exceeded that at site 3 which in turn was greater than that at site 2, thereby leading to the production of significant amounts of large NT besides NT and large NN (Table 1).

3.2 Processing in Cell Lines

A number of neuroendocrine cell lines, endogenously express pro-NT/NN and have provided good models to study pro-NT/NN processing. The endocrine rat medullary thyroid carcinoma (rMTC) 6-23 cell line was shown to express high levels of pro-NT/NN following dexamethasone induction (Zeytinoglu et al. 1980; de Nadai et al. 1993). Studies of precursor processing in this cell line showed that sites 2, 3, and 4 were readily cleaved to generate almost stoichiometric amounts of NT and NN and minor quantities of large NT and large NN (Bidard et al. 1993) (Table 1). Thus, the processing pattern of pro-NT/NN in rMTC 6-23 cells resembles that observed in the brain. The endocrine BON cell line, derived from a human pancreatic carcinoid tumor, was shown to constitutively express pro-NT/NN and process this precursor essentially at sites 3 and 4, leading to the formation of NT and large NN as the major products (Carraway et al. 1994) (Table 1). Such a processing pattern is reminiscent of that described in the gut. The rat pheochromocytoma PC12 cell line, a widely used model of neuronal cells, was shown to express high levels of pro-NT/NN in response to a combination of inducers comprising nerve growth factor, dexamethasone, forskolin, and lithium (Kislauskis and Dobner 1990). However, in contrast to rMTC and BON cells, PC12 cells poorly processed pro-NT/NN at any of its dibasic sites, which resulted in the presence of high concentrations of intact precursor in these cells (Carraway et al. 1993; Rovère et al. 1993).

Table 1 Proneurotensin/neuromedin N cleavage sites and maturation products in tissues, cell lines, and prohormone convertase-transfected PC12 cells

	Extent of cleavage at sites 1–4	Relative abundanc of major products						
Tissues	1	2	3	4	NT	NN	Large NT	Large NN
Brain	Minor	Major	Major	Major	High	High	Low	Low
Gut		Minor	Major	Major	High	Low	Very low	High
Adrenals		Minor	Intermediate	Major	High	Low	High	High
			Cell lines					
RMTC 6-23		Major	Major	Major	High	High	Low	Low
BON		Minor	Major	Major	High	Low	Low	High
ColoDM320		Minor	Intermediate	Major	High		High	High
PC12			Minor	Minor	Very low			
			PC-transfected PC12 cells					
PC2–PC12		Major	Major	Major	High	High	Very low	Very low
PC1–PC12		Minor	Major	Major	High	Low		High
PC5A–PC12		Minor	Intermediate	Major	High	Very low	High	High

Nonendocrine cell lines derived from human colon, pancreatic, prostate, and lung cancers have also been shown to produce NT and/or to express pro-NT/NN (Evers 2006; Carraway and Plona 2006). Precursor processing was studied in 13 human colon cancer cell lines that all express pro-NT/NN and it was shown that only approximately 50% of the cell lines were able to generate biologically active products (Rovère et al. 1998). Analysis of pro-NT/NN processing pattern revealed that site 2 was not cleaved at all whereas sites 3 and 4 were unequally and partially cleaved to an extent that varied depending on the cell line. This often resulted in the formation of large NT and large NN in amounts that exceeded that of NT, as illustrated in Table 1 for the coloDM320 cell line. Recently, the release of pro-NT/NN from small cell lung carcinoma cell lines was reported and it was suggested that the precursor protein could be a useful marker of small cell lung carcinoma (Ogura et al. 2008). However, the processing of pro-NT/NN in these cell lines was not investigated.

4 Pro-NT/NN Processing by Prohormone Convertases

PCs are a family of subtilisin-like proteases that excise biologically active proteins and peptides from their precursors by cleaving at the C-terminal side of the basic residues that flank the sequence of the mature proteins and peptides (Mains et al. 1990). Most often, the cleavage site is a cluster of basic residues, usually a pair, and more seldom a single basic residue. Among the PCs, some are specialized in the processing of neuropeptide and hormone precursors in the regulated secretory pathway of neuroendocrine cells. Thus, PC1 and PC2 are exclusively found in compartments of the regulated secretory pathway (Golgi and secretory vesicles) in neuroendocrine cells. PC1 and PC2 differ in their specificity towards dibasic sites and may be expressed either separately or together in endocrine and neuronal cells, which may result in differential precursor processing. Besides PC1 and PC2, PC5-A has a widespread distribution not only in nonendocrine tissues but also in neuroendocrine cells where it is localized in the regulated secretory pathway (De Bie et al. 1996). The mode of cleavage by PCs will leave at the C-terminus of the excised peptides one or two basic residues that, in neuroendocrine cells, are subsequently removed by carboxypeptidase E (CPE), an enzyme also expressed in the regulated secretory pathway (Fricker et al. 1986).

The ability of PC1, PC2, and PC5-A to process pro-NT/NN in neuroendocrine cells was studied in the PC12 cell line. As mentioned above, PC12 cells express but poorly process pro-NT/NN. Interestingly, it was shown that PC12 cells lacked PC1 and PC2 expression and expressed only low levels of PC5-A (Rovère et al. 1996a). This offered the possibility to determine and compare the processing patterns of pro-NT/NN by these proteases by stably transfecting each of them in PC12 cells and analyzing the content of pro-NT/NN-derived products in the transfected cell lines. These studies showed that all three convertases were expressed and sorted to the regulated secretory pathway of transfected PC12 cells where they efficiently, albeit differentially, processed pro-NT/NN (Barbero et al. 1998; Rovère et al. 1996a).

Thus, pro-NT/NN processing by PC2-transfected PC12 cells readily occurred to a similar extent at sites 2, 3,and 4, thereby generating equivalent amounts of NT and NN. In PC1-transfected PC12 cells, cleavage at site 3 was slightly more efficient than cleavage at site 4 which in turn was more efficient than cleavage at site 2. This processing pattern predominantly generated NT and large NN and produced only small amounts of NN. Finally, PC5-A-transfected PC12 cells exhibited yet another pro-NT/NN processing pattern as they cleaved site 4 to a greater extent than site 3, and were virtually inefficient in cleaving site 2. This resulted in the production of NT, large NT, and large NN in comparable amounts and of virtually no NN .This data is recapitulated in Table 1.

To summarize, it appears that (1) all three convertases are capable of generating NT; (2) PC5-A, unlike PC1 and PC2, has the capablity to generate substantial amounts of large NT; (3) PC1 and to a lesser extent PC5-A are able to generate large NN; and (4) PC2 differs from both PC1 and PC5-A in its ability to generate NT and NN in similar amounts and its inability to generate the large forms. It is further apparent that PC2 mimics the pro-NT/NN processing pattern observed in the brain and in rMTC 6-23 cells, that PC1 reproduces the pattern observed in the gut and in BON cells, and that PC5-A generates a pattern similar to that found in the adrenals and in some colon cancer cell lines (Table 1).

5 Involvement of PCs in Pro-NT/NN Processing in Tissues and Cell Lines

5.1 PC2 in Brain and rMTC 6-23 Cells

From what has been said above, PC2 appears as a good candidate for processing pro-NT/NN in brain neurons. Supporting this hypothesis is the observation that PC2 is the most abundant prohormone convertase in the brain. It was therefore not unexpected to find that PC2 immunoreactivity colocalized extensively with immunoreactive NT in neuronal populations that express pro-NT/NN (Villeneuve et al. 2000a). Interestingly, subsets of NT/PC2-immunopositive neurons were also positive for PC1 and PC5-A, but, in general, those neurons that expressed PC1 did not contain PC5-A and vice versa (Villeneuve et al. 2000a, b). That PC2 plays a major role in processing brain pro-NT/NN was further demonstrated in $PC2^{-/-}$ mice in which brain levels of NN were reduced by more than 50% with a compensatory increase in the levels of large NN, indicating that cleavage at site 2 was impaired, as would be expected from the cleavage specificity of PC2 (Villeneuve et al. 2002). There was also a 20% reduction in NT levels in $PC2^{-/-}$ mice, which is less than would be expected had PC2 been the sole pro-NT/NN convertase in brain, and which strongly supports the idea that PC1 and PC5-A were also active in processing brain pro-NT/NN.

The involvement of PC2 in pro-NT/NN processing in rMTC 6-23 was directly demonstrated (Rovère et al. 1996a, b). First, of the three PCs discussed here, only

PC2 was expressed in this cell line. Second, stable expression of an antisense PC2 mRNA in rMTC 6-23 cells resulted in a drastic decrease in PC2 protein synthesis (>90%) that was accompanied by a marked reduction in pro-NT/NN cleavage (>80%) at sites 2, 3, and 4.

5.2 PC1 in Gut and BON Cells

PC1 is an obvious candidate for pro-NT/NN processing in the gut and in BON cells. In the gut, immunoreactive NT is chiefly localized to a subset of endocrine cells, designated N cells, located in the mucosa of the small intestine. It was observed that immunoreactive NT and PC1 colocalized in the N cells (Kitabgi 2006). However, a direct demonstration of PC1 involvement in pro-NT/NN processing in the gut using $PC1^{-/-}$ mice is lacking. Similarly, BON cells were shown to express PC1 and reduction of PC1 expression using antisense strategy markedly impaired the processing of chromogranin A, a protein that is stored in secretory vesicles (Udupi et al. 1999). It would be of interest to investigate the processing of pro-NT/NN in antisense PC1 mRNA-expressing BON cells. In any case, the above observations strongly favor PC1 as the major pro-NT/NN converting enzyme in the gut and in BON cells.

5.3 PC5-a in Adrenals and Colon Cancer Cells

The adrenals and some colon cancer cell lines were shown to produce substantial amounts of large NT, a product that only PC5-A could generate. It is therefore of interest that the enzyme was found to be particularly abundant in the adrenals (De Bie et al. 1996), and that human colon cancer cell lines such as the coloDM320 cell line were shown to express PC5-A whereas they were devoid of PC1 and PC2 (Rovère et al. 1998). Although this is no direct demonstration that PC5-A does process pro-NT/NN in these systems, the above observations strongly support a role for the protease in pro-NT/NN precursor cleavages that lead to the formation of large NT and possibly other pro-NT/NN-derived products in the adrenals and in colon cancer cell lines.

6 Carboxypeptidase E and Dibasic Removal

As mentioned above, PCs cleave at the C-terminal side of dibasics and will therefore generate peptides with a C-terminal dibasic extension which has then to be removed by CpE in the regulated secretory pathway of neuroendocrine cells before the mature peptides can be secreted in the extracellular medium. The importance of this step in prohormone processing was demonstrated in *fat/fat* mice. These animals are characterized by a marked hyperproinsulinemia and late-onset obesity. The *fat* mutation

was shown to map to the gene encoding CpE and result in inactivation of the enzyme leading to the production of poorly active diarginyl insulins (Naggert et al. 1995). In the case of pro-NT/NN, studies of precursor-derived products in the brain of *fat/fat* mice showed that both brain and hypothalamic levels of authentic NT and NN were reduced approximately tenfold and replaced by equimolar amounts of C-terminally extended NT and NN bearing a C-terminal Lys–Arg sequence (Rovère et al. 1996b). As these products are pharmacologically inactive, NT-ergic transmission might be expected to be markedly impaired in *fat/fat* mice. Hypothalamic NT having well established anorexigenic effects (Beck 2000), this data might explain in part the obese phenotype of these animals.

7 Physio-Pathological Implications

We have seen that processing of pro-NT/NN leads to the production of NT and NN in the brain where both neuropeptides have well established neuromodulator properties. In the periphery, pro-NT/NN processing gives rise mostly to NT and large precursor forms such as large NT and large NN. A paracrine role for NT in regulating intestinal functions has been well documented (Zhao and Pothoulakis 2006). On the other hand, little is known about the biological properties of large NN and large NT. The question then arises as to what might be the physiological relevance of the large peptides.

While a paracrine role for intestinal NT is well accepted, there has been debate as to whether the peptide could exert endocrine actions on target organs such as the stomach or the pancreas that are located at distance from its jejuno-ileal sites of release. Indeed, NT has been shown to undergo rapid enzymatic degradation both locally at sites of release and in the circulation, resulting in barely increased circulating levels of intact NT following meal ingestion (Leeman and Carraway 1982). Could large NT and large NN exert hormonal neurotensinergic actions? Several lines of evidence suggest that this is the case. Thus, besides the fact that they are produced in endocrine tissues and cell lines of peripheral origin as reviewed here, both large NT and large NN were found to be released in the hepatic portal circulation after small intestine exposure to oleic acid (Carraway et al. 1992). Actually, basal large NN concentrations exceeded those of NT and stimulated levels were three times higher and plateaued longer with large NN than with NT. Partially purified large NN was shown to have biological activity on isolated guinea-pig ileum preparations (Carraway and Mitra 1991; Carraway et al. 1992). Pure recombinant large NT and large NN were shown to bind to and activate the NTS1 NT receptor which is thought to mediate most of the peripheral and central effects of NT (Friry et al. 2002). Large NT was quite potent in this regard. Finally, large NT and large NN were much more resistant to degradation than their small counterparts when exposed to cultured cells, which may have explained their longer life span in the circulation. Altogether, these observations lend strong support to the proposal that both large NT and large NN might be considered as bona fide peripheral hormones endowed with NT-like activity.

More recently, human pro-NT/NN (1-117), a large precursor form that ends N-terminally to site 2 (Fig. 1), was detected in the human circulation, found to be increased following a meal and shown to have great metabolic stability (Ernst et al. 2006). Lacking both the NN and NT sequences, it is unlikely that this product might activate NT receptors. However, due to its stability, it might provide a useful index of the release of pro-NT/NN-derived peptides in the general circulation.

In addition to their existence in normal tissues, the presence of large forms in several cancers has been reported. Thus, as reviewed here, large NN was found in high concentrations in BON cells, a cell line derived from a human pancreatic carcinoid tumor, and both large NT and large NN were present, sometimes in greater amounts than NT, in human colon cancer cell lines. NT has been proposed to act as a trophic factor in colon, pancreas, lung, and prostate cancers (four). The facts that large NT and large NN potently activate the NTS1 receptor and are more metabolically stable than small NT clearly point to these proteins as also being good candidates for inducing tumor growth. They could act either as autocrine trophic factors released by target cancer cells or as circulating trophic factors originating from tissues such as the gut or the adrenals.

8 Conclusions

Like many polypeptide hormone or neuropeptide precursors, pro-NT/NN undergoes tissue-specific processing, which gives rise to different combinations of active peptides. This appears to be due to the differential distribution of PC1, PC2, and PC5-A in pro-NT/NN-expressing tissues and to the differential ability of the convertases to cleave dibasic sites within the precursor. Most studies on neurotensinergic systems have focused on NT and NN, the small mature products of pro-NT/NN. The recognition that large forms such as large NT and large NN can be produced and released in substantial amounts by peripheral tissues and that they are endowed with biological activities strongly suggests that they could fulfill physiological functions. Therefore, it appears necessary to determine the content not only of NT and NN but also of large NT and large NN in tissues and cell lines that express pro-NT/NN in order to draw conclusions as to the potential of these systems to activate neurotensinergic pathways. In particular, such considerations may have pathophysiological relevance in cancers where growth-promoting effects might be exerted by large forms that are often produced in greater amount than NT by cancer cell lines.

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Biosynthesis of Cardiac Natriuretic Peptides

Jens Peter Goetze

Abstract Cardiac-derived peptide hormones were identified more than 25 years ago. An astonishing amount of clinical studies have established cardiac natriuretic peptides and their molecular precursors as useful markers of heart disease. In contrast to the clinical applications, the biogenesis of cardiac peptides has only been elucidated during the last decade. The cellular synthesis including amino acid modifications and proteolytic cleavages has proven considerably more complex than initially perceived. Consequently, the elimination phase of the peptide products in circulation is not yet well characterized. An ongoing characterization of the molecular heterogeneity will help appreciate the biosynthetic capacity of the endocrine heart and could introduce new diagnostic possibilities. Notably, different biosynthetic products may not be equal markers of the same pathophysiological processes. An inefficient post-translational prohormone maturation will also affect the biology of the cardiac natriuretic peptide system. This review aims at summarizing the myocardial synthesis of natriuretic peptides focusing on B-type natriuretic peptide, where new data has disclosed cardiac myocytes as highly competent endocrine cells. The structurally related atrial natriuretic peptide will be mentioned where appropriate, whereas C-type natriuretic peptide will not be considered as a cardiac peptide of relevance in mammalian physiology.

1 Introduction

Ernest H. Starling would have enjoyed the endocrine heart. Dr. Starling contributed immensely to modern physiology by discovering peptide hormones (Bayliss and Starling 1902) as well as the relationship between venous return and cardiac output (Patterson and Starling 1914). Most likely, he would have been intrigued by the existence of cardiac natriuretic peptides that combine the essence of his own discoveries.

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The heart not only biomechanically responds to volume loading but also regulates volume homeostasis by peptide hormones released from the cardiac myocytes. Thus, the heart muscle promptly adapts to changing hemodynamic demands and, in turn, regulates the workload as a consequence of this demand.

An endocrine phenotype of the heart muscle was suggested by anatomical findings half a century after the principal findings of Dr. Starling. In the 1960s, electron microscopy revealed granules in the cytoplasm of atrial myocytes, which structurally resembled secretory granules in known peptide hormone producing cells (Kisch 1963; Jamieson and Palade 1964). In 1981, the Canadian physiologist Adolfo de Bold and his colleagues reported that infusion of atrial tissue extracts elicits renal excretion of sodium and water (de Bold et al. 1981). Moreover, a rapid decrease in blood pressure and increase in blood hematocrit was observed and the substance was named atrial natriuretic factor. This factor was soon after identified as a peptide comprising 28 amino acid residues (Flynn et al. 1983; de Bold and Flynn 1983) and renamed atrial natriuretic peptide (ANP). This discovery paved the way for identification of two structurally related peptides in the porcine brain, i.e. brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP; Sudoh et al. 1988, 1990). However, BNP is mainly expressed in the heart (Minamino et al. 1988b; Saito et al. 1989; Kambayashi et al. 1990; Hino et al. 1990), and the name BNP is now often replaced by B-type natriuretic peptide (BNP; Fig. 1). CNP is expressed in the invertebrate heart and can be considered the ancestor gene for the natriuretic peptide family (Inoue et al. 2003). Nevertheless, the CNP gene is not expressed to the same extent in mammalian hearts

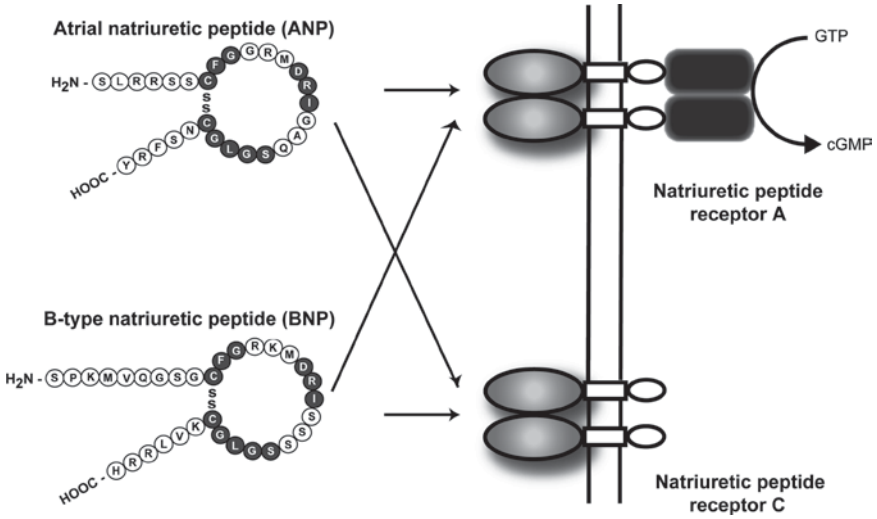

Fig. 1 Schematic presentation of human atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) with their principal receptors. Homologue amino acid residues between the natriuretic peptides are marked in bold circles. The NPR-A receptor mediates ANP and BNP signal transduction through induction of cGMP, while the NPR-C receptor lacks the intra-cellular domain and has been classed primarily as a clearance receptor

and should not be considered as a cardiac-derived peptide in humans, where the gene dominantly is expressed in other tissues including the vasculature and the male reproductive glands (Schulz 2005; Nielsen et al. 2008).

The endocrine heart gained clinical interest when it was reported that patients with cardiac disease display increased concentrations of ANP in plasma (Burnett et al. 1986). In parallel, BNP circulates in highly increased concentrations in patients suffering from congestive heart failure (Mukoyama et al. 1990a, b). The concept of a quantitative plasma marker in the heart failure syndrome was thereby introduced and has been intensely pursued with a strong focus on the clinical applications. In addition to the bioactive end-products, N-terminal fragments from the precursor peptides (proANP and proBNP) were also shown to circulate in heart failure plasma and provided new molecular targets for biochemical detection (Buckley et al. 1990; Hunt et al. 1995). As of today, proBNP-derived peptides have become the preferred routine markers in heart failure diagnostics and prognosis, and the clinical relevance of peptide measurement is frequently and extensively being reviewed (Cowie et al. 2003; de Lemos et al. 2003; Ruskoaho 2003; Baxter 2004; Clerico and Emdin 2004; Costello-Boerrigter and Burnett 2005; Daniels and Maisel 2007; Rubattu et al. 2008; Januzzi et al. 2008).

In contrast to the clinical focus on the diagnostic possibilities, much less is known concerning the biosynthesis of proBNP-derived peptides (Goetze 2004). The post-translational phase of gene expression and the cellular secretion still remain incompletely characterized. The first data on the molecular composition in tissue and plasma suggested an overall simple cellular maturation. As cardiac myocytes possess a biosynthetic apparatus including enzymes for propeptide processing, cardiac prohormone maturation has proven to be much more complex than initially assumed. Clinical studies have also revealed that plasma concentrations of the different proBNP-derived peptides vary greatly, which suggest that cardiac myocytes do not always release the different biosynthetic products on a simple equimolar basis (Hunt et al. 1997a, b); Goetze et al. 2004). A more comprehensive understanding of the biochemical structure may, accordingly, be of both analytical and biological relevance.

This review aims at recapitulating the present understanding of the post-translational phase of cardiac BNP gene expression where new data has changed the perception on the cardiac synthesis. The clinical potential of selective measurement of specific prohormone products will also be summarized.

2 Peptide Nomenclature and Biosynthesis

Some confusion has arisen due to an incoherent nomenclature on cardiac natriuretic peptides. In some ways, this confusion reflects the underlying lack of knowledge of the biosynthetic products, which has led researchers to apply unspecific terms for the measured substances. A stringent nomenclature, however, is essential for a uniform understanding of peptide structure and function (Rehfeld et al. 2004).

If the measured peptide is not readily distinguishable by its name, simple comparisons of reported concentrations may confuse and at worst lead to incorrect decisions. For instance, some of the abbreviations used do not identify the measured peptide(s), which should be the primary goal with the name. A common abbreviation "NT-proBNP" is used for one particular analytical method, which refers to the measurement of non-glycosylated proBNP 1–76 against which the immunoanalysis is calibrated. But the abbreviation does not provide specific information on the primary structure that is actually measured, which includes the intact precursor (proBNP 1–108) and with some cross-reactivity to the glycosylated forms (see later). Other investigators have used broader terms for plasma measurement as, for instance, "N-BNP," which refers to measurement of intact proBNP 1-108 as well as its N-terminal fragment(s). However, this abbreviation leaves the less astute user with the impression that it is the N-terminus of BNP that is measured. The use of abbreviations like "BNP 77–108" is simply incorrect. A rational nomenclature needs to be structurally informative and should see the names in relation to their origin, i.e. with insight into and reference to biosynthesis of the precursor. If this information is not available, then that should be stated. In the following, a nomenclature based on these premises will be attempted.

3 The Pre-Translational Phase of B-Type Natriuretic Peptide Gene Expression

Human ANP and BNP are encoded by small genes located on chromosome 1 (Yang-Feng et al. 1985; Arden et al. 1995). In rodents, the genes are located on chromosome 4 (mouse) or chromosome 5 (rat). The overall gene structure is simple and resembles other peptide hormone genes in size and composition with three exons separated by two introns (Fig. 2). For both ANP and BNP, the major part of

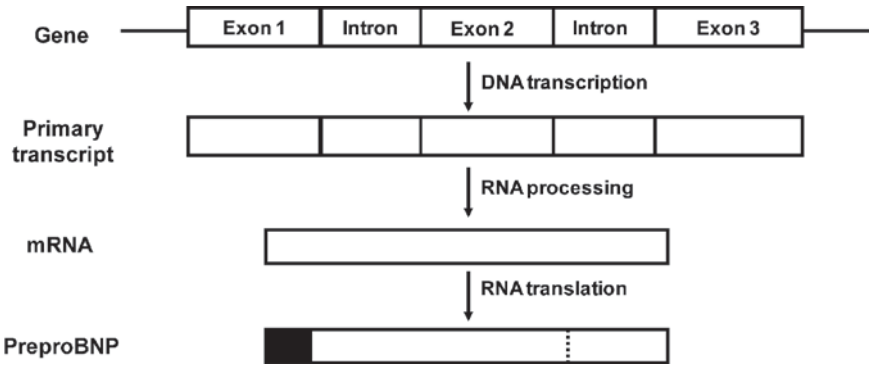

Fig. 2 The gene, RNA, and proprotein in BNP expression. The gene consists of three exons separated by two small introns. The coding sequence mostly resides in exon 2

the coding sequence resides in exon 2. Genetic polymorphisms and mutations have been reported in both genes as well as in the natriuretic peptide receptor genes (Lanfear 2008). Although the impact of genetic variation in the ANP and BNP systems remains to be fully established, it seems reasonable to suspect that it may affect the plasma concentrations in a heritable manner, which has in fact been reported in the general population (Wang et al. 2003). Associations between mutations and risk of disease, however, are more interesting. On that note, a common polymorphism in the BNP promoter region has been associated with the prevalence of type 2 diabetes (Meirhaeghe et al. 2007). Genetic variance in cardiac natriuretic peptides may thus be involved in the pathophysiology of a common metabolic disorder. To complicate matters further, diabetes mellitus induces increased risk of cardiovascular disease with concomitant changes in cardiac natriuretic peptide expression (Christoffersen et al. 2007). In addition to the promoter polymorphism, a frameshift mutation in the ANP gene has been reported in heritable atrial fibrillation, where the frameshift introduces a C-terminally extended ANP peptide (Hodgson-Zingman et al. 2008). Whether the "mutant" ANP exerts stimulatory or inhibitory effects (or no changes) on the natriuretic peptide receptors is still a fascinating question for future experimental studies.

The mature ANP and BNP transcripts consist of approximately 500–700 base pairs. Although a review of intra-cellular regulation of natriuretic gene transcription is beyond the scope of this review (see LaPointe 2005; McGrath and de Bold 2005; McGrath et al. 2005; Gardner et al. 2007 for expert reviews), both genes seem regulated by the same transcriptional factors, including p38 mitogen-activated protein kinase (MAPK). p38 MAPK activates the transcription factor NF-κB and subsequently ANP and BNP gene transcription (Thuerauf and Glembotski 1997; Liang and Gardner 1999). Vasoactive substances like catecholamines and angiotensin-II increase natriuretic gene transcription in a p38 MAPK dependent manner. Myocyte stretching increases the intra-cellular calcium concentration and modulates calcium-binding proteins that regulate downstream modulators including calcineurin, which stimulates myocyte natriuretic gene expression (Kudoh et al. 2003). Thus, cardiac natriuretic gene expression can be modulated by blocking the p38 MAPK and the calcineurin pathways. Finally, the hypoxia inducible transcription factor (HIF) 1-alpha activates both ANP and BNP transcription, which is of importance in ischemic heart disease (Chun et al. 2003; Weidemann et al. 2008). One particular scenario where the ANP and BNP genes seem not to be co-regulated is in inflammation driven by specific cytokines where BNP gene expression increases but ANP gene expression is unaffected (Ma et al. 2004). This differential gene expression has led to the suggestion that ANP and BNP gene products might be clinically useful when measured in concert during conditions with both hemodynamic changes and a pro-inflammatory drive on the cardiac myocytes – as seen after cardiac transplantation (de Bold 2007).

One feature of ANP and BNP mRNA regulation should be recapitulated. Although gene expression is regulated at the transcriptional level, another relevant mechanism is changes in the mRNA stability and the half-life of the transcripts. In fact, this regulatory mechanism has been demonstrated for BNP mRNA through stimulation of alpha-adrenergic receptors (Hanford et al. 1994; Hanford and

Glembotski 1996). The BNP mRNA stabilization is thought to be mediated through the AU-rich elements in the 3´ untranslated region that is not present in the ANP gene. Consequently, mRNA stabilization seems only to involve BNP and not ANP mRNA, which differentiates the BNP gene as a "primary response gene" and the ANP gene as a "secondary response gene."

4 The Primary Structure of ProBNP

Human proBNP comprises 108 amino acid residues (Fig. 3). The primary structure is slightly shorter in mouse but with a similar C-terminal region including the bioactive, receptor binding motif. Mammalian precursor sequences have been deduced from cDNA sequences that encode the entire preprostructure (Sudoh et al. 1989; Steinhelper 1993; Asano et al. 1999; Liu et al. 2002). Amino acid homology between species is largely confined to the amino- and carboxy-terminal regions, whereas the remaining prostructure varies considerably between animals. Moreover, the principal motifs for amino acid modifications and enzymatic prohormone processing are not well conserved between species.

In addition to proBNP, human preproBNP contains an N-terminal hydrophobic signal peptide of 26 amino acid residues (Fig. 2). As with most regulatory peptides, this sequence is removed during translation before synthesis of the C-terminal part of the precursor is completed. PreproBNP does, therefore, not exist as a separate entity but is only a theoretical structure. On the other hand, proBNP is likely to be an existing polypeptide, which has been indicated by chromatographic profiling and sequence-specific immunoassays (Hunt et al. 1995; Schulz et al. 2001; Goetze et al. 2002; Giuliani et al. 2006; Seferian et al. 2007; Liang et al. 2007). The precursor molecule still remains to be purified together with the processing-intermediates thereof, apart from the C-terminal cleavage product, i.e. BNP-32, and the N-terminal region of the precursor (Hino et al. 1990; Minamino et al. 1988a, b; Flynn et al. 1989; Aburaya

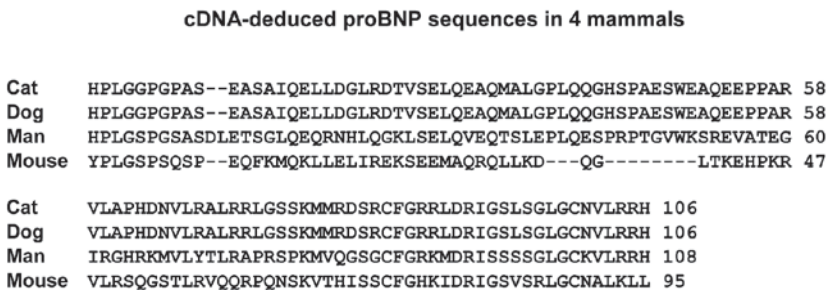

Fig. 3 The primary structure of proBNP in 4 mammals. The human proBNP sequence comprises 108 amino acid residues. The precursor sequence is evolutionarily conserved in the C-terminal region that comprises the bioactive natriuretic peptide. In contrast, the cleavage site corresponding to position 73–76 in the human sequence is not well conserved

et al. 1989; Kambayashi et al. 1990). Thus, whenever the primary proBNP structure is mentioned, it still refers to the cDNA-deduced sequence combined with antibody-based data from chromatographic elution, western blotting, or by immunoassays.

5 The Post-Translational Phase of B-Type Natriuretic Peptide Gene Expression

The post-translational phase of BNP expression has only recently become a subject of interest. One contributing factor may relate to the troublesome lack of useful in vitro cellular models. Although neonatal atrial myocytes can be cultured for short periods of time, they do not anatomically or functionally resemble differentiated atrial, or for that matter ventricular, myocytes. Moreover, only a few immunoassays have been available for characterizing the molecular heterogeneity of the processing-intermediates. Recent advances through mass spectrometry combined with the development of sequence-specific antibodies have, nevertheless, revealed a complex cardiac synthesis of natriuretic peptides.

5.1 Disulphide Bond Formation

The proBNP structure appears simple (Figs. 2 and 3). In humans, it is divided into two principal regions by a cleavage site in position 73–76 (Arg-Ala-Pro-Arg). The first region is the N-terminal fragment proBNP 1–76, and the second region is the C-terminal BNP-32 (proBNP 77–108). In contrast to other prohormones, proBNP does not contain a C-terminal flanking region. The C-terminal region contains a ring structure formed by a disulphide bond between the cystyl residues in positions 86 and 102, respectively (Fig. 1). The ring formation is essential for receptor binding and biological activity (Misono et al. 1984). This crucial modification in ANP and BNP synthesis takes place in the endoplasmic reticulum and may be considered the first step in post-translational processing. The protein disulphide isomerase family and thiol-disulphide oxidoreductases are likely candidate enzymes involved in cardiac myocyte disulphide bond formation. Interestingly, cardiac expression of the protein disulphide isomerase transcript was recently reported to be up-regulated in cardiac disease (Severino et al. 2007). Cellular experiments further suggest a direct cardioprotective effect of this regulation. It may even be that not all cardiac natriuretic peptides are activated through this enzymatic process, which introduces the earliest possible regulatory step in natriuretic peptide biosynthesis and hormone activation. Regulation of protein disulphide isomerase has been classified as "endoplasmic reticulum stress," which is a hallmark of several pathological disorders including diabetes mellitus, neurodegenerative disorders, and ischemic heart disease (Azfer et al. 2006). Other regions of the precursors may also be involved in the disulphide bond formation. For instance, it has been shown for insulin biosynthesis

that alterations in the proinsulin sequence can result in incorrect disulphide bonding and synthesis of insulin with altered chemical and biological properties (Liu et al. 2003; Steiner 2004). For the cardiac natriuretic peptides, the recently reported frame shift mutation in the human ANP gene that generates an elongated ANP peptide (C-terminally extended with the amino acid sequence RITAREDKQWA-COOH) could be a relevant peptide candidate to look for alterations in disulphide bond formation (Hodgson-Zingman et al. 2008).

5.2 Glycosylation

Larger forms of BNP than the purified BNP-32 were first suspected from gel filtration studies of cardiac tissue extracts and plasma from patients with severe cardiac disease (Hunt et al. 1995; Schulz et al. 2001; Goetze et al. 2002; Shimizu et al. 2002, 2003). Some data even suggested molecular forms larger than the predicted precursor. Independently, several groups observed immunoreactive forms with molecular masses of 25–45 kDa in cardiac tissue and plasma (Fig. 4). Intact proBNP, however, has an expected mass of ~11 kDa based on the primary structure. One report suggested that proANP and proBNP oligomerize through a leucine-zipper like motif in the mid-region (Seidler et al. 1999). Whether the peculiar elution patterns were in vitro artefacts or represented peptide binding to other molecules were nevertheless put aside when it was shown that human proBNP exists as an O-linked glycoprotein (Schellenberger et al. 2006). In the precursor structure, the mid-region (proBNP 36-71) contains seven seryl and threonyl residues, where O-linked glycosylation occurs either fully or partially (Fig. 4). This major modification of a polypeptide does not apparently affect the overall structure of the precursor (Crimmins and Kao 2008). On the other hand, the presence of carbohydrate groups clearly affects immunodetection if the epitope recognition resides within this region (Seferian et al. 2008). No specific immunoassay has yet been developed against the glycosylated forms, and the ratio between glycosylated versus non-glycosylated proBNP products can only be deduced from assays that specifically measure the non-glycosylated forms or cross-react with both forms. Whether O-linked glycosylation is an "unlimited" post-translational modification or is affected by increased BNP gene expression, as in heart disease, will be an important question for future studies. It should also be noted that the ANP precursor may be subject for glycosylation. In addition, the proBNP sequence varies considerably between species in the mid-region (Fig. 3), which probably renders glycosylation a species-specific modification. Finally, it is not known whether atrial and ventricular myocytes possess the same biosynthetic capacity to glycosylate natriuretic precursor peptides.

Glycosylation could perhaps be a biochemical target for diagnostic applications if the modification is affected by cardiac disease and/or reflects changes in BNP gene expression. Most captivating, however, is the potential impact of early biosynthetic glycosylation on the cellular sorting and the subsequent precursor processing. As O-linked glycosylation can occur close to the principal maturation site in position 74–76 (on the threonyl residue in position 71), it should be suspected that the presence

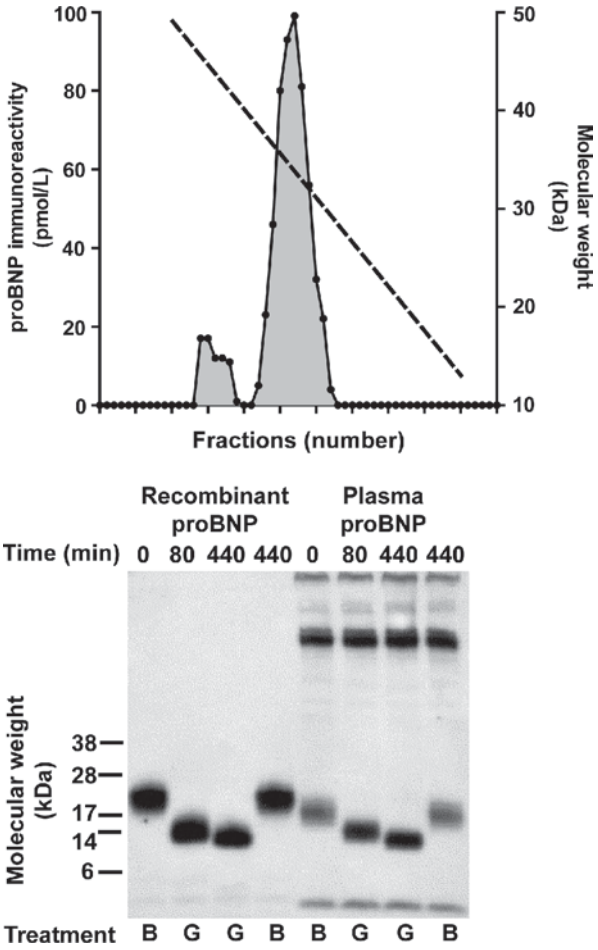

Fig. 4 The upper panel shows a chromatographic profile of proBNP immunoreactivity in human atrial tissue. Cardiac tissue extract was subjected to size-exclusion HPLC. Molecular size calibrators were eluted in a separate run. The proBNP immunoreactivity eluted in positions approximately three times higher than the theoretical molecular weight of intact proBNP. The *lower panel* displays western blotting of recombinant (*left*) and patient (*right* (proBNP)) in buffer (B) or after deglycosylation (G). The incubation time is also listed (Modified from Schellenberger et al. (2006) with permission from the publisher)

of carbohydrate groups affects processing and hormonal maturation. In turn, this modification could regulate prohormone cleavage by either blocking or guiding endoproteolytical enzymes, which may leave the propeptide with reduced or no biological activity. Such post-translational regulation has been shown for other regulatory peptides, as for instance IGF-II (Daughaday et al. 1993). Conceptually, immunoreactive BNP with little or no biological activity has been nicknamed "junk-BNP." This "junk" may, nevertheless, prove to be the most useful peptide forms for diagnostic measurement.

5.3 Endoproteolysis

Human proBNP was first suggested to be cleaved by the ubiquitous endoprotease furin as the furin and the BNP gene are co-expressed in cardiac myocytes (Sawada et al. 1997a, b). The Arg-Ala-Pro-Arg motif in position 73–76 in human proBNP has been shown to be a target for furin-mediated cleavage. In fact, endoproteolytical processing can be blocked in vitro by inhibition of furin, and furin has been shown to be essential for maturation of the structurally related CNP (Wu et al. 2003). A novel protease named corin has been identified in human heart cDNA (Yan et al. 1999; Hooper et al. 2000). Corin is a serine protease that can cleave both proANP and proBNP in vitro, presumably at a similar cleavage site (Yan et al. 2000; Wu et al. 2002). Corin contains a transmembrane domain anchored in the cell membrane and is thought to cleave the precursors upon secretion. The enzymatic activity, however, does not require the transmembrane domain, as a mutant soluble form is also capable of processing proANP (Knappe et al. 2003). The role of corin in the biosynthesis of cardiac natriuretic peptides in vivo has been substantiated by genetic coupling of corin mutations to clinical phenotypes that can be explained by reduced ANP and BNP bioactivity in circulation, i.e. hypertension (Dries et al. 2005; Wang et al. 2008). Corin thus seems to be a relevant candidate for cardiac biosynthesis of natriuretic peptides generating N-terminal processing fragments and C-terminal bioactive peptides (Wu 2007). Of note, no study has yet demonstrated exactly where corin cleaves the proBNP structure. Moreover, atrial post-translational processing of proANP and proBNP is likely to differ from ventricular processing as isolated atrial granules have been reported to contain both unprocessed proANP and mature BNP-32 (Yokota et al. 1995). Corin activity alone can therefore not fully explain the endoproteolytical maturation of cardiac natriuretic propeptides. The putative corin site in the BNP precursor is not conserved between mammals (Fig. 3)and it would be interesting to examine whether human corin can cleave precursor peptides from other mammalian species.

A well-established family of intra-cellular processing enzymes involved in prohormone maturation is the proprotein/prohormone convertases – or the PCs. In addition to the already mentioned furin, the subtilisin-like endoproteases PC1/3 and PC2 are also expressed in the mammalian heart (Bloomquist et al. 1991; Beaubien et al. 1995), and PC1/3 expression has been demonstrated both in normal and pathological human cardiac tissue (Dschietzig et al. 2001). Atrial myocytes transfected with an adenoviral vector expressing PC1/3 processes proANP to both mature ANP and to a truncated form (Marx and Mains 1997). Although the precise cleavage site was not established and the processing capacity was somewhat inefficient, this singular report does underscore the possibility that other proteases than furin and corin may be involved in the post-translational endoproteolysis of proANP and proBNP. PC1/3 is active in secretory granules and could be an important regulator of atrial proBNP processing. Cardiac PC1/3 expression has been reported to be up-regulated at the transcriptional level in heart disease (Jin et al. 2004). Unfortunately, there is no data on other proBNP-derived fragments stemming from endoproteolytical processing. This may reflect the lack of specific tools for identifying

such peptide fragments, which require antibodies directed at other epitopes than the ones used so far for biochemical identification. Sandwich-based immunoassays are usually not ideal for this type of experiments. The precursor sequence contains several basic amino acid residues that potentially could represent cleavage sites for the PCs, and the molecular characterization may not be complete when it comes to identifying processing-intermediates from the natriuretic peptide precursors.

5.4 Exoproteolysis

N-terminal trimming of proBNP-derived peptides seems to be a biological feature, as both the N-terminus of the biosynthetic precursor and the C-terminal bioactive BNP product contain an amino acid motif for aminopeptidase recognition and cleavage. Both the N-terminus of proBNP and BNP-32 (proBNP 77–108) contain a prolyl residue in position 2 (His-Pro and Ser-Pro, respectively). While prolyl residues are important for peptide structure and folding, they can also be involved in exoproteolytic trimming if located near the N-terminus (Vanhoof et al. 1995). N-terminal trimming has in fact recently been demonstrated for BNP in vitro (Brandt et al. 2006). Synthetic BNP-32 (proBNP 77–108) incubated in the presence of the dipeptidyl peptidase (DPP) IV removes the N-terminal Ser-Pro residues. DPP-IV is an enzyme located mainly on endothelial cells and in the circulation with a preference for cleaving N-termini with either prolyl or alanyl residues in the second position (Ahrén 2007). Thus, this DPP-IV cleavage in BNP-32 cannot per se be considered as part of the biosynthetic maturation but is rather related to the elimination phase. An N-terminally trimmed form of proBNP lacking the His-Pro residues in positions 1–2 has also been reported in heart failure patients (Lam et al. 2007). This report disclosed that a truncated proBNP 3–108 form circulates in increased concentrations in heart failure patients. Experiments in our laboratory show that the human proBNP N-terminus can be trimmed by DPP-IV in vitro and blocked by inhibition of DPP-IV. In this context, it is noteworthy that the initial report on glycosylated proBNP in a recombinant expression system (CHO cells) also identified a truncated proBNP 3–108 form in cell extracts (Schellenberger et al. 2006). While this finding may be explained by experimental handling of extracts and medium, it could also imply that N-terminal exoproteolysis is a biosynthetic event. N-terminal trimming as part of peptide biosynthesis has, for instance, been demonstrated for melittin, which is a secretory peptide produced in honeybee venom glands (Kreil et al. 1980; Kreil 1990). In mammalian cells, intracellular aminopeptidase has been reported in compartments different from the lysosomes suggesting N-terminal trimming as a possible part of the biosynthetic peptide maturation (Underwood et al. 1999; Chiravuri et al. 2000). Several regulatory peptides in human physiology are deactivated by amino-terminal cleavage in the circulation, where two clinically relevant peptides are gastrin-inhibitory polypeptide (GIP) and glucagon-like-peptide (GLP)-1 (Holst 2004). Whether the trimming of BNP and its molecular precursor serves an actual regulatory function in cardiac natriuretic peptide physiology remains an open question for future experimental research.

One could speculate that amino-terminal trimming affects the metabolic fate of the peptides and thus their turnover in circulation. There is, however, no data available on actual biological relevance of these trimmings. Comparison between mammalian species reveals homology at the N-terminus of proBNP – but not at the N-terminus of BNP (Fig. 3), which indicates that the N-terminus of the precursor has been subjected to phylogenetic conservation through some sort of selection process, perhaps related to the correct removal of the signal peptide.

Carboxy-terminal exoproteolysis of BNP-32 has not been demonstrated. Formation of C-terminal amides by oxidative scission of the N-C bond of a glycyl residue is catalyzed by peptidyl-amino-monooxygenase – or PAM. Interestingly, PAM is present in the secretory granules of cardiac mycoytes (Eipper et al. 1988; Stoffers et al. 1989; Maltese and Eipper 1992). No peptide target though has been identified for cardiac PAM. In the primary BNP-32 (proBNP 77–108) structure, the amino acid sequence NH2-SPKMVQGSGCFGRK constitutes the N-terminal region. If proBNP folding through disulphide bonding in the endoplasmic reticulum is not achieved, such a peptide could be generated in the secretory granules through endoprotelysis at the C-terminal dibasic cleavage site and harbor a potential target for PAM and C-terminal amidation. PAM may also be involved in the amidation of yet unidentified peptide products that are co-expressed in the cardiac myocytes. It has been estimated that almost half of the mammalian neuropeptides become biologically active through conversion of their COOH-terminus into an essential α-amide moiety (Prigge et al. 2000).

6 Cellular Storage and Secretion

BNP gene expression is a feature of both atrial and ventricular myocytes. In the normal heart, the main site of BNP expression is in the atrial regions (Luchner et al. 1998; Christoffersen et al. 2002). Ventricular BNP gene expression increases drastically in cardiac disease that affects the ventricles, i.e. congestive heart failure (Mukoyama et al. 1991). The observation of ventricular BNP gene expression in ventricular disease may have given rise to the common statement that BNP is dominantly a ventricular hormone. Atrial and ventricular myocytes, however, differ considerably with respect to their endocrine phenotypes, and it is reasonable to expect major differences in peptide storage and secretion patterns (Doyama et al. 1998; Goetze et al. 2006). For instance, it is well-established that atrial myocytes contain intra-cellular granules for peptide storage and maturation, which even contributed to the primary hypothesis of the endocrine heart (Kisch 1963; Jamieson and Palade 1964). Atrial granules contain both intact precursors and biosynthetic end-products, i.e. bioactive ANP-28 and BNP-32. In contrast, normal ventricular myocytes do not seem to express such granules, and neither do normal ventricular myocytes contain proBNP-derived peptides (Christoffersen et al. 2002). A few reports have observed granules and proBNP-derived peptides in ventricular myocytes sampled from pathological hearts (Hasegawa et al. 1993; Nicolau et al. 1997; Takemura et al. 1998).

Thus, ventricular myocytes not only regulate the BNP gene at the transcriptional and post-translational level but also seem to be able to differentiate with respect to the biosynthetic apparatus per se. One report suggests the presence of different classes of granules, where one class contains only ANP-related products and the other contains both ANP and BNP peptides (Hasegawa et al. 1991). In this context, the amidation enzyme PAM (see Section 5.4) has been suggested to be involved in granule formation in concert with the N-terminal proANP sequence (Labrador et al. 2004). The proANP structure has also been implicated in granule formation through Calcium-mediated aggregation in the trans-Golgi network, where substitution of the acidic residues in the N-terminal region changes the size and shape of intra-cellular vesicles and their ability to dock with the plasma membrane (Canaff et al. 1996; Baertschi et al. 2001). In extension to these findings, it should be recapitulated that atrial myocytes from ANP gene deficient mice do not contain secretory granules on electron microscopy (John et al. 1995). Cardiac BNP expression in ANP deficient mice is also affected with decreased BNP mRNA contents in the atria and increased expression in the ventricles (Tse et al. 2001). BNP peptide contents in these tissues paralleled the mRNA findings with no peptide in atrial regions and borderline detectable contents in ventricular samples. The formation of granules in atrial and ventricular myocytes consequently differs and may be dependent on the two cardiac natriuretic peptide systems. To fully understand these mechanisms, further experiments addressing the role of the proBNP-derived peptides in granule formation and docking should be pursued. Characterization of ANP expression in BNP deficient animals could also prove informative, as atrial granules have been observed in these animals (Tamura et al. 2000). At present, the general perception of cardiac secretion nevertheless refers to atrial release as a regulated process, while ventricular release resembles constitutive or constitutive-like secretion.

An acidic protein class involved in granule formation is the chromogranins (Taupenot et al. 2003). Chromogranins, or just granins, comprise at least three proteins (A, B and C) that posses aggregation characteristics suggesting a function in the formation of secretory granules. Cardiac expression of chromogranin A and B has been established (Steiner et al. 1990; Tota et al. 2008; Heidrich et al. 2008). The focus on cardiac chromogranins, however, has mainly been on the potential biological activity of chromogranin-derived fragments (the vasostatins) or on plasma measurement for diagnostic purposes. Whether cardiac chromogranins are involved in the biosynthesis of ANP and BNP through formation of granules remains an obvious area for future studies. Notably, chromogranin A deficient mice do not reveal obvious changes in granule formation in, for instance, adrenal chromaffin cells (Hendy et al. 2006). It would nevertheless be interesting to characterize the biosynthesis of cardiac natriuretic peptides in these mice. Cardiac chromogranin B has also been suggested to be directly involved in BNP gene expression through a Ca^{2+}-dependent induction of the BNP promoter (Heidrich et al. 2008). Further in vitro experiments targeted at proANP and proBNP maturation in cardiac cell systems devoid of chromogranin A and B may consequently reveal a specific role for the granins in storage and secretion of natriuretic peptides.

Finally, other cells within the heart also express the BNP gene. For instance, cardiac fibroblasts have been shown to produce and secrete cardiac natriuretic

peptides (Tsuruda et al. 2002), and the coronary vasculature expresses all three natriuretic peptide genes, at least in coronary atherosclerosis (Casco et al. 2002). The biosynthetic capacity in these cells still remains to be explored.

7 ProBNP-Derived Peptides in Plasma

ProBNP-derived peptides are secreted by cardiac myocytes and circulate in plasma. Their molecular heterogeneity has primarily been characterized by chromatography in combination with sequence-specific immunoassays. Much of our present conception of the cellular synthesis is in fact derived from the plasma phase, which represents the sum of secretion and metabolism. The picomolar concentrations in plasma limit the possibilities for full biochemical identification and underscore a careful understanding of epitope recognition by the immunoassays. With this in mind, it is established that bioactive BNP is secreted from the heart and circulates without binding to plasma proteins (Hawkridge et al. 2008). Synthetic BNP-32 (proBNP 77–108) is trimmed when incubated in whole blood generating a BNP form lacking the two N-terminal amino acid residues (Shimizu et al. 2002; Hawkridge et al. 2005). As mentioned earlier, this molecular form can also be generated in vitro by enzymatic trimming by DPP-IV (Brandt et al. 2006) and possibly by other aminopeptidases. Further processing of plasma BNP seems to involve degradation with a loss of bioactivity through disruption of the ring structure mediated by neutral endopeptidase (NEP 24.11) – or by receptor mediated cellular uptake. While this has been known for some time, the therapeutic potential of inhibiting neutral endopeptidase with increased plasma concentrations of "beneficial" natriuretic peptides is still an appealing strategy (Corti et al. 2001). The metabolic fate of BNP-32 has been reported to be 13–20 min (Richards et al. 1993; Smith et al. 2000). Immunoreactive BNP is also excreted in urine, but the precise contribution of renal excretion to renal metabolism is not yet clarified. A minor degree of hepatic clearance has also been shown, which is not significantly altered in patients with liver failure (Henriksen et al. 2003).

In addition to bioactive BNP, other proBNP-derived fragments circulate in plasma (Goetze 2004; Mair 2008). These fragments are commonly referred to as "N-terminal proBNP," but the molecular heterogeneity also includes the intact precursor, in particular in heart failure patients (Hunt et al. 1995, 1997b; Goetze et al. 2005). Cardiac secretion of proBNP and its N-terminal fragments has been demonstrated by blood sampling from the coronary sinus. The molar ratio of secreted proBNP 1–76 to intact proBNP is not yet fully clarified but is likely to depend on cardiac status, i.e. more unprocessed precursor compared to biosynthetic cleavage products in severe heart failure (Fig. 5). On the metabolic phase, there are still major discrepancies in the suggested half-life of N-terminal precursor fragments, which at least partially reflect the epitope recognition in the assays. Theoretically, the half-life of proBNP 1–76 in circulation should be around 25 min (Kroll et al. 2007) and thus not differ greatly from the established metabolism of BNP-32 (proBNP 77–108).

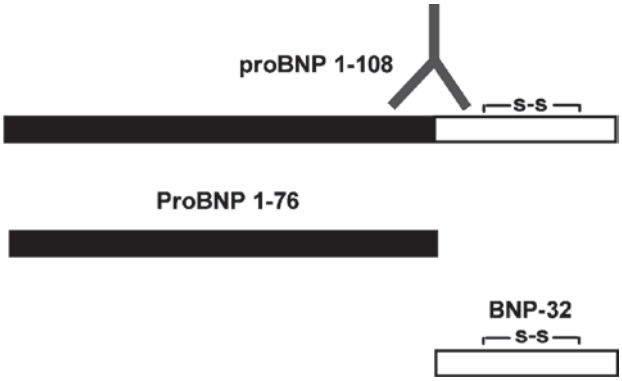

Fig. 5 Immunoassay for detection of unprocessed human proBNP. The assay utilizes antibody recognition of an epitope spanning the Arg-Ala-Pro-Arg site (proBNP 74–76) thought to be cleaved by Corin (Giuliani et al. 2006)

One report, however, suggested a considerably longer half-life (~90 min after cardiac pacing), which would fit well with the higher plasma concentrations of N-terminal proBNP fragments compared to bioactive BNP in healthy individuals and in cardiac patients (Pemberton et al. 2000). As our perception of the molecular heterogeneity in plasma has changed radically over the last years, there is an urgent need for new pharmacokinetic experiments to separate the biosynthetic phase from the peripheral elimination. Ideally, such experiments should be performed by classical peptide infusion strategies with measurement across organ beds.

8 Biosynthesis and Assay Calibration

Elucidation of the cardiac natriuretic peptide biosynthesis has disclosed a complex post-translational maturation that produces a variety of peptides targeted for cellular secretion (Fig. 6). The different phases of gene expression are not only region-specific but also depend on changes within the secretory apparatus in cardiac myocytes. The main clinical applications of the peptides today strongly relates to plasma measurement in cardiovascular diagnostics and prognosis. The immunoassays thus need to be designed with insight into the biosynthesis of the peptides. Another defining aspect of immunoassay measurement is the choice of calibrator. This aspect has so far not been scrutinized by researchers, apart from the observation of disturbingly large molar discrepancies between the different assays (Rehfeld and Goetze 2003). On the other hand, it has not been possible to raise meaningful assay calibration issues before now when the existence of a complex molecular heterogeneity has been established. One way of by-passing this lack of information has been introduced as a “processing-independent assay,” which in principle quantifies one in vitro cleavage product that represents all the secreted precursor molecules.

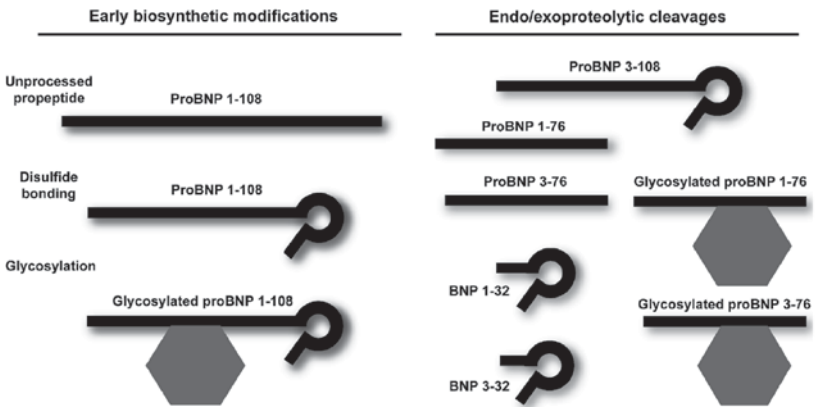

Fig. 6 Schematic presentation of the possible proBNP-derived peptide products. Note that most peptides are not chemically identified but rather suggested from biochemical methods that rely on antibody recognition

This assay can then be calibrated with the specific cleavage product and assay measurement performed on a stoichiometric correct basis. If one is to choose a proBNP-derived calibrator peptide for plasma measurement, it becomes somewhat more blurred. As the ratio of bioactive BNP to intact precursor shifts toward less processed biosynthetic products, one would perhaps choose the dominant "disease" form over the more prevalent forms in healthy individuals. However, large comparative studies have still not revealed major differences between BNP or proBNP measurements in terms of overall clinical performance. One report on assay calibration has shown that assays directed at the C-terminal BNP region do not really cross-react with the larger biosynthetic products (Luckenbill et al. 2008). Plasma measurement based on assays directed against the N-terminal proBNP fragment is, however, greatly influenced by the degree of O-linked glycosylation. Clearly, this issue is far from settled, and our present perception of "normal" concentrations of the different biosynthetic products may still have to be redefined.

9 Inefficient Prohormone Maturation in Heart Failure

Heart failure patients display highly increased plasma concentrations of bioactive ANP and BNP. With a dramatic upregulation of the gene expression and concomitant high concentrations of immunoreactive ANP and BNP in plasma, it seems reasonable to expect increased natriuresis. The common presentation of heart failure, however, is congestion, sodium retention, and edema. Although heart failure is a complex syndrome with both activation and inhibition of multiple neurohumoral systems, the paradoxical lack of ANP and BNP bioactivity is still compelling. Heart failure patients respond to intravenous administration of chemically synthesized

ANP and BNP, which has led to the introduction of a new and potent drug: Nesiritide (Mohammed et al. 2008). This BNP analogue is a potent drug in heart failure, which has raised serious concerns regarding patient safety through induction of unwanted hypotension (Topol 2005). Experts have further explored the possibilities of natriuretic peptide drugs by constructing structurally related peptides that possess natriuretic effects but without the undesirable hypotension (Lisy et al. 2008; Dickey et al. 2008). Obviously, this research area could prove of major relevance to medical therapy, as all the different physiological effects of natriuretic peptides could have specific roles in modern treatment of heart failure and other cardiovascular pathologies.

The endocrine paradox of sodium and water retention in heart failure, where the gold standard biomarkers are the cardiac natriuretic peptides, relates to insufficient post-translational maturation of the biosynthetic precursors (Goetze et al. 2003). A well-established analogy to this phenomenon is enhanced secretion of proinsulin over mature insulin in patients with type 2 diabetes. In the early stages of the disease, selective proinsulin measurement is a valuable tool in evaluating pancreatic β-cell dysfunction. A shift towards secretion of unprocessed precursors in cardiac disease may also represent early involvement of ventricular expression and secretion, as efficient precursor maturation seems to be an endocrine feature of atrial biosynthesis. In support of this explanation, the intra-cellular processing enzymes involved in ANP and BNP maturation are dominantly expressed in atrial myocytes. Moreover, the ventricular myocytes do not, at least in the early stages of disease, contain secretory granules for peptide storage and maturation. The post-translational processing of ventricular precursors may not be efficient in the production of needed natriuretic potency, while immunoassays cross-react to various degrees with the unprocessed biosynthetic products. Although speculative, there may even be large individual differences in the cardiac ability to mature the precursor peptides, which could help explain the highly variable heart failure phenotypes. The ratio of mature BNP to unprocessed proBNP might also be of diagnostic relevance in parallel with the present application of proinsulin to insulin measurement. If specific assays for the various forms are applied in concert, it may be possible to define an early endocrine hallmark of the heart failure syndrome that could aid clinicians in tailoring diuretic therapy according to the patient-specific ability to ameliorate congestion through secretion of bioactive natriuretic hormones.

10 Concluding Remarks

Since the discovery of the cardiac natriuretic peptides, a tremendous amount of research has identified proANP and proBNP-derived peptides as useful plasma markers in heart failure. In contrast, our present understanding of cardiac synthesis, secretion, and elimination is still incomplete. Cellular expression including post-translational maturation has revealed a complex biosynthetic phase with both regional as well as major cellular differences in storage and secretion between health

and disease. Focusing on the molecular heterogeneity will provide biological insight into the endocrine heart and could introduce new diagnostic possibilities. The different biosynthetic products will not all be equal markers of the same pathophysiological processes. With an urgent need for sensitive and specific biomarkers in modern medicine, the biosynthetic phase of cardiac natriuretic peptide expression is likely to introduce new molecular targets for clinical evaluation of cardiovascular disease.

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Glucagon and Glucagon-Like Peptides 1 and 2

Jens Juul Holst

Abstract The glucagon gene is expressed not only in the alpha cells of the pancreatic islets but also in the endocrine cells of the intestinal epithelium (so-called L-cells), and in certain neurons of the brain stem. Whereas in the pancreas, glucagon, the hyperglycaemic hormone, is cleaved out of the 160 amino acid precursor, proglucagon, leaving behind proglucagon fragments (PG 1-30 and PG 72-158, the so-called major proglucagon fragment (MPGF)) that are probably inactive, the intestinal processing leads to the formation of glicentin (PG 1-69; action uncertain) and glucagon-like peptides 1 (PG 78-107amide, a potent incretin homone, regulating insulin secretion, glucagon secretion, gastrointestinal motility and appetite) and 2 (PG 126-158, a regulator of gut mucosal growth and integrity). The two prohormone convertases PC2 and PC1/3, respectively, are responsible for the differential processing. After their release, the hormones are eliminated mainly in the kidneys, but both GLP-2 and in particular GLP-1, but not glucagon, are metabolized both locally and in the circulation and liver by dipeptidyl peptidase 4 (DPP-4) which inactivates the peptides, suggesting that GLP-1 acts locally rather than in an endocrine manner. A number of transcription factors have been identified that can at least partly explain the differential cellular expression of the glucagon gene as well as the differential tissue-specific processing of the precursor.

1 Introduction

The glucoregulatory hormone, glucagon, was discovered in 1923 as a hyperglycemic substance present in pancreatic extracts (Murlin et al. 1923). Subsequent research indicated that hyperglycaemic substances were also present in the extracts of the gastrointestinal mucosa, and Sutherland and DeDuve suggested in 1948, based on

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bioassays, that (Sutherland and De Duve 1948) the gastric extracts might contain glucagon, a finding that was confirmed in several subsequent studies. Furthermore, endocrine cells that resemble the pancreatic A-cells were reported to be present in the gastrointestinal mucosa (Orci et al. 1968). Upon the advent of the radioimmunoassay for glucagon – one of the first radioimmunoassays to be developed (Unger et al. 1961) – it was confirmed that the gastrointestinal tract contains substances with glucagon immunoreactivity, i.e., reacting with the antibodies employed in the radioimmunoassay (Unger et al. 1966). In addition, in immunohistochemical studies, some intestinal endocrine cells could be stained using glucagon antibodies (Grimelius et al. 1976), but it was also shown that these cells differed from the pancreatic A-cells with respect to granule morphology (Grimelius et al. 1976) and they were hence designated as "L-cells" (Buffa et al. 1978). Furthermore, in 1968 it was established (Unger et al. 1968) that the glucagon-like immunoreactive material that was secreted in response to an oral glucose load differed from true glucagon both physicochemically and biologically. Furthermore, it was demonstrated that this "gut glucagon-like immunoreactivity" was heterogenous, consisting of at least two distinct moieties differing in molecular size (Valverde et al. 1968). Through contributions from independent groups, it was eventually demonstrated that both the two predominant molecular forms of gut glucagon-like immunoreactivity contain the entire 29-amino acid glucagon sequence (Bataille et al. 1982; Holst 1980, 1982; Thim and Moody 1981) (Fig. 1). One has a C-terminal octapeptide extension, and the other has the same C-terminal extension plus an N-terminal extension of 30 amino acids. The former molecule was named oxyntomodulin because of its effects on the oxyntic mucosa in some species (Bataille et al. 1982), and the latter was designated glicentin, partly because of its Glucagon-Like Immunoreactivity, and partly because it was first thought to consist of 100 amino acids (Sundby et al. 1976). Full sequence analysis, however, revealed it to consist of 69 amino acids (Thim and Moody 1981). The N-terminal extension of glucagon in glicentin was identified also in extracts from the pancreas, from which it was shown to be secreted in parallel with glucagon (Moody et al. 1981; Thim and Moody 1982). Conceivably, therefore, glicentin was a proglucagon – a biosynthetic precursor for glucagon – which in the pancreas was cleaved to glucagon and equimolar amounts of the N-terminal extension peptide, which was named glicentin-related pancreatic polypeptide (GRPP) (Thim and Moody 1982).

From studies of the biosynthesis of glucagon in the pancreatic islets, however, it was evident that a large molecule (MW around 10,000), subsequently designated "the major proglucagon fragment" (MPGF) that did not contain the glucagon sequence, was also formed in parallel with glucagon (Patzelt and Schug 1981). As the structures of glucagon-encoding mRNA and the glucagon gene were determined (Bell et al. 1983a, b), it became evident that in mammals, this major fragment of proglucagon contains two glucagon-like sequences, now designated glucagon-like peptides 1 and 2, GLP-1 and GLP-2. For a while it was thought that the GLP-2 moiety was an evolutionary late addition to the gene, since fish and bird proglucagon appeared to contain only a single glucagon-like peptide corresponding to GLP-1 (Lund et al. 1982). Subsequent research, however, established that the

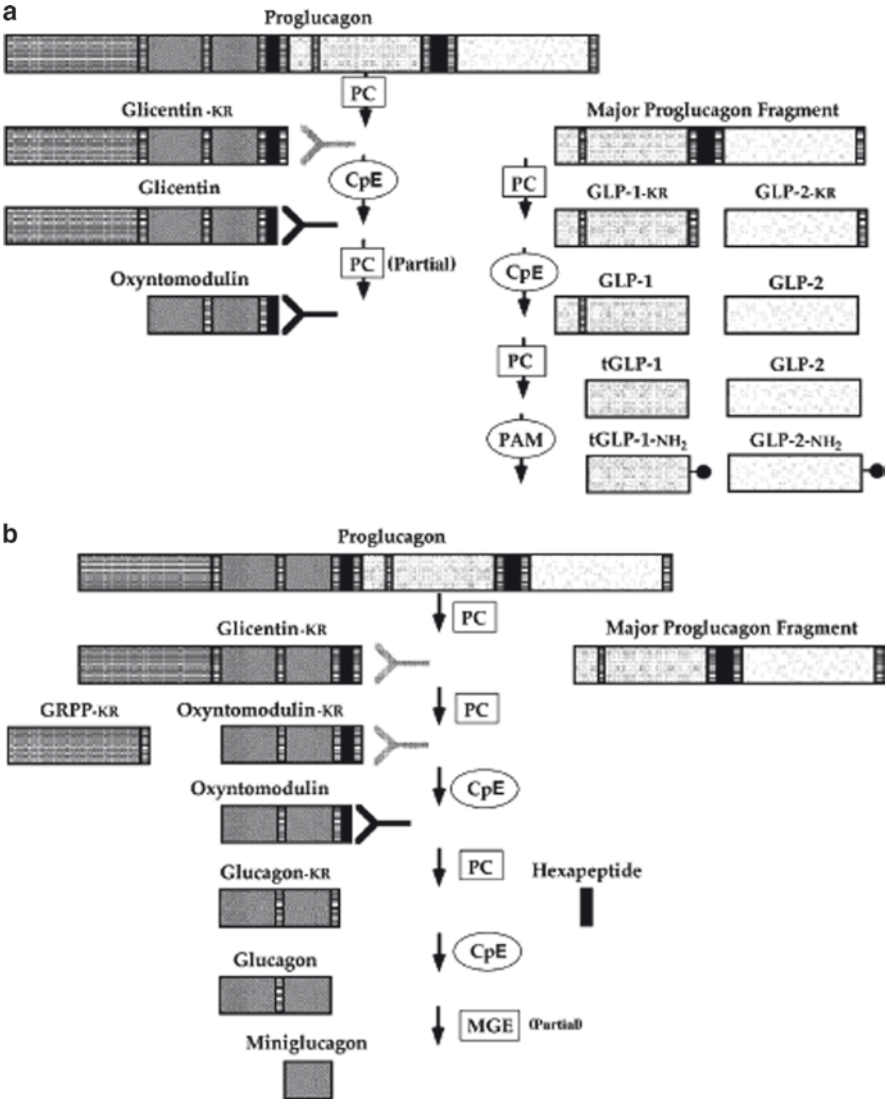

Fig. 1 Processing of proglucagon in intestinal L cells (and presumably also neurons of the brain stem) (**a**) and in the alpha cells of the islets of Langerhans (**b**). *PC* prohormone convertases; *CpE* carboxypeptidase-E; *PAM* peptidylglycine alpha-amidating monooxygenase; *MGE* miniglucagon generating endopeptidase; *GRPPm* glicentin-related pancreatric polypeptidew; *GLP-1* glucagon-like peptide. From Bataille (2007)

proglucagon genes in these species (there are two glucagon encoding genes in fish) also contain the GLP-2 encoding sequence, which, however, is sequestered by differential splicing upon pancreatic expression of the gene (Irwin and Wong 1995) but remains in sequence upon intestinal expression.

In further studies of the processing and secretion of proglucagon products in humans and other mammals, it was confirmed that, in the pancreas, the two glucagon-like peptides are contained in a single large molecule, the MPGF, secreted in parallel with glucagon (Holst et al. 1994; Mojsov et al. 1986; Orskov et al. 1986, 1987). In the intestinal mucosa, however, the two glucagon-like peptides are formed and secreted separately, whereas the N-terminal part of the precursor, i.e., the part that corresponds to glicentin, remains uncleaved (Mojsov et al. 1986; Orskov et al. 1986) or partly cleaved into GRPP and oxyntomodulin (Fig. 1).

2 Proglucagon Gene Expression

The structure of proglucagon was deduced from the sequence of the cDNA encoding hamster proglucagon (Bell et al. 1983b) and the human proglucagon gene (Bell et al. 1983a). Also the murine and bovine genes were cloned (Heinrich et al. 1984; Lopez et al. 1983). Apparently, only a single gene encodes proglucagon in mammalian species, and identical mRNAs are produced in the pancreas and the intestines (Mojsov et al. 1986; Novak et al. 1987). The differences in the proglucagon products in these tissues are, therefore, due to tissue-specific, differential, posttranslational processing of proglucagon (Mojsov et al. 1986; Orskov et al. 1986). The proglucagon gene is also expressed in certain neurons in the nucleus of the solitary tract in the brain stem (Larsen et al. 1997) and a recent report found expression of both the GLP-1 receptor and proglucagon in mouse skin, suggesting a role for skin GLP-1 in skin development and folliculogenesis (List et al. 2006). An impressive number of in vitro studies have demonstrated that at least 6 homeodomain proteins are able to interact with the glucagon gene promoter and activate its expression (Jin 2008). For some of them (Cdx-2, Brn-4 and Nkx6.2) gene deletion studies carried out in vivo had no apparent effect on pancreatic alpha cells. This may indicate that several proteins normally cooperate in regulation glucagon gene expression so that loss of one of them can be compensated for by the action of the others. The role of Pax-6 and Pbx-1, however has been established in knock-out studies (Jin 2008). In addition, glucagon gene expression appears to be regulated protein kinase A and Epac signaling pathways in response to cAMP elevation both in intestinal L-cells (Lotfi et al. 2006) and in pancreatic alpha cells (Ma et al. 2005).

Recent studies have shed some light on the mechanisms that result in tissue specificity and the mechanisms that regulate the expression of the proglucagon gene in the gut in vivo. Thus, it has been established that the transcription factor, pax6, is expressed in intestinal endocrine cells and activates proglucagon transcription (Trinh et al. 2003). Mice with dominant negative pax6 mutation exhibit total absence of gut endocrine cells expressing GLP-1 or GLP-2. Disruption of the pax6 gene disrupts both islet development and selectively eliminates the endocrine cell population of the small and large bowel, including the proglucagon-producing cells (Hill et al. 1999; Trinh et al. 2003). Yi and

colleagues (Yi et al. 2005) recently demonstrated that beta-catenin, the major effector of the Wnt signaling pathway, activates proglucagon expression in intestinal, but not in islet cells. Furthermore, this effect was mediated by the transcription factor TCF-4, which is highly expressed in intestinal endocrine cells, but not in islets. Both TCF-4 and beta-catenin bind to the proglucagon gene promoter, and a dominant negative TCF-4 repressed proglucagon expression in an intestinal cell line that expresses proglucagon and produces GLP-1 (Yi et al. 2005). It is of interest that approximately 1,250 nucleotides of the rat proglucagon gene promoter direct the expression to pancreatic islets and neurons of the brain, whereas additional upstream sequences extending to −2,250 are required for expression of the gene in intestinal endocrine cells (Lee et al. 1992). However, the mechanisms regulating tissue specific proglucagon gene expression in humans appear to differ from those described for the rat gene (Nian et al. 1999, 2002), and unfortunately little is known about the regulation of the human gene. A recent study identified conserved sequences within intron 1 of the human proglucagon gene as being important for the pancreatic expression (Zhou et al. 2006).

The TCF-4 mediated regulation of proglucagon expression in the gut is of considerable interest in view of the recent demonstration in several unrelated populations of a genetic association of a specific single nucleotide polymorphism (SNP) – the microsattelite DG10S478 – in the intron 3 of the TCF4 gene (now designated TCF7L2), and the development of type 2 diabetes. These findings raise the possibility that this SNP influences disease susceptibility through modulation of intestinal progucagon gene expression and hence possibly plasma levels of GLP-1 (Grant et al. 2006). However, subsequent studies revealed (Pilgaard et al. 2009; Schafer et al. 2007) that GLP-1 levels were unaffected in the high risk group, whereas the insulinotropic actions of the incretin hormones were impaired, supporting the importance of TCF7L2 for beta cell function. Indeed, decreased incretin receptor levels have been found to correlate with beta cell TCF7L2 expression in diabetic models (Shu et al. 2009).

Yi et al. recently reported that insulin in pathological dosages stimulated glucagon gene mRNA expression in intestinal L-cells and provided evidence for a crosstalk between insulin and Wnt singaling pathways. There are no reports supporting an effect of insulin on GLP-1 secretion in vivo and there was no effect of insulin on GLP-1 secretion from isolated perfused pig intestine (Hansen et al. 2004). In the pancreatic alpha cells, insulin has repeatedly been demonstrated to repress glucagon gene expression (Philippe 1991), and recent studies have pointed to PKB (Schinner et al. 2005) and FOXO1 (McKinnon et al. 2006) to possibly transmit the actions of insulin. This is important because it is generally believed that insulin exerts a paracrine intra-islet inhibitory control of glucagon secretion. On the other hand, glucagon secretion may also be regulated in the absence of insulin, e.g., by GLP-1 in patients with type 1 diabetes and no residual beta cell function (Creutzfeldt et al. 1996), suggesting that other mechanisms, including a pracrine control exerted by neighboring somatostatin producing cells (de Heer et al. 2008) may be of importance.

3 Posttranslational Processing and Chemical Structures

The *differential posttranslational processing* of proglucagon in the pancreas and gut results *in the pancreas* in the formation of glucagon, the GRPP, a small fragment corresponding to the proglucagon sequence 64-69 and the MPGF corresponding to PG 72-160 (Holst et al. 1994) (Fig. 1), and all of these peptide products are secreted in parallel upon stimulation (Holst et al. 1994). The processing in the alpha cells is due to the coexpression of the prohormone convertase PC2, which has been demonstrated to be both necessary and sufficient for cleaving proglucagon as outlined (Rouille et al. 1994). In agreement with this, animals in which the PC2 gene is disrupted cannot cleave proglucagon in the alpha cells (Furuta et al. 1997) and, as a result, have lower glucose levels than wild type animals, and improved tolerance to glucose, and they develop alpha cell hyperplasia, features that also characterize mice with a deletion of the glucagon receptor gene (Gelling et al. 2003).

The *intestinal processing* results in the formation of glicentin, corresponding to proglucagon residues no 1-69, part of which may be cleaved further to oxyntomodulin, corresponding to proglucagon 33-69 (Baldissera and Holst 1984; Orskov et al. 1986). The proglucagon sequence that corresponds to the MPGF contains pairs of basic amino residues, canonical cleavage sites for the prohormone convertases, flanking both the glucagon-like peptide sequences in the human cDNA, and it was therefore predicted that the prohormone might be cleaved at these sites (Orskov et al. 1986) and that the sequences of GLP-1 and GLP-2 would therefore correspond to PG 72-108 and 126-158, 126-159, or 126-160 (the nucleotide codon encoding residue no 160 is found in a separate exon and was therefore overlooked in the first cloning experiments). However, sequencing of the naturally occurring peptides extracted from human gut, revealed that the structure of native GLP-1 corresponds to PG 78-107 (Holst et al. 1987). This turned out to be of utmost importance, since the truncated peptide was found to be a potent stimulator of glucose induced insulin secretion, whereas full length GLP-1 was inactive (Holst et al. 1987; Mojsov et al. 1987). The truncation also had consequences for the nomenclature, since the naturally occurring peptide was henceforth designated either truncated GLP-1 or GLP-1 7-36amide (or GLP-1 7-37). In current literature, the unqualified designation GLP-1 covers only the truncated peptide. It was also found that the Gly corresponding to PG 108 serves as substrate for amidation of the carboxyterminal Arg (Orskov et al. 1989), but the biological consequences of the amidation are unclear. The amidated and the Gly-extended forms have similar bioactivities and overall metabolism (Orskov et al. 1993) although the amidated form may exhibit slightly improved stability towards plasma enzymes (Wettergren et al. 1998). In humans, almost all of the GLP-1 secreted from the gut is amidated (Orskov et al. 1994), whereas in many animals (rodents, pigs) part of the secreted peptide is GLP-1 (7-37) (Hansen et al. 2000; Mojsov et al. 1990). This poses special problems with respect to the measurement of GLP-1 secretion in these species (see below). The sequence of naturally occurring GLP-2, was found to correspond to PG 126-158 (Buhl et al. 1988; Hartmann et al. 2000b) in pigs (Buhl et al. 1988),

but larger GLP-2 containing fragments were also identified. The sequence of human GLP-2 was found to exclusively correspond to PG 126-158 with no signs of further posttranslational modifications (Hartmann et al. 2000b). The so-called intervening peptide 2 (Fig. 1) was found to correspond to PG 111-123 and also occurs in an amidated form (Buhl et al. 1988). This peptide is also secreted in parallel with GLP-1 and GLP-2, but nothing is currently known about its possible actions. A comparison of GLP sequences among species shows that the GLP-1 sequence is preserved 100% in all mammals, where this has been studied, and the sequence homology is pronounced across other classes of animals (Kieffer and Habener 1999). GLP-2 shows more variation with, for example, 4 substitutions between human and porcine GLP-2. The Ala in position 7 of human intervening peptide 2 is also variable with Thr or Asn in, for example, the porcine and bovine peptides. A recent database search suggested that porcine proglucagon does not contain basic amino acid residues at position 159 and 160, and isolation and sequencing of the natural peptide revealed a peptide of 35 amino acids with Ser and Leu at the C-terminus (Pedersen et al. 2008). Porcine GLP-2 had a shorter plasma half-life in pigs, but had otherwise similar effects and receptor interactions to human GLP-2, comfirming that the C-terminus is less important for receptor activation.

The processing of proglucagon in the intestinal L-cells results from the actions of co-expressed prohormone convertase 1/3, which is both necessary and sufficient for the complete processing (Ugleholdt et al. 2004; Zhu et al. 2002). Interestingly, the N-terminal cleavage site of GLP-1 is not a classical pair of basic amino acids, but represents a single Arg residue; however, in vitro co-expression of PC1/3 and proglucagon has demonstrated efficient cleavage at this site (Rouille et al. 1997). In agreement with this, mutations in PC-1/3 lead to abnormalities in GLP-1 processing and secretion, associated with multiple endocrinopathies (Jackson et al. 2003) (undoubtedly because PC 1/3 is important for processing of many regulatory peptides/hormones), and mice with a targeted deletion of the PC-1/3 gene are unable to process proglucagon to GLP-2 and GLP-1 (Ugleholdt et al. 2004; Zhu et al. 2002). Interestingly, it was recently demonstrated that adenovirus-mediated expression of PC 1/3 in the pancreatic alpha cells increases islet GLP-1 secretion, resulting in improved glucose-stimulated insulin secretion and enhanced survival in response to cytokine treatment as well as enhanced performance after transplantation to mouse models of type 1 diabetes – a finding of considerable clinical interest (Wideman et al. 2006).

A fragment of glucagon corresponding to its C-terminal part (residues no 19-29), also designated "miniglucagon," is reported to be present in the pancreas in amounts corresponding to a few % of the total glucagon content (Bataille 2007). The processing is thought to be due to a "miniglucagon-generating endopeptidase" made of a metalloendoprotease called N-arginine dibasic convertase or nardilysin that cleaves on the N-terminus of basic doublets or singlets and of a specialized aminopeptidase of the B-type (aminopeptidase-B or ApB) that trims the excess basic amino acid (s) (Bataille 2007). Miniglucagon has been speculated to play a role in the intraislet intereactions between the alpha and the beta cells, where miniglucagon may block secretagogue effects (including that of glucagon) on the beta

cells by stabilizing their membrane potential (Bataille 2007). The physiological importance of this is unclear.

In both the pancreas and the gut small amounts of a peptide corresponding to proglucagon 1-61 may be found (Baldissera and Holst 1984; Holst et al. 1994). With conventional C-terminally directed assays for glucagon, this component will be co-determined. Normally it represents a neglible fraction of immunoreactive glucagon, but in pancreatectomized patients and in renal failure it may reach higher concentrations (Baldissera and Holst 1986; Holst et al. 1983b).

N-terminally extended GLP-1, corresponding to proglucagon 72-107amide or 108 amide, may also be formed particularly in the pancreas (Holst et al. 1994), and may in conditions with hyperglucagonaemia make significant contribution to the circulating GLP-1 concentrations (Gelling et al. 2003). Although small amounts of fully processed GLP-2 may be formed in the pancreas (Holst et al. 1994), very little GLP-1 7-37/7-36amide is normally found in the adult pancreas. However, in the rat pancreas and in the fetal and/or neonatal pancreas, some alpha cells appear to produce PC1/3, and GLP-1 7-36/37 may be formed (Heller and Aponte 1995; Masur et al. 2005; Wilson et al. 2002). In addition, increased expression of PC1/3 associated with increased pancreatic levels of GLP-1 have been reported in diabetic rats (Nie et al. 2000). Given that GLP-1 has potent trophic effects on the beta cells (Drucker 2006), this might suggest that a local GLP-1 production plays a role in the development, maintenance, and function of beta cells. However, it should be noted that beta cells form and function normally in mice with deletion of the GLP-1 receptor gene, even in diabetic animals with beta cell hyperplasia (Flamez et al. 1998; Scrocchi et al. 2000).

4 Localization of Proglucagon Expression

It has been known since 1968 that endocrine cells resembling pancreatic A-cells occur in the intestinal mucosa (Orci et al. 1968). Later research demonstrated cells that are indistinguishable at the electron microscopic level from the pancreatic A-cells in the oxyntic mucosa of some species (Sundler et al. 1976). Such cells are abundant particularly in dogs and appear to secrete apparently true glucagon in appreciable amounts (Lefebvre and Luyckx 1980). Similar cells have not been found in humans, however (Holst et al. 1983a). The majority of the intestinal proglucagon-derived peptides are secreted from the L-cells, which differ clearly from the A-cells by their granule morphology (Orci et al. 1983). A-cell granules show a distinct halo of less electron dense material surrounding a core of dense material, whereas the granules of the L-cells are homogeneous without halo formation. The L-cell is an open-type endocrine cell with a slender triangular form with the base resting on the basal lamina and a long cytoplasmic process reaching the gut lumen. This process is equipped with microvilli that protrude into the lumen. It may be via these microvilli that the L-cell can sense the presence of nutrients in the lumen and transform this information into a stimulation of secretion. The peptide products of

the A- and L-cells have been identified at the cellular level by immunocytochemistry. As expected, antisera directed against the mid region of glucagon ("sideviewing") stain the pancreatic A-cells as well as the intestinal L-cells, whereas antibodies against the free C-terminus of glucagon (which is not exposed in the L-cells) only stain the A-cells. Antisera against the N-terminal, non-glucagon part of glicentin stain both the A-cells and the L-cells because they react with GRPP in the pancreas and with glicentin in the gut (Orci et al. 1983). As expected from their common origin, both GLP-1 and GLP-2 show complete coexistence with glucagon (sideviewing antisera) upon immunohistochemical examination (Mojsov et al. 1986; Orskov et al. 1986, 1987). GLP-1 and GLP-2 immunoreactivity have been co-localized with glucagon in the electron dense core of the A-cells, presumably due to their presence in the MPGF (Varndell et al. 1985).

The density of L-cells shows maximum in the ileum in most species (Bryant et al. 1983; Eissele et al. 1992); no or very few cells are present proximal to the ligament of Treitz in humans and other primates. A considerable number is present in the colon (Knudsen et al. 1975), particularly the distal part. Surprisingly, the entirely unrelated peptide, PYY has also been localized to the L-cells in all mammal studies so far (Bottcher et al. 1986) and was even found to be localized to the same granules as glicentin by immuncytochemistry at the electron microscopic level (Bottcher et al. 1986). The distribution was reexamined in a recent study involving combined in situ hybridization, peptide chemistry, and immunohistochemistry. In this study of endocrine cells of the porcine rat, and human small intestines (Mortensen et al. 2003), GLP-1 nearly always (92%) co-localized with either the incretin hormone GIP (glucose-dependent insulinotropic polypeptide, formerly known as gastric inhibitory polypeptide) or the enterogastrone hormone, PYY (peptide YY). GIP and PYY were rarely co-localized. In the mid small intestine, 55–75% of the cells staining for either GLP-1 or GIP also expressed the other incretin hormone. As mentioned, the proglucagon gene is also expressed in the central nervous system. Thus cells immunoreactive for GLP-1, glucagon, and glicentin have been demonstrated in the nucleus of the solitary tract of the brain stem of rats, monkeys, and man (Drucker and Asa 1988; Jin et al. 1988; Kauth and Metz 1987). These neurons project to many regions in the brain, in particular the nuclei of the hypothalamus, including the arcuate and the paraventricular nuclei (Larsen et al. 1997). The processing of proglucagon has been examined both at the level of the cell bodies and at the levels of the fibers projecting to the hypothalamus (Larsen et al. 1997). The pattern was intestinal with a pronounced contribution of processed oxyntomodulin.

5 Postsecretory Fate of Proglucagon-Derived Peptides

GLP-1 is eliminated extremely rapidly from plasma in both humans and experimental animals. GLP-1 is extremely susceptible to the catalytic activity of the enzyme dipeptidyl peptidase IV, which cleaves off the two N-terminal amino acids

(Deacon et al. 1995). The metabolite thus generated, GLP-1 9-36amide or GLP-1 (9-37), is inactive with respect to insulin secretion (but may have other actions, see below) and may even act as a competitive antagonist at the GLP-1 receptor (Deacon et al. 1995; Knudsen and Pridal 1996) although its formation does not seem to result in antagonism in vivo (Zander et al. 2006). In studies of GLP-1 secretion from isolated perfused porcine ileum, it has been shown that a very large part of the GLP-1 that leaves the gut is already degraded to the inactive metabolite (Hansen et al. 1999, 2000). About 25% of newly secreted GLP-1 leaves the gut in an intact, active form. A similar degradation amounting to about 40–50% takes place in the liver (Deacon et al. 1996), and it can therefore be calculated that only about 10–15% of newly secreted GLP-1 reaches the systemic circulation in the intact form. This is in contrast to the fact that virtually all of GLP-1 stored in the granules of the L-cells is intact (Hansen et al. 1999). It has been shown that the degradation is due to the actions of the enzyme dipeptidyl peptidase IV, which is expressed not only in the enterocyte brush border but also in the endothelial cells lining the capillaries of the lamina propria (Hansen et al. 1999). In agreement with this, inhibitors of DPP-IV can completely prevent this degradation (Hansen et al. 1999). DPP-IV activity is also responsible for the extremely rapid initial whole-body metabolism of GLP-1, which results in an apparent half-life for intact GLP-1 in plasma of 1–2 min and a metabolic clearance rate exceeding cardiac output by a factor of 2–3 (Deacon et al., 1996; Vilsboll et al. 2003). The metabolite is also cleared rapidly, mainly in the kidneys, with a half-life of 4–5 min (Meier et al. 2004; Vilsboll et al., 2003). It has been established that GLP-1 is also a substrate for the enzyme, neutral endopeptidase 24.11 (Hupe-Sodmann et al. 1997), and recent studies have shown that inhibition of this enzyme will enhance survival of both endogenous and exogenous GLP-1 in vivo (Plamboeck et al. 2005). However, the enhanced survival will only be apparent if the N-terminal degradation by dipeptidyl peptidase IV has been prevented. Because of the extensive degradation, the plasma concentrations of the intact hormone are very low, and may not even rise significantly in response to small meals (Vilsboll et al. 2001). However, determination of the concentrations of the metabolite, which will be much higher, may reveal that a stimulation of the L-cells has nevertheless taken place. It follows that for the estimation of *L-cell secretion* it is best to measure the sum of the intact hormone and the primary metabolite. In humans, this can be accomplished with assays for the amidated C-terminus of the molecule, which is common to the intact hormone and the metabolite, because in humans, all of the GLP-1 released from the gut is amidated (Orskov et al. 1994). Such assays are frequently designated "total"GLP-1 assays. Clearly, for estimation of the impact of circulating intact GLP-1 for insulin secretion via the endocrine route, it is necessary to measure the concentration of the intact hormone, which may be accomplished with sandwich assays (often designated "active GLP-1 assays"). The degradation rate of GLP-2 is slower than that of GLP-1. Thus, the half-life of GLP-2 is 7 min (Hartmann et al. 2000a) and the clearance about 7 ml $kg^{-1} min^{-1}$. The elimination mainly takes place in the kidneys (Pedersen et al. 2008). In agreement with the much weaker susceptibility towards DPP-IV of GLP-2 compared to GLP-1, all of the newly released GLP-2 that leaves

the gut is still in the intact form (unpublished studies). The metabolite of GLP-2 is cleared from the plasma with a half-life of 27 min and its clearance corresponds to what is lost by glomerular filtration in the kidneys (Hartmann et al. 2000a).

Glucagon itself may be degraded by DPP-4 in vitro, but in vivo it is not appreciably metabolized by this enzyme, at least in pigs (Deacon et al. 2003). In contrast, neutral endopeptidase plays a major role and is involved in both peripheral and renal degradation (Trebbien et al. 2004). It is often thought that the liver is responsible for a major part of glucagon clearance, but careful measurements of portal-hepatic venous gradients have documented that there is no significant hepatic uptake or measurable degradation of this hormone (Holst 1991), the elimination of which is mainly renal (Deacon et al. 2003; Holst 1991).

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Somatostatin and Somatostatin Receptors

Ujendra Kumar and Michael Grant

1 Introduction

The biological effects of somatostatin (SST) were first encountered unexpectedly in the late 1960s in two unrelated studies, one by Krulich et al. (1968) who reported on a growth hormone (GH)-releasing inhibitory substance from hypothalamic extracts, and the other, by Hellman and Lernmark (1969), on the presence of a potent insulin inhibitory factor from the extracts of pigeon pancreatic islets. However, the inhibitory substance was not officially identified until 1973 by Guillemin's group (Brazeau et al. 1973). In both synthetic and naturally occurring forms, this tetradecapeptide, originally coined as somatotropin release-inhibitory factor (SRIF, SST-14) was shown by Brazeau et al. to be the substance controlling hypothalamic GH release. This single achievement not only pioneered SST research but was also duly recognized, as Guillemin shared the 1977 Nobel Prize in Medicine. The following years bequeathed an exponential increase in SST-related studies. It soon became clear that SST-synthesis was not restricted to the hypothalamus. Its production is widely distributed throughout the central nervous system (CNS), peripheral neurons, the gastrointestinal tract, and the pancreatic islets of Langerhans (Luft et al. 1974; Arimura et al. 1975; Dubois 1975; Hokfelt et al. 1975; Orci et al. 1975; Pelletier et al. 1975; Polak et al. 1975; Patel and Reichlin 1978). In fact, SST-like immunoreactivity can be found throughout various tissues of vertebrates and invertebrates, including the plant kingdom (Patel 1992; Tostivint et al. 2004). Given its broad anatomical distribution, it is no wonder that SST produces a wide spectrum of biological effects. Generally regarded as an inhibitory factor, SST can function either locally on neighboring cells or distantly through the circulation, to regulate such physiological

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processes as glandular secretion, neurotransmission, smooth muscle contractility, nutrient absorption, and cell division (Reichlin 1983a, b; Patel 1992, 1999; Patel et al. 2001; Barnett 2003).

These extraordinary efforts into the biology of SST could not have been possible if it were not for the availability of stable and potent analogs, given that SST has an extremely short plasma half-life (Weckbecker et al. 2003). In 1978, while working at Sandoz (later Novartis, Basel, Switzerland), Vale et al. reported on the first SST-analog, an octapeptide with full SST-like biological activity, derived from a cyclic cysteine-bridged hexapeptide backbone (Vale et al. 1978). Further modification of the peptide by the introduction of two D-amino acid isomers and L-threoninol at the C-terminal, provided increased metabolic stability even in the midst of aggressive media such as gastric juices at elevated temperatures (Bauer et al. 1982). This improved octapeptide was coded as SMS 201-995 or octreotide, and developed under the name Sandostatin®. In 1988, Sandostatin gained its first FDA approval for the symptomatic treatment of gastroenteropancreatic tumors. Today, the inhibitory actions of SST-analogs are applied in several clinical scenarios, including, the suppression of tumoural hormone hypersecretion (acromegaly, neuroendocrine tumors (NETs), pancreatic tumors, carcinoid tumors), gastrointestinal bleeding, dumping syndrome, and pancreatitis (Lamberts et al. 1996; Weckbecker et al. 2003).

Despite the marked achievement in the development of octreotide, it was not until the early 1990s, that the structure of the first SST receptor (SSTR) emerged by molecular cloning (Yamada et al. 1992). Subsequent cloning revealed five distinct SSTR genes which was a greater number than predicted from pharmacological and biochemical criteria at the time (Patel et al. 1995; Reisine and Bell 1995; Patel 1997). The conceptualization behind the development and successful deployment of octreotide, was only later identified by its preferential-binding to SSTR2 (Reisine and Bell 1995; Patel 1997; Weckbecker et al. 2003), as many tumors express this receptor-subtype (Lamberts et al. 2002; Hofland and Lamberts 2003; Gardette et al. 2004). Rational approaches to developing peptide and nonpeptide analogs that bind more selectively soon followed (Weckbecker et al. 2003). However, it soon became clear that SSTRs would often show overlapping patterns of distribution in a tissue specific manner, which raised questions on the relevance of receptor-coexpression and the importance of target specificity, given the similarities in receptor-signaling. Nevertheless, reports have surfaced describing activation of putative second messengers as well as differential cellular and physiological responses in cells bearing more than one receptor-subtype when treated with SST agonists (Shimon et al. 1997a, b; Cattaneo et al. 2000; Jaquet et al. 2000; Danila et al. 2001; Saveanu et al. 2001; Tulipano et al. 2001; Bruns et al. 2002; Florio et al. 2003b; Ren et al. 2003; Zatelli et al. 2004, 2005b; Ben-Shlomo et al. 2005; Jaquet et al. 2005; Saveanu et al. 2006; Fedele et al. 2007). Recently, the trafficking and desensitization of SSTR2 following its selective activation was shown to be affected when SSTR5 was coexpressed; however the mechanism for this behavior had yet to be described (Sharif et al. 2007).

All five SSTR subtypes are members of the superfamily of G-protein coupled receptors (GPCRs) (Patel 1999; Olias et al. 2004). An abounding amount of reports has challenged the age-old notion that GPCRs exist and function as monomeric entities

at the cell surface. It is now clear that many (Franco et al. 2003; Kroeger et al. 2003; Bai 2004; Breitwieser 2004; Hansen and Sheikh 2004; Terrillon and Bouvier 2004; Prinster et al. 2005; Milligan 2008) but not all GPCRs function exclusively as dimers (Patel et al. 2002; Gripentrog et al. 2003; Grant et al. 2004b; James et al. 2006; Meyer et al. 2006; Bayburt et al. 2007; Rasmussen et al. 2007; Whorton et al. 2007, 2008). SSTRs are no exception, as several laboratories have demonstrated their ability to form both homo- and heterodimers, with members of the same or distantly related receptor-families (Rocheville et al. 2000a, b; Pfeiffer et al. 2001, 2002; Patel et al. 2002; Grant et al. 2004a, b; Baragli et al. 2007; Duran-Prado et al. 2007; Grant et al., 2008a; Watt et al. 2008). This chapter describes the functional and pharmacological properties of SST and SSTR subtypes and their possible clinical implication.

2 Somatostatin Processing

It has been over three decades since the discovery of SSTs - a family of cyclopeptide hormones -which are mainly produced by normal endocrine, gastrointestinal, immune, and neuronal cells (Brazeau et al. 1973; Reichlin, 1983a, b; Patel 1992, 1999; Patel et al. 2001; Barnett 2003). SST is synthesized as two bioactive products, the form originally identified in the hypothalamus consisting of 14 amino acids, SST-14, and its congener, SST-28, subsequently discovered to contain an extension at the N-terminus (Pradayrol et al. 1980) (Fig. 1a and b). Elucidation of the biosynthesis of both forms of SST, like other protein hormones (Hook et al. 1994),

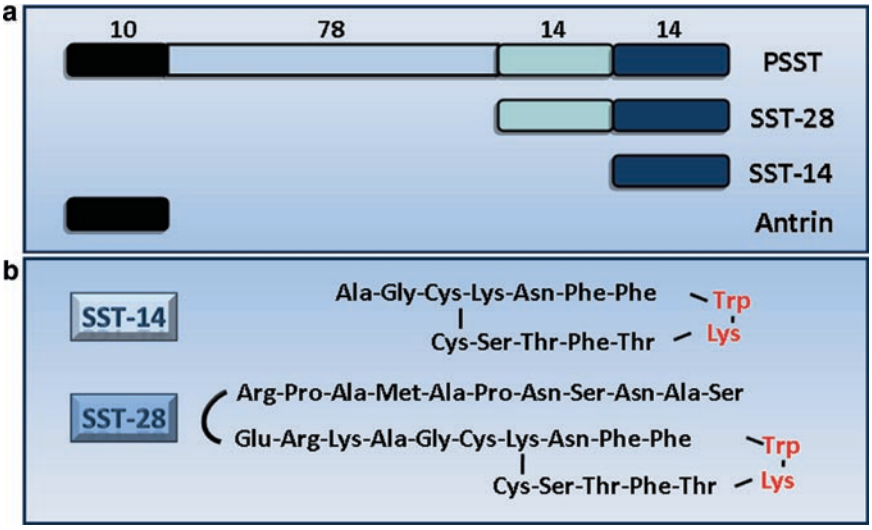

Fig. 1 Somatostatin processing. (**a**) Prosomatostatin is processed into two bioactive forms, SST-14 and SST-28. (**b**) Amino acid sequence of somatostatin isoforms depicting the cysteine bridge maintaining its cyclic structure and the pharmacophore

revealed a larger inactive precursor molecule, preprosomatostatin (PPSST), which is processed by post-translational enzymatic cleavage to yield the active polypeptides (Goodman et al. 1980; Joseph-Bravo et al. 1980; Oyama et al. 1980; Patzelt et al. 1980; Shields 1980; Goodman et al. 1981; Zingg and Patel 1982). In the early 1980s, development of recombinant DNA technology, allowed for the isolation and cloning of human and rat cDNAs encoding PPSST (Goodman et al. 1982; Shen et al. 1982; Funckes et al. 1983). This work revealed the sequence and structure of PPSST, a polypeptide consisting of 116 amino acids. Enzymes implicated in the processing of PSST belong to the subtilisin/kexin-related Ca^{2+}-dependent class of serine proteinases, collectively termed precursor convertases (Seidah and Chretien 1999; Zhou et al. 1999). Although there are seven known mammalian precursor convertases, a select few have been shown important in PSST processing (Mouchantaf et al. 2001, 2004a, b). Processing of PSST primarily occurs at the C-terminal end generating the two bioactive forms. SST-14 is generated by dibasic cleavage at an Arg-Lys residue, whereas endoproteolysis of a monobasic Arg site produces SST-28 (Patel and O'Neil 1988; Bersani et al. 1989). In addition, a secondary monobasic site was determined in PSST, cleavage of which results in the generation of a 10-amino acid peptide termed antrin ($PSST_{1\text{-}10}$), named after its initial discovery in the gastric antrum, for which it showed the highest concentration (Benoit et al. 1987). Although antrin has no known function, it has been isolated in all SST-producing tissues (Ravazzola et al. 1989; Rabbani and Patel 1990)

Due to differential processing of PSST, various mixtures of SST-14 and SST-28 are produced in mammalian tissues (Patel et al. 1981). SST-14 is largely present in pancreatic islets, stomach, and neural tissues; it is the prominent form in the retina, peripheral nerves, and enteric neurons (Patel et al. 1981). In the brain, SST-28 accounts for approximately 20–30% of total SST-like immunoreactivity. In the periphery, SST-28 synthesis predominates in intestinal mucosal cells as the terminal biosynthetic product following PSST processing (Patel et al. 1981; Baskin and Ensinck 1984). Although only SST-14 and SST-28 are the known biologically active forms of PSST, other products have been identified in circulation following processing;however their biological function remains uncertain as they are devoid of any known activity (Patel et al. 1981; Shoelson et al. 1986; Patel and O'Neil 1988; Ensinck et al. 1989; Ravazzola et al. 1989; Rabbani and Patel 1990).

3 Somatostatin Distribution

The production of SST occurs at high densities in cells throughout the CNS, the peripheral nervous system, the endocrine pancreas, and the gut, in addition to small numbers in the thyroid, adrenals, submandibular glands, kidney, prostate, placenta, blood vessel walls, and immune cells (Arimura et al. 1975; Dubois 1975; Hokfelt et al. 1975; Pelletier et al. 1975; Polak et al. 1975; Patel and Reichlin 1978; Finley et al. 1981; Reichlin 1983a, b; Johansson et al. 1984; Fuller and Verity 1989; Aguila et al. 1991; Patel 1992). Within the CNS, neurons and fibers positive for

SST are abundantly dotted, the notable exception being the cerebellum (Finley et al. 1981; Johansson et al. 1984). More specifically, brain regions such as the hypothalamus, the deep layers of the cortex, the limbic system, and all levels of the major sensory pathway are rich in SST-producing neurons (Kumar 2007). In a subpopulation of C cells in the thyroid, SST coexists with calcitonin (Reichlin 1983a, b). At least in rats, total body SST can be divided as follows: gut accounts for the majority of SST, approximately 65%, the brain for approximately 25%, the pancreas for approximately 5%, while the remaining organs account for the residual 5% (Patel and Reichlin 1978).

4 Somatostatin Physiology

The physiological role of hypothalamic SST is well established [reviewed in (Patel 1992, 1999; Barnett 2003)]. As early as week 10 of gestation, SST is detected in the fetal hypothalamus (Bugnon et al. 1978). It is there that its release regulates the secretion of GH from the pituitary, the counterbalance being a growth hormone-releasing hormone (GHRH), which is detected in the hypothalamus at week 18 of gestation (Bresson et al. 1984). In adults, GH secretion occurs at a basal rate throughout the day. The major role of hypothalamic SST is the tonic inhibition of both basal and GHRH-stimulated secretion of GH from anterior pituitary somatotrophs (Barinaga et al. 1985). Somatostatinergic neurons emanate from the anterior hypothalamus and project to the median eminence, where SST is released into hypophyseal portal vessels to interact with pituitary somatotrophs (Patel 1992; Barnett 2003). SST and GHRH pathways interact with each other at both their point of convergence at the level of the pituitary and through direct neural connections within the hypothalamus (Horvath et al. 1989). Thus, SST inhibits the secretion of GH via a direct interaction on the pituitary and indirectly through suppression of GHRH release (Katakami et al. 1988; Tannenbaum et al. 1990). Two secretory feedback loops exist that modulate SST release: the short loop, where SST is negatively regulated by GHRH (Katakami et al. 1988) but subject to positive-feedback by GH (Berelowitz et al. 1981a); the long loop, where insulin growth factor type 1 (IGF-I) produced by GH acting on the liver, provides a positive influence (Berelowitz et al. 1981b). This mechanism in regulating GH release is further supported in SST knockout mice, as nadir GH levels are consistently higher in these animals compared to their wild-type counterparts (Low et al. 2001). In addition, secretion of hypothalamic SST can be further promoted by dopamine, substance P, neurotensin, glucagon, hypoglycemia, various amino acids, acetylcholine, α_2-adrenergic agonists, vasoactive intestinal polypeptide (VIP), and cholecystokinin; it is however inhibited by glucose (Chihara et al. 1979; Berelowitz et al. 1982; Reichlin 1983b). Similar mechanisms also exist in the hypothalamic control of thyroid-stimulating hormone (TSH) secretion (Siler et al. 1974; Vale et al. 1975; Arimura and Schally 1976; Ferland et al. 1976; Tanjasiri et al. 1976; Rodriguez-Arnao et al. 1981; Reichlin 1983a, b; Samuels et al. 1992; James et al. 1997).

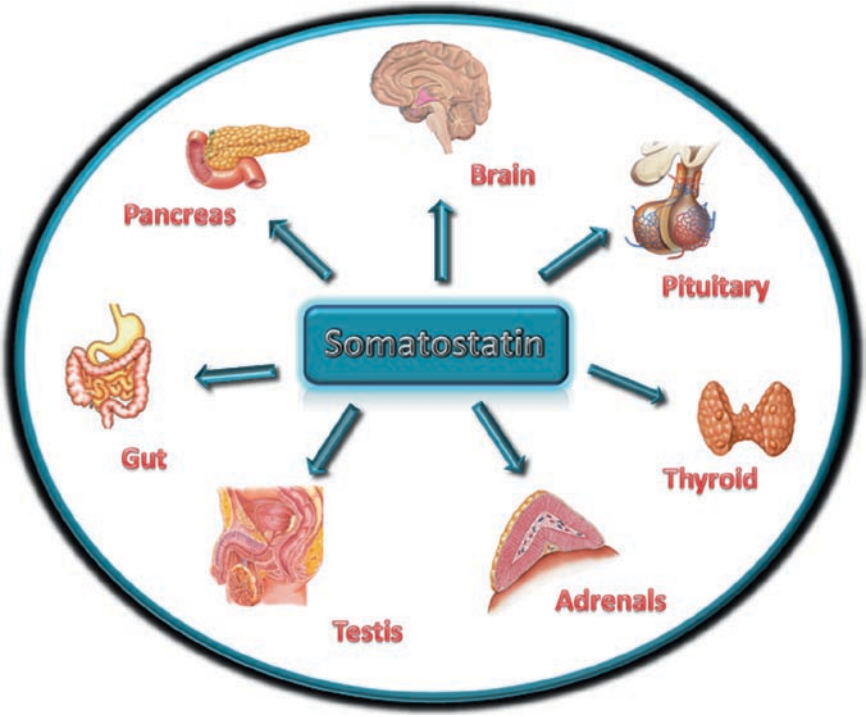

Fig. 2 Schematic depicting the hormonal actions of somatostatin

In addition to its actions on the pituitary, SST functions as a neurotransmitter in the brain with effects on cognition, locomotor, sensory, and autonomic functions (Reichlin, 1983a, b; Patel 1992; Epelbaum et al. 1994; Barnett 2003) (Fig. 2). SST inhibits the release of dopamine from the midbrain, the secretions of norepinephrine, thyroid-releasing hormone,and corticotrophin-releasing hormone including its own secretion from the hypothalamus. As previously indicated, it inhibits both the basal and stimulated secretion of GH and TSH, but has no effects on the release of luteinizing hormone, follicle-stimulating hormone, prolactin, or adrenal corticotrophin hormone under normal physiological conditions. SST has direct effects on the thyroid by inhibiting the release of T4, T3, and calcitonin from thyroid parafollicular cells stimulated by TSH. It acts on the adrenals to inhibit angiotensin II stimulated aldosterone secretion and acetylcholine stimulated medullary catecholamine secretion. SST inhibits the secretion of renin in the kidneys when stimulated by hypovolemia, including the inhibition of antidiuretic hormone-mediated water absorption. Within the gastrointestinal tract, virtually every gut hormone has been shown to be inhibited by SST including gastric acid, pepsin, bile, and colonic fluid. SST also has a generalized suppressive effect on the motor activity within the gastrointestinal tract, such that it inhibits gastric emptying, gallbladder contraction, and small intestinal segmentation. In the pancreas, SST is an endogenous islet hormone. Its actions on the pancreas were first noted within the year of its discovery by two groups,

following infusion in humans and baboons (Alberti et al. 1973; Koerker et al. 1974). SST regulates the secretion of hormones from several tissues, including neurotransmission. When synthesized and released from δ cells of pancreatic islets, SST causes suppression of the synthesis and secretion of both insulin and glucagon, including the inhibition of pancreatic polypeptide (German et al. 1990; Zhang et al. 1991; Philippe 1993; Nelson-Piercy et al. 1994; Redmon et al. 1994; Kendall et al. 1995; Kleinman et al. 1995; Ballian et al. 2006) (Fig. 2). SST is also known to block the release of several growth factors and cytokines (Blum et al. 1992; Hayry et al. 1993; Elliott et al. 1994). More recently, the antisecretory properties of SST were demonstrated to affect ghrelin release (Barkan et al. 2003). Additional effects of SST include vasoconstriction and an antiproliferative effect on immune, intestinal mucosal, cartilage, and bone precursor cells (Weiss et al. 1981; Reichlin, 1983a, b; Patel 1992; Karalis et al. 1994; Aguila et al. 1996; Takeba et al. 1997). Interestingly, a down regulation in SST and SSTRs expression has been associated with Alzheimer's disease (Kumar 2005). The brains of mice which were deficient in SST showed a greater accumulation of $A\beta_{42}$, the main contributor to Alzheimer's disease, due to a decrease in neprilysin activity (Saito et al. 2005). When either SST was administered or neprilysin was directly activated, decreases in the aggregation of $A\beta_{42}$ was observed (Saito et al. 2005). In addition, SST release is impaired in the presence of Aβ (Geci et al. 2007). Although the role of SST in Huntington's disease is controversial, it is believed that SST positive neurons are selectively spared in disease. In an experimental model of Huntington's disease, selective sparing of SST positive neurons has been shown and blocking SST by using SST antisense oligonucleotides potentiates neuronal cell death in quinolinic acid and NMDA induced excitotoxicity (Kumar et al. 1997; Kumar 2004, 2008). These studies strongly link SST with pathophysiology of Huntington's disease.

5 Somatostatin Regulation

Given its widespread distribution and interaction with various bodily systems, it is no wonder that SST can be regulated by a broad array of secretagogues - from ions and nutrients to neuropeptides, neurotransmitters, hormones, growth factors, and cytokines (Reichlin, 1983a, b; Patel 1992; Patel et al. 2001; Barnett 2003). For instance, membrane depolarization stimulates SST release from both neurons and peripheral SST-secreting cells. However, the effects of nutrients such as glucose, amino acids, and lipids, on SST secretion appears to be tissue-specific, a predominant feature in the triggering of SST release from δ cells in pancreatic islets. Contrarily, the secretion of hypothalamic SST is inhibited by glucose but insensitive to aminogenic agents. On the other hand, gut SST is promoted by luminal but not circulating nutrients. The effects of glucocorticoids are distinct, however, and employ a biphasic effect on SST secretion: stimulatory at low doses and inhibitory at high doses. Almost every neurotransmitter or neuropeptide tested has been shown to exert some sort of effect on SST secretion with a certain degree of tissue

specificity. In particular, glucagon, GH-releasing hormone, neurotensin, corticotrophin-releasing hormone, calcitonin gene-related peptide, and bombesin are potent stimulators of SST secretion, while opiate and GABA are inhibitors (Patel 1992; Epelbaum et al. 1994; Patel et al. 2001). With regard to the hormones investigated, thyroid, GH, IGF-I, and insulin augment SST release from the hypothalamus (Patel 1992; Patel et al. 2001; Barnett 2003); insulin, leptin, and epinephrine inhibits its release from the pancreas and hypothalamus respectively (Patel 1992; Patel et al. 2001; Barnett 2003). Inflammatory mediators have also shown differential effects on SST secretion: IL-1, IL-6, IL-10, INF-γ, and TNF-α stimulate SST release while TGF-β inhibits it (Scarborough et al. 1989; Quintela et al. 1997; Elliott 2004).

In addition to modulating SST secretion, many of the same agents also regulate gene expression. For instance, various members of the growth factor and cytokine family - glucocorticoids, testosterone, estradiol, insulin, leptin,TGF-β, and NMDA receptor agonists - affect steady state SST mRNA levels (Patel 1992, 1999; Patel et al. 2001). The typical transcriptional unit of a mammalian SST gene consists of two exons separated by an intron (Patel et al. 2001; Vallejo 2004). Several intracellular mediators are known to affect SST gene function and include, cAMP, cGMP, nitric oxide, Ca^{2+}, and activators of protein kinase C (Kanatsuka et al. 1981; Frankel et al. 1982; Montminy et al. 1986; Patel et al. 1991; Aguila 1994). Immediately upstream of the start transcription site is a variant of the TATA box element, followed by a cAMP response element (CRE), two glucocorticoid response element (GRE), nonconsensus sequences, and an insulin response element. Tissue-specific promoter elements are also present that work in concert with the CRE to impart high levels of constitutive gene activity. Finally, two silencer elements located within the promoter mediate repression of SST gene transcription (Patel et al. 2001; Vallejo 2004).

6 A New Member in the Somatostatin Family?

A little over a decade ago, cDNA encoding a peptide was cloned from rat brain tissue with structural similarity to SST (Tostivint et al. 1996). This new peptide termed cortistatin (CST), due to its predominantly cortical expression, is synthesized from a larger precursor molecule, preprocortistatin. Enzymatic cleavage gives rise to two products, CST-14 and CST-29. Of the fourteen amino acids pertaining to CST-14, eleven are identical to SST-14. A human form was also identified, but unlike the rat homolog, it contains seventeen residues (hCST-17) (Fukusumi et al. 1997). However, unlike SST, CST has potent sleep-promoting activities when infused into rat brain ventricles, a property achieved by its antagonizing effect on the neurotransmitter acetylcholine on cortical excitability (de Lecea et al. 1996). Recently, CST mRNA has been demonstrated in various peripheral organs and hence,it is not restricting its expression to the CNS (Papotti et al. 2003; Dalm et al. 2004; Xidakis et al. 2007). Furthermore, a biological relevance for CST outside the CNS has been recently

confirmed, as similar observations have been obtained in comparison to SST-analogs in measures of endocrine function (Gottero et al. 2004).

7 Somatostatin Receptors

The identification of high-affinity plasma membrane SSTRs, was first described in 1978, using the rat pituitary GH_4C_1 cell line by whole-cell binding analysis (Schonbrunn and Tashjian 1978). However, it was soon apparent that more than one class of SSTR existed, based upon differential binding affinities and potencies for SST-14 and SST-28 in brain, pituitary, and islet cells (Mandarino et al. 1981; Srikant and Patel 1981). These studies including one by Tran et al. further categorized SSTRs into two subclasses based on their affinity for the then available SST-analogs octreotide and seglitide: SRIF I, that bound SST-analogs and SRIF II, the group that was insensitive to these compounds (Tran et al. 1985; Reisine and Bell 1995). Using a variety of techniques such as binding analysis, covalent crosslinking, and purification of solubilized receptor including in vivo and in vitro autoradiography, the expression of SSTRs was demonstrated at various densities in the brain, gut, pituitary, thyroid, adrenals, endocrine and exocrine pancreas, kidneys, and immune cells (Patel et al. 1995; Reisine and Bell 1995; Patel 1997, 1999; Olias et al. 2004). Several tumor cell lines have also demonstrated to be rich sources of SSTRs and include AtT-20 mouse pituitary tumor cells, hamster insulinoma and Rin m5F islet tumor cells, AR42J and Mia PaCa pancreatic tumor cells and human breast cancer, neuroblastoma, glioma, leukemic, and myeloma cell lines (Patel et al. 1995; Reisine and Bell 1995; Patel 1997, 1999; Kumar 2005). Photoaffinity labeling and purification studies, revealed the existence of several SSTR species in the range of 32–85 kDa in a tissue-specific manner (Patel et al. 1990, 1995; Reisine and Bell 1995).

Fourteen years following the discovery of high-affinity SSTR binding sites on whole-cell membranes (Schonbrunn and Tashjian 1978), the first SSTR sequence was resolved by molecular cloning (Yamada et al. 1992). It was not long before the identity of five distinct SSTR genes became available (Patel et al. 1995; Reisine and Bell 1995; Patel 1997, 1999; Olias et al. 2004). Using the mRNA from human islets, the first two SSTRs were cloned and termed SSTR1 and SSTR2 (Yamada et al. 1992). The sequences of the remaining SSTRs were soon elucidated (SSTR3, SSTR4 and SSTR5) as identified in human and rodent tissue (Patel et al. 1995, 1996; Patel 1997). SSTRs encoded from the human genome are all nonallelic, and map to separate loci on different chromosomes. With the exception of SSTR2, which gives rise to two spliced variants, SSTR2A and SSTR2B, SSTRs are intronless. SSTR2A and SSTR2B differ only in the length of their carboxy-terminal segments (C-terminus). All SSTR subtypes display seven α helical transmembrane (TM) segments typified by GPCR topology (Fig. 3). GPCRs are grouped into three distinct families, A, B, and C on the basis of their sequence similarity. Family A, the largest group, also known as the rhodopsin-like family, includes rhodopsin, the

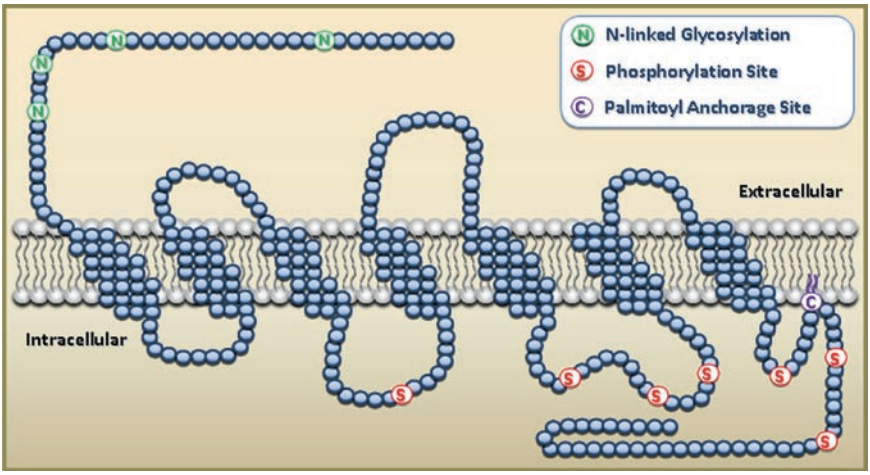

Fig. 3 Schematic representation of the structure of SSTR2A. Possible glycosylation and phosphorylation sites including a palmitoyl membrane-anchorage site are shown

adrenergic receptors, the olfactory, and many other nonolfactory members including the SSTR family. Family B consists of approximately two dozen members including the gastrointestinal peptide hormone receptor family (secretin, glucagon, vasoactive intestinal peptide, and growth-hormone releasing hormone), corticotrophin-releasing hormone, calcitonin, and parathyroid hormone receptors. Family C contains only a few members including, the metabotropic glutamate receptor family, the $GABA_B$ receptor, and the calcium-sensing and taste receptors. This family of GPCRs is typified by a large extracellular amino terminus, which appears critical for ligand binding.

SSTRs range in size from 356 to 391 amino acid residues and have an overall sequence identity of 39–57%, with most of their divergence presented in the amino- and C-terminal segments (Reisine and Bell 1995; Patel et al. 1996; Patel 1997, 1999). A highly conserved motif, YANSCANPI/VLY, in the seventh TM has been identified in all SSTR subtypes in every species, and serves as a signature sequence for this family of receptors. N-linked glycosylation sites have been identified within the amino-terminus and second extracellular loop (ECL) of all five human (h) SSTRs. Several putative phosphorylation recognition sites have been identified in the C-terminus, second and third intracellular loops (ILs) for protein kinase A, protein kinase C, and calmodulin kinase II for all hSSTRs. Interestingly, hSSTR3 is the only hSSTR that does not contain a cysteine residue downstream from the seventh TM for purposes of palmitylation and hence membrane anchoring however, it does possess an unusually long C-terminus, which may be a characteristic of its unique signaling properties (Sharma et al. 1996, 1999; Sharma and Srikant 1998b). In addition to these classical GPCR features (Pierce et al. 2002; Qanbar and Bouvier 2003), various others have been identified including a PDZ (postsynaptic density-95/discs large/ZO-1) recognition domain in the C-terminus of all SSTR subtypes (Kreienkamp et al. 2004). Several PDZ interacting proteins have been

discovered, specific to each of the five subtypes, presumably implicated in the chaperoning, scaffolding, and transport of SSTRs (Kreienkamp et al. 2004).

8 Development of Somatostatin Receptor Ligands

All five hSSTR subtypes bind SST-14 and SST-28 with nanomolar affinity; the exception is hSSTR5, which binds SST-28 with a 5- to 10-fold higher affinity than SST-14 (see Table 1). CST also interacts with all five SSTRs with nanomolar affinities (Spier and de Lecea 2000). Administration of SST produces a wide spectrum of effects that occur mainly at the site of injection and are short-lived. This is the result of peptidases found in blood and tissues (Benuck and Marks 1976; Marks et al. 1976), making the circulation half-life of SST extremely short (1.1–3 min) (Schusdziarra et al. 1977). Not surprisingly, circulating SST levels are relatively low, ranging between 14 and 32.5 pg ml^{-1} (Peeters et al. 1981; Penman et al. 1981; Tsuda et al. 1981; Vasquez et al. 1982; Skamene and Patel 1984; Shoelson et al. 1986; Gyr et al. 1987; Ensinck et al. 1989). An intense investigation has surrounded the development of compounds with selective actions and metabolic stability to be used in both investigational and clinical settings (Lamberts et al. 1991; Reisine and Bell 1995; Weckbecker et al. 2003). Various hexa- and octapeptide derivatives were synthesized, the most potent of which maintained the β-turn of the original SST molecule - the biologically active core or pharmacophore. Structure-function studies determined that amino acid residues Phe^7, Trp^8, Lys^9, and Thr^{10}, are necessary for biological activity, although residues Phe^7 and Thr^{10} could undergo minor substitution. The first FDA approved SST analog SMS 201-995 (octreotide, Sandostatin®), an octapeptide, BIM 23014 (lanreotide, Somatuline®), eventually followed. These analogs are prepared in long-acting formulations for diagnosis and treatment of various disorders including, gastrointestinal, islet cell, gut, and pituitary tumors (Lamberts et al. 1991, 1996; Weckbecker et al. 2003). Both lanreotide and octreotide exhibit high-affinity binding to SSTR2 and intermediate binding to SSTR3 and SSTR5 (see Table 1). In 2005, RC160 (vapreotide, Sanvar®), an SST-analog with similar binding affinities to SSTR2, 3, and 5 like lanreotide and octreotide, but moderate affinity to SSTR4 (Patel 1999), was granted approval for indication of acute oesophageal variceal bleeding secondary to portal hypertension (Patch and Burroughs 2002). In an attempt to reduce size but maintain metabolic stability, an SST mimic was achieved based on a cyclohexapeptide template termed MK-678 (seglitide), showing slightly higher-affinity and selectivity to SSTR2 than SSTR3 and SSTR5. As previously mentioned, SSTR2 through -5 can be categorized as group SRIF I, based upon their ability to bind octapeptide analogs; however, analogs that bind receptors in group SRIF II (SSTR1 and SSTR4), would only become available in the mid 90s (Liapakis et al. 1996). The analog Des-$AA^{1,2,5}$[$D\text{-}Trp^8$ $IAMP^9$] SST (SCH-275), was reported to have high-affinity for SSTR1 and moderate affinity for SSTR4 (Liapakis et al. 1996; Patel 1997) (see Table 1). Recently, the highly potent and stable

Table 1 Binding-affinities of endogenous, synthetic and nonpeptide somatostatin agonists. Adapted from (Florio 2008; Patel 1999)

	Binding constants (nM)				
	Receptors				
Agonists	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Endogenous					
SST-14	0.1–2.26	0.2–1.3	0.3–1.6	0.3–1.8	0.2–0.9
SST-28	0.1–2.2	0.2–4.1	0.3–6.1	0.3–7.9	0.05–0.4
hCST-17	7	0.6	0.6	0.5	0.4
Synthetic					
Octreotide	290–1140	0.4–2.1	4.4–34.5	>1000	5.6–32
Lanreotide	500–2330	0.5–1.8	43–107	66–2100	0.6–14
Vapreotide	>1000	5.4	31	45	0.7
SCH-275	3.2–4.3	>1000	>1000	4.3–874	>1000
SOM-230	9.3	1	15	100	0.16
Nonpeptide					
L-797,591	1.4	>1000	>1000	170	>1000
L-779,976	>1000	0.05	729	310	>1000
L-796,778	>1000	>10000	24	>1000	>1000
L-803,087	199	>1000	>1000	0.7	>1000
L-817,818	3.3	52	64	82	0.4

cyclohexapeptide SOM-230 (pasireotide), designed by Novartis, is a near-universal agonist, the first of its kind, demonstrating high-affinity for SSTR1, 2, 3, and 5 (Bruns et al. 2002; Weckbecker et al. 2002). SOM-230 has demonstrated to be affective in regulating pituitary control in rats, dogs, and monkeys including its control in patients with acromegaly and Cushing disease (Bruns et al. 2002; Weckbecker et al. 2002, 2003; Labeur et al. 2006). Despite the achievement of SSTR-analogs with group selectivity, there has been moderate success in the development of peptide analogs with receptor-specificity. Several analogs have been devised however; their specificity in targeting receptor-subtype ranges between 20-and 50-fold (Patel 1999; Weckbecker et al. 2003; Olias et al. 2004). A breakthrough in SST agonist design came from the Merck Research Group, using the backbone of peptide agonists for molecular modeling; they constructed subtype-selective nonpeptide agonists by combinatorial chemistry (Rohrer et al. 1998). Of the five nonpeptide agonists, three of the compounds, L-797,591, L-779,976, and L-803,087 display high-selectivity and low nanomolar binding affinity for SSTR1, SSTR2, and SSTR4 respectively. The compound L-796,778 binds to SSTR3 with approximately 50-fold selectivity while the SSTR5 subtype-agonist, L-817,818, displays dual selectivity for SSTR1 (see Table 1).

With respect to the development of SST antagonists, the field has been lagging. The first SST peptide antagonist developed, CYN-154806, a cyclic octapeptide,which displayed high-affinity for SSTR2 however, exhibited intermediate affinity for SSTR5 (Bass et al. 1996; Feniuk et al. 2000). Unfortunately, follow up studies

demonstrated near full agonism in a cAMP-accumulation assay (Nunn et al. 2003). Using the same backbone design as CYN-154806, a high-affinity SSTR3 antagonist was developed that inhibited the effects of SST-14 in a functional assay for cAMP (Reubi et al. 2000). In a unique design, open-chain octapeptide antagonists BIM 23056, BIM 23627, and BIM 23454 were selected for their preferentially binding to SSTR5 and SSTR2 respectively; however, both compounds do show partial affinities for the other subtypes to various degrees (Shimon et al. 1997b; Tulipano et al. 2002). The first high-affinity nonpeptide antagonist was designed for SSTR3 with greater than 1000-fold selectivity (Poitout et al. 2001). An SSTR1 nonpeptide antagonist SRA880, was recently characterized in vitro to have modest selectivity of up to 100-fold (Hoyer et al. 2004).

The high density of SSTRs on tumor cells, particularly the SSTR2 subtype, allowed the possibility of visualizing them and their metastases by scintigraphy. Studies involving SSTR scintigraphy were initially performed using ^{123}I-^{3}Tyr-octreotide in the late 80s (de Herder and Lamberts 2005). The use of iodinated SST-analogs are no longer in practice for radiodiagnostic purposes; instead, octreotide and lanreotide have been suited with metal chelators for stable coupling to various α- and β-emitting isotopes such as [^{111}In-DTPA^{0}]octreotide, [^{90}Y-DOTA^{0}Tyr^{3}]octreotide, [^{177}Lu-DOTA^{0}Tyr^{3}]octreotate, [^{111}In-DOTA^{0}]lanreotide, and [^{90}Y-DOTA^{0}]lanreotide (de Herder and Lamberts 2005). Although [^{111}In-DTPA^{0}]octreotide is the standard in the radioimaging of NETs, the ^{90}Yttrium (^{90}Y) and ^{177}Lutetium (^{177}Lu) conjugated derivatives have been effectively demonstrated in peptide receptor radionuclide therapy (PRRT) of patients with inoperable or metastasized NETs (Van Essen et al. 2007).

9 Somatostatin Receptor Localization

The expression of SSTR subtypes has been well characterized in human and rodent tissue including various tumors and tumor cell lines by a multitude of techniques such as Northern blot, RT-PCR, ribonuclease protection assay, *in situ* hybridization, and immunocytochemistry and has been extensively reviewed elsewhere (Patel et al. 1995, 1996; Reisine and Bell 1995; Patel 1997, 1999; Barnett 2003; Moller et al. 2003; Gardette et al. 2004; Kreienkamp et al. 2004). The distribution is widespread, with localization throughout the CNS, periphery, often overlapping in subtype expression depending on tissue- and species-type. The mRNA expression of SSTR1-5 in the rat has been localized to brain regions such as the cerebral cortex, striatum, hippocampus, amygdale, olfactory bulb, and preoptic area (Bruno et al. 1993). Comparing the individual expression patterns of each receptor-subtype revealed SSTR1 to predominate in the brain, with expression in the pituitary, islets, and adrenals. SSTR2 is also abundantly expressed throughout the brain, including the pituitary, islets, and adrenals. SSTR3 is densely expressed in the cerebellum but in moderate amounts throughout the rest of the brain. However, it is highly expressed in the spleen, kidneys, and the liver. Compared to the other SSTR subtypes, SSTR4

is poorly expressed in the brain. It is however abundant in the heart and occurs at moderate levels in the lungs and islets. SSTR5 is sparsely expressed throughout the brain but is especially prominent in the pituitary, intestine, and islets.

As previously mentioned, coexpression of SSTR subtypes is often seen in various degrees depending on tissue and cell type (Patel et al. 1995, 1996; Reisine and Bell 1995; Patel 1997, 1999; Barnett 2003; Moller et al. 2003; Gardette et al. 2004; Kreienkamp et al. 2004). Overlapping patterns of SSTR distribution have been demonstrated throughout the CNS. Colocalization of SSTR1 and SSTR2 mRNA can be found in GHRH-producing arcuate neurons (Tannenbaum et al. 1998). In the adult human pituitary, SSTR1, 2, 3, and 5 are expressed whereas all five are found in the pituitary of rats (Bruno et al. 1993; Day et al. 1995; O'Carroll and Krempels 1995; Panetta and Patel 1995). Although the five receptors have been identified in the pituitary, the primary subtypes expressed are SSTR5 and SSTR2 (Day et al. 1995; Kimura et al. 1998). In the periphery, human pancreatic islets were shown to express all five subtypes but colocalization was strongly identified for SSTR1 and SSTR5 in insulin-secreting β-cells - to a lesser extent for SSTR1 and SSTR2 (Kumar et al. 1999). The same authors also reported on the colocalization of SSTR2 and SSTR5 in glucagon-producing α-cells, an occurrence that was only identified in up to a third of the population. Elsewhere, in rat testis, SSTR1-3 displayed overlapping distribution patterns in Sertoli and germ cells, a property that was dependent on the stage of the seminiferous epithelium cycle (Zhu et al. 1998).

The expression of SSTRs in neoplastic tissue has been in the forefront of current day investigation, as their densities are found to be much higher than in normal tissue. The first evidence of the expression of SSTRs in human tumors appeared as early as 1984 in GH-secreting pituitary adenomas (Reubi and Landolt 1984). The identification of differential SSTR subtypes initially appeared in 1987 from autoradiographic studies on NETs (Reubi et al. 1987). Since then, many tumors have been shown to express SSTRs, a characteristic often exploited for both diagnostic and treatment purposes (Patel et al. 1995, 1996; Reisine and Bell 1995; Patel 1997, 1999; Barnett 2003; Moller et al. 2003; Gardette et al. 2004; Kreienkamp et al. 2004; Reubi et al. 2004). SSTRs are often highly expressed in NETs, in particular, GH-secreting pituitary adenomas and gastroenteropancreatic tumors. Several other tumors known to express SSTRs include neoplasias of the brain, breast carcinomas, lymphomas, renal cell cancers, mesenchymal tumors, prostatic, ovarian, gastric, hepatocellular, and nasopharyngeal carcinomas. Although the most prevalent subtype expressed in human tumors is hSSTR2, the appearance of other subtypes is often found - a property originally identified in pituitary adenomas (Greenman and Melmed 1994a, b; Miller et al. 1995; Panetta and Patel 1995; Schaer et al. 1997) and gastroenteropancreatic tumors (Jais et al. 1997; Schaer et al. 1997; Wulbrand et al. 1998). Over the past several years, a profusion of studies have been published addressing the variable expression of SSTR subtypes in a large variety of cancers (Patel et al. 1995, 1996; Reisine and Bell 1995; Patel 1997, 1999; Barnett 2003; Moller et al. 2003; Gardette et al. 2004; Kreienkamp et al. 2004; Reubi et al. 2004). The knowledge gained by these studies has been instrumental not only for investigational purposes but also to decipher the use of SST-analogs in both diagnostic (tumor

imaging by radio-labeled analogs) (Kwekkeboom et al. 2004) and therapeutic applications.

10 Regulation of Somatostatin Receptor Genes

One major factor affecting the potency of SST is the expression of cell surface receptors. Hormones have been shown to have a profound impact on SSTR gene regulation. For instance, oestrogen induces SST binding sites in cultured rat prolactinoma cells via upregulation of SSTR2 and SSTR3 (Visser-Wisselaar et al. 1997). Similarly, both in vitro and in vivo studies have demonstrated the ability of oestrogen to induce the transcription and upregulation of SSTR2 and SSTR3 in rat pituitary cells (Djordjijevic et al. 1998; Kimura et al. 1998). While SSTR1 transcripts were found to be upregulated by both oestrogen (Kimura et al. 1998) and testosterone (Senaris et al. 1996) in the rat pituitary, SSTR5 mRNA was in fact downregulated (Kimura et al. 1998). In MCF-7 breast cancer cells, oestrogen was reported to simulate SSTR2 gene expression (Xu et al. 1996). In two other breast cancer cell lines T47D and ZR75-1, oestrogen was found to increase and decrease SSTR binding sites respectively (Van Den Bossche et al. 2004). Investigation on the subtypes involved by Western blotting revealed an upregulation of SSTR2 in T47D cells and a downregulation of SSTR5 in ZR75-1 cells following oestrogen treatment (Van Den Bossche et al. 2004). In mouse TtT-97 thyrotrophic tumor cells, thyroid hormone increases the synthesis of SSTR1 and SSTR5 transcripts (James et al. 1997). The effects of glucocorticoids on SSTR gene expression are somewhat unique: transient exposure induces SSTR1 and SSTR2 mRNA, while prolonged exposure inhibits transcription (Xu et al. 1995). Other factors affecting SSTR gene transcription include cAMP, gastrin, epidermal growth factor, and even SST itself (Patel et al. 1993; Bruno et al. 1994a; Vidal et al. 1994). Finally, food deprivation and even diabetes in rat models have shown decreases in mRNA transcripts for SSTR1, 2, and 3 in the pituitary and SSTR5 in the hypothalamus (Bruno et al. 1994b). Investigation on the promoters of each subtype and specific elements involved in their regulation has been described (Meyerhof 1998; Patel 1999; Moller et al. 2003; Olias et al. 2004).

11 Somatostatin Receptor Signaling

The signal transduction pathway of SSTRs is rather complex, for most part,and it begins by activation of G-proteins. In the classical model of GPCR activation, agonist-binding induces a conformational change that transcends to G-proteins resulting in their activation [reviewed in (Pierce et al. 2002; Lefkowitz 2004)]. The G-protein is comprised of three subunits: the α subunit (Gα) and the β ,and γ subunits (G$\beta\gamma$) which are tightly bound. There are fifteen α subunits, five β subunits

and fourteen γ subunits known to date. Activation of the G-protein heterotrimer is preceded by the nucleotide exchange of GDP for GTP, resulting in the dissociation of the complex and allowing the Gα and Gβγ subunits to be free to propagate their signal. The G-proteins are generally referred to by their Gα subunits and therefore, can be classified under four categories based on function: $G\alpha_s$, stimulate adenylate cyclase; $G\alpha_{i/o}$, inhibit adenylate cyclase and activate inwardly rectifying potassium channels; $G\alpha_q$, activate phospholipase Cβ; and G_{12}, activate Rho guanine-nucleotide exchange factors.

Binding of SSTRs by SST ligands modulates the activates of several key enzymes, including adenylate cyclase, phosphotyrosine phosphatases (PTPases), and mitogen-activated protein kinase (MAPK) along with changes in the intracellular levels of calcium and potassium ions, as typified by activation of calcium and potassium channels, including the regulation of the sodium/proton antiporter (Fig. 4) (Patel et al. 1995, 1996; Reisine and Bell 1995; Patel 1997, 1999; Csaba and Dournaud 2001; Barnett 2003; Moller et al. 2003; Olias et al. 2004). The type of signal that prevails is dependent on several factors such as the SSTR subtype(s) expressed, signaling elements, SSTR internalization, desensitization, and/or receptor crosstalk. The ability of SST to block regulated secretion from various cell types is typified in part by its effects on the synthesis and release of two important mediators, cAMP and calcium respectively. Adenylate cyclase was the first effector

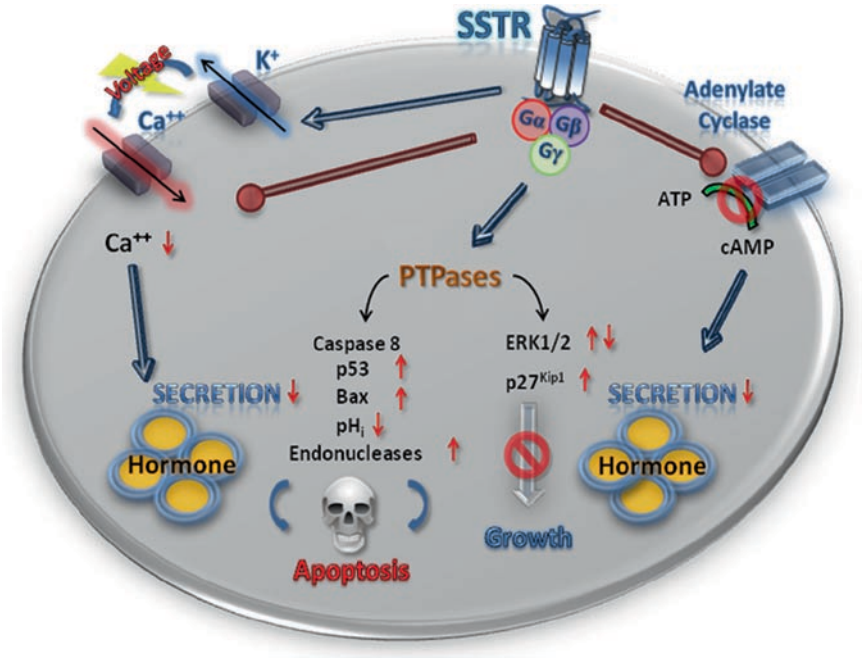

Fig. 4 Schematic representation of SSTR signaling pathways. SSTR signaling cascades leading to the modulation of hormone secretion, cell growth and apoptosis are shown

enzyme to be identified and regulated by SSTRs (Patel et al. 1994). All five SSTR subtypes negatively couple to the enzyme by activating pertussis-toxin (PTX) sensitive $G\alpha_i$ G-proteins, a property observed in various cell types (Meyerhof 1998). In an attempt to elucidate the most relevant G-protein subtypes involved in SSTR-mediated inhibition of adenylate cyclase, the subtypes $G\alpha_{i1}$, $G\alpha_{i2}$,and $G\alpha_{i3}$ were identified, as determined by targeted-disruption using either antiserum or G-protein antisense plasmids (Tallent and Reisine 1992; Liu et al. 1994; Gu and Schonbrunn 1997). SSTRs are coupled to several types of potassium channels and include the delayed rectifier, inward rectifier, ATP-sensitive potassium channels, and large-conductance calcium-activated BK channels (de Weille et al. 1989; Wang et al. 1989; Sims et al. 1991; White et al. 1991; Akopian et al. 2000). The G-protein subtype $G\alpha_{i3}$ and possibly its interacting $\beta\gamma$ dimer pair are implicated in potassium channel regulation (Takano et al. 1997). SSTRs have also been shown to directly modulate high-voltage-dependent calcium channels via $G\alpha_{o2}$ (Ikeda and Schofield 1989; Kleuss et al. 1991). SSTRs may also inhibit calcium currents by activation of cGMP protein kinase, through the induction of cGMP to regulate channel phosphorylation (Meriney et al. 1994). Aside from regulating channels to control ion flux, SSTRs have also been shown to couple to Na^+/H^+ exchangers (NHEs) (Barber et al. 1989; Hou et al. 1994; Smalley et al. 1998; Ye et al. 1999; Lin et al. 2003) to modulate such features as cell adhesion, migration, and proliferation (Putney et al. 2002). SSTR1 was the first subtype to specifically-regulate NHE-1, decreasing the extracellular acidification rate (ECAR) when transfected in either fibroblast Ltk^- or HEK-293 cells (Hou et al. 1994). It was later determined that SSTR3 and SSTR4 also contribute, but not SSTR2 and SSTR5 (Lin et al. 2003).

SSTRs activate a number of phosphatases that have been implicated in cell growth [reviewed in (Patel 1999; Csaba and Dournaud 2001; Moller et al. 2003; Olias et al. 2004)]. For instance, the SH2 domain containing tyrosine phosphatases, SHP-1 and SHP-2, which play a role in cell growth and differentiation, are known to be recruited by various SSTR subtypes. Both phosphatases are rapidly recruited to the membrane of breast cancer cells upon stimulation with SST (Srikant and Shen 1996). More specifically, SHP-1 has been demonstrated to coprecipitate with SSTR2 in a constitutive manner, suggesting its importance in the formation of signaling complexes (Lopez et al. 1997; Hortala et al. 2003). Furthermore, the activation of SHP-1 was shown to be highly dependent on the recruitment of SHP-2 through phosphorylation of tyrosine residues present at the C-terminal portion of the receptor, impressing the importance of both phosphatases in SSTR signaling (Ferjoux et al. 2003). A receptor-like PTP known as PTPη, has also been demonstrated to be an important player in the cytostatic effects of SST, particularly since its expression is necessary for the control of thyroid tumor and human glioma cells (Florio et al. 1997, 2001; Massa et al. 2004a, b). Recently, a signaling complex involving JAK2, SHP-2, and c-src was demonstrated in the SSTR1-mediated activation of PTPη (Arena et al. 2007). In addition to tyrosine phosphatases, the activation of serine/threonine phosphatases has also been demonstrated to be recruited by SSTRs. Modulation of the N- and L-type calcium channels and potassium channels have been shown to be dependent on the activation of phosphatase 2A (PP2A) and cal-

cineurin (PP2B) in several cell types such as sympathetic neurons, pancreatic alpha cells, and pituitary tumor cells. Evidence of the importance of PP2B recruitment by SSTR activation comes from studies on the regulation of neurotransmitter release and exocytosis in sympathetic neurons and pancreatic alpha cells respectively (White et al. 1991; Zhu and Yakel 1997; Gromada et al. 2001).

Several important yet recently identified signaling cascades found downstream of SSTR activation are the MAPKs (Patel 1999; Bousquet et al. 2001; Csaba and Dournaud 2001; Moller et al. 2003; Weckbecker et al. 2003; Olias et al. 2004). SSTR activation (Florio et al. 1999, 2001, 2003a; Lahlou et al. 2003) or inhibition (Dent et al. 1997; Cattaneo et al. 1999; Douziech et al. 1999) of MAPKs has been demonstrated to be mediated by PTPases. In case of SSTR5, the inhibition of MAPKs was related to the activation of a cGMP-dependent pathway when expressed in CHO-K1 cells (Cordelier et al. 1997). In addition to PTPases, recruitment of phosphoinositide-3 kinase has also been shown fundamental in the activation of MAPKs. For instance, studies involving human SSTR4 (Smalley et al. 1999), rat SSTR2B (Sellers et al. 2000) or mouse SSTR2A (Lahlou et al. 2003) have underscored its relevance.

12 Relevance of SSTRs in Cancer

As previously mentioned, a number of cancer cells overexpress SSTRs, with more than one subtype often being expressed. As early as the 1980s, the antiproliferative effects of the SST-analog octreotide were being recognized for treatment of hypersecreting tumors of the pancreas, intestine, and pituitary (Lamberts et al. 1991, 1996; Weckbecker et al. 1993). It was noted that not only was treatment blocking hormone secretion but it was also causing variable tumor shrinkage through a distinct antiproliferative effect. The antiproliferative effects of SST was demonstrated in normal dividing cells such as intestinal mucosal cells, activated lymphocytes, inflammatory cells, as well as in experimental tumor models for example solid tumors of transplanted rat mammary carcinomas and finally cultured cells derived from both endocrine and epithelial tumors (pituitary, thyroid, breast, prostate, colon, pancreas, lung, and brain) (Patel 1999; Csaba and Dournaud 2001; Lamberts et al. 2002; Moller et al. 2003; Weckbecker et al. 2003; Olias et al. 2004; Zatelli et al. 2006). The antiproliferative effects of SST on normal or transformed cells can be directed by cell growth arrest and/or apoptosis; several SSTR signaling pathways have been implicated (Fig. 3).

A large body of evidence implicates PTPs as central mediators in the antiproliferative effects of SSTRs. SSTR subtypes activate different PTPs, resulting in varying effects on downstream effectors such as MAPKs, ultimately regulating the induction of cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1/Waf1}$. In the mid 80s, a role for PTPases in SST-mediated antiproliferation of pancreatic cancer cells was postulated on the basis of an inhibition toward epidermal growth factor receptor phosphorylation patterns (Hierowski et al. 1985). All five receptor subtypes display

some capacity to activate PTPs (Buscail et al. 1994; Florio et al. 1994; Sharma et al. 1996; Reardon et al. 1997; Sharma et al. 1999), whether the PTPases are cytosolic (Srikant and Shen 1996; Reardon et al. 1997; Bousquet et al. 1998; Florio et al. 2000) or membrane localized as demonstrated with PTP (Florio et al. 1997, 2001; Massa et al. 2004a, b). An increase in the PTP SHP-1 has been reported in several different cancer cell lines including pituitary adenomas, pancreatic cancers, medullary, breast, and prostate carcinomas following SST treatment (Douziech et al. 1999; Thangaraju et al. 1999; Ferjoux et al. 2000; Zapata et al. 2002; Zatelli et al. 2005a; Theodoropoulou et al. 2006). Consequently, SHP-1 activation is a critical factor in SSTR2-mediated cell growth arrest (Lopez et al. 1997; Bousquet et al. 1998; Theodoropoulou et al. 2006). In fact, a multieffector complex between c-src and SHP-2 was determined central in the recruitment and activation of SHP-1 following stimulation of SSTR2 (Ferjoux et al. 2003). Ultimately, SHP-1 activation results in growth factor receptor signaling inhibition by dephosphorylating its substrates (Lopez et al. 1997; Bousquet et al. 1998). Other inhibitory pathways of SHP-1 include the activation of nNOS by its dephosphorylation, resulting in an increase in cGMP formation and subsequent induction of $p27^{Kip1}$ and cell cycle arrest (Lopez et al. 2001).

In a similar vein, activation of SSTR1 has also been shown to recruit SHP-2 and c-src for its antiproliferative activity (Reardon et al. 1997; Florio et al., 1999). Activation of SHP-2 by SSTR1 was reported to orchestrate antiproliferation by mediating the dephosphorylation of growth factor receptors for EGF, insulin, and platelet derived growth factor (PDGF), with the consequent inhibition of Ras and MAPK activity (Cattaneo et al. 2000). However, unlike SSTR2, the final effector PTP for SSTR1 is not SHP-1 but the membrane-bound PTPη (Florio et al. 1997, 2001; Massa et al. 2004a, b; Arena et al. 2007). Recently, SSTR2 was shown to inhibit the activity of phosphatidyl inositol 3 kinase (PI3K), thereby, preventing the activation of AKT in both pituitary and insulinoma tumor cells (Theodoropoulou et al. 2006; Grozinsky-Glasberg et al. 2008c). The PI3K/Akt signaling pathway has been demonstrated to promote the survival, proliferation, angiogenisis, and motility of tissue invasion of cancer cells and therefore, provides an important target in tumor control (Altomare and Testa 2005). Although typically involved in cell growth and proliferation (Dhanasekaran et al. 1995), the activation of the MAPKs as demonstrated via distinct SSTRs can be associated with cell growth inhibition (Florio et al. 1999; Sellers et al. 2000; Alderton et al. 2001; Lahlou et al. 2003). Stimulation of SSTR2 was shown to inhibit the proliferation of CHO-K1 cells by activating two members of the MAPK family - extracellular-regulated kinase-1 and -2 (ERK1/2) and p38 - and upstream the activation of the cyclin-dependent protein kinase inhibitor $p21^{cip}1$/WAF1 (Sellers et al. 2000; Alderton et al. 2001). Similar findings were also reported upon activation of SSTR1 (Florio et al. 1999). Contrarily, the antiproliferative actions of SSTR5 do not require activation but rather inhibition of MAPKs (Cordelier et al. 1997). Pathways suggested to be implicated in SSTR5-mediated antiproliferation include one involving phospholipase C/inositol phospholipid/Ca^{2+} (Buscail et al. 1995) and the other involving the induction of the retinoblas-

toma tumor suppressor protein (Rb) and p21 (Sharma et al. 1999). In rare instances, SST may actually stimulate cell growth - an anomaly shown to occur by MAPK activation via human SSTR4 (Sellers et al. 2000).

In addition to the cytostatic effects of SST, apoptosis or programmed cell death has also been observed to contribute to the antiproliferative response following treatment. Apoptosis was first demonstrated in AtT-20 and MCF-7 cells when treated with octreotide (Pagliacci et al. 1991; Srikant 1995; Sharma and Srikant 1998a). In MCF-7 cells, SHP-1 is necessary for SSTR-mediated apoptotic signaling (Sharma and Srikant 1998a; Liu et al. 2000). Because both cell types express more than one SSTR, it is not possible to assign the subtype that may be contributing to apoptosis. When CHO-K1 cells were individually transfected with each receptor-subtype, apoptosis was uniquely triggered by human SSTR3 (Sharma et al. 1996). The events preceding apoptosis following hSSTR3 activation include activation of tumor suppressor protein p53 and proapoptotic protein Bax (Sharma et al. 1996). However, recent reports have described p53-independent apoptosis via SSTR2 in HL-60, human pancreatic adenocarcinoma,and human somatotroph tumor cells (Teijeiro et al. 2002; Guillermet et al. 2003; Ferrante et al. 2006).

Unlike the direct effects of SST on tumor cell proliferation mentioned above, SST can indirectly control tumor growth and development by inhibiting angiogenesis. Antiangiogenic activity was first described by Woltering et al. using a chicken corioallanthoic membrane model, a property that was further supported by the findings conducted in vitro and in vivo with SST and its analogs (Barrie et al. 1993; Danesi and Del Tacca 1996; Danesi et al. 1997; Albini et al. 1999; Dasgupta and Mukherjee 2000; Garcia de la Torre et al. 2002; Koizumi et al. 2002; Zalatnai and Timar 2002; Florio et al. 2003a; Murray et al. 2004). Three different pathways have been proposed and may operate concurrently to achieve the antiangiogenic activity of SSTRs. First, activation of SSTRs may directly inhibit the proliferation, migration, and invasion of endothelial cells to the tumor. Second, SST may regulate the secretion of angiogenic promoting factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Third, SST may modulate the activation of monocytes, cells which are important in the immune response,but whose migration in the peritumoral region can produce proangiogenic factors resulting in neovascularization (Florio 2008). SST may also indirectly regulate tumor growth by inhibiting the synthesis and/or secretion of growth factors and hormones such as EGF, transforming growth factor, insulin, prolactin, GH, and IGF-I (Susini and Buscail 2006).

The first conclusive evidence that SST-analogs can have antiproliferative properties in the clinic came from a multicenter randomized trial recruiting 32 acromegalic patients with hypersecreting pituitary adenomas (Thapar et al. 1997). These patients demonstrated an 83% reduction in mean growth fraction when compared to untreated controls, suggesting that octreotide had exerted an antineoplastic effect on somatotroph adenomas. In a separate study by Losa et al. the Ki-67 index, a nuclear protein expressed only in dividing cells, was significantly lower in the GH hypersecreting adenomas of patients pretreated with octreotide compared to untreated controls (Losa et al. 2001). Many trials have since been undertaken dem-

onstrating the effects of SST-analog therapy on tumor shrinkage in acromegalic patients. Typically, patients receiving SST-analogs as primary therapy show reductions of up to 50% in tumor volume (Bevan 2005; Melmed et al. 2005). With regard to the antiproliferative effects of SST-analogs in the treatment of patients with other types of tumors, evidence is scanty. In approximately half of patients with gastrointestinal NETs, stabilization of tumor growth was apparent for duration of 8–16 months; however, tumor shrinkage was achieved in only 10–20% of cases (Eriksson and Oberg 1999). In a study with patients diagnosed with malignant gastrinoma, treatment with the long-acting formulation of octreotide demonstrated an antiproliferative response in approximately 50% of the subjects (Shojamanesh et al. 2002). Although SST-analogs are effective in the symptomatic treatment of NETs, a family of tumors which originate from various endocrine glands including the pituitary, parathyroid, adrenals, endocrine islets, in addition to exocrine cells from the digestive and respiratory tracts (Grozinsky-Glasberg et al. 2008a, b), SST-analog therapy is rarely curative.

13 Agonist-Regulation of Somatostatin Receptors

The initial responses following activation of SSTRs diminish with continued exposure to SST (Patel 1997, 1999; Csaba and Dournaud 2001; Moller et al. 2003; Olias et al. 2004). This feature is shared by many GPCRs and is a requirement for terminating signaling. This process can be divided into two general steps: desensitization and internalization. Desensitization is the result of rapid attenuation of receptor function, usually by phosphorylation of its c-tail, causing uncoupling of the receptor from its respective G-protein. This property can be mediated by second-messenger kinases, such as protein kinase A or protein kinase C, or through a distinct family of G-protein-coupled receptor kinases termed as GRKs and is typically followed by internalization (Pierce et al. 2002; Premont and Gainetdinov 2007). Internalization is a process by which the receptor is redistributed away from the surface and brought into the cell, also known as endocytosis (Claing et al. 2002; Pierce et al. 2002). The process of internalization can be divided into three different pathways: clathrin-coated pits, caveolae, and uncoated vesicles (Claing et al. 2002; Pierce et al. 2002). The least understood method by which GPCRs internalize involves caveolae. This mechanism involves membrane invaginations that are rich in both caveolin and cholesterol (Nichols 2003). Several GPCRs have been demonstrated to undergo caveolae-dependent internalization and include the endothelin and vasoactive intestinal peptide receptors (Claing et al. 2000) in addition to the chemokine receptors (Neel et al. 2005). The most investigated and best understood mechanism involved in GPCR internalization is the β-arrestin-dependent mediated pathway, which occurs via clathrin-coated vesicles (Claing et al. 2002; Pierce et al. 2002; Lefkowitz and Shenoy 2005). There are two subtypes of β-arrestin, β-arrestin-1 and β-arrestin-2, both of which are ubiquitously expressed. A third type of arrestin known as visual arrestin is exclusively expressed in the retina where it was originally identified

(Pierce et al. 2002; Lefkowitz and Shenoy 2005). This process of internalization is initiated by the recruitment of β-arrestin to the phosphorylated portion of the receptor (Lohse et al. 1990). This in turn engages the receptor into the clathrin-coated pit machinery, as β-arrestin is known to interact with several components involved in this process including the heavy chain of clathrin itself, the β2-adaptin subunit of the clathrin adaptor protein AP-2, the small guanosine triphosphatase ARF6, and its guanine nucleotide exchange factor ARNO, the *N*-ethylmaleimide-sensitive fusion protein (NSF) in addition to constituents of the inner leaflet of the cell membrane itself (Claing et al. 2002; Pierce et al. 2002; Lefkowitz and Shenoy 2005). The final step to internalization requires the actions of a GTPase known as dynamin, as it is responsible for pinching off the pits to generate endosomes. There are two general types of β-arrestin-mediated internalization that depend on its avidity for the receptor: class A, β-arrestins bind transiently to the receptor, target it to clathrin-coated pits, and dissociate during receptor-internalization; class B, β-arrestin remains tightly bound to the receptor and does so throughout the internalization process for extended periods of time, from which the receptor can be sorted to lysosomes where it is degraded. The end result is that class A receptors such as the β_2-adrenergic receptors, are recycled more quickly to the cell surface, as their fate is not tied to β-arrestin sorting; whereas class B receptors, for example, the V_2R vasopressin receptors, are slowly recycled and often end up being degraded (Claing et al. 2002; Pierce et al. 2002; Lefkowitz and Shenoy 2005).

In the early 1980s, it was appreciated that SSTRs can undergo agonist-induced uncoupling from their G-proteins, a property demonstrated in AtT-20 cells (Reisine and Axelrod 1983). It wasn't long before agonist-induced internalization was documented and shown to occur in cells from the rat anterior pituitary and islet, mouse AtT-20 cells, and human pituitary and islet tumor cells (Morel et al. 1985; Amherdt et al. 1989; Hofland et al. 1995, 1999). However, a rather unusual occurrence developed following prolonged agonist exposure (24–48 h) in GH_4C_1 and Rin m5f cells; SSTRs were found to increase at the cell surface (Presky and Schonbrunn 1988; Sullivan and Schonbrunn 1988). Although the underlying mechanisms are still unclear, agonist-induced upregulation has been observed by several other GPCRs (Presky and Schonbrunn 1988; Cox et al. 1995; Hukovic et al. 1996; Ng et al. 1997; Tannenbaum et al. 2001) and may play a role in long-term drug therapy. A hunt for the specific receptor-subtypes mitigating these events is underway. The results are confounding, as studies have revealed differences that were not only dependent on receptor-subtype but also on the species from which the receptor is derived. Despite these differences, two important conclusions can be made based upon the subgroup of SSTRs examined: SRIF1 receptors (SSTR2, SSTR3 and SSTR5) internalize readily following agonist treatment, whereas SRIF2 receptors (SSTR1 and SSTR4) are rather resistant to agonist-induced internalization.

Initial evidence for the desensitization and internalization of SSTR2 following agonist treatment in vivo came from rat brain slices (Boudin et al. 2000). Around the same time, it was also observed that SSTR2 internalized when activated in primary cultured hippocampal neurons using fluorescently-labeled SST ligands (Stroh et al. 2000). Stereotactic injections of the SST-analog octreotide in the rat parietal cortex

(Csaba et al. 2001) and endopiriform nucleus (Csaba and Dournaud 2001) demonstrated that SSTR2 internalized via a clathrin-mediated pathway. Similar mechanisms were also described for the internalization of SSTR2 in vivo, as demonstrated by studies in the rat forebrain (Schreff et al. 2000), dorsolateral septum (Csaba et al. 2002), and arcuate nucleus of the hypothalamus (Csaba et al. 2003). When transfected in either CHO-K1, HEK-293, or even pancreatic β-cells, human and rat SSTR2 internalize in response to SST stimulation (Hukovic et al. 1996; Roosterman et al. 1997; Roth et al. 1997b; Cescato et al. 2006), via a clathrin-dependent pathway. Furthermore, endocytosis of SSTR2 was also demonstrated in glioma and neuroblastoma cells that endogenously express the receptor (Koenig et al. 1997; Krisch et al. 1998). In other cell types, the densitization, internalization, and phosphorylation of rat SSTR2 was observed (Hipkin et al. 1997, 2000; Roosterman et al. 1997). The phosphorylation of SSTR2 was related to its internalization of clathrin-coated pits and shown to occur at both the C-terminal portion and the IL of SSTR2. Both protein kinase A and protein kinase C play a role in the phosphorylation and internalization of SSTR2 (Hipkin et al. 2000; Oomen et al. 2001). Interestingly, although β-arrestin subtype-1 was found to desensitize mouse SSTR2 transfected in CHO-K1 cells, it was not implicated in its internalization. Recently, both GRK2 and β-arrestin subtype-2 were shown to be actively involved in the phosphorylation and clathrin-mediated internalization of the receptor when expressed in HEK-293 cells, respectively (Tulipano et al. 2004). The same authors also described a region in the C-terminal portion of the receptor as a recognition site for GRK2 phosphorylation. SSTR2 can therefore be classified a class B receptor, as SSTR2 forms stable associations with β-arrestin throughout its sequestration and localization in endosomes (Tulipano et al. 2004).

The regulation of SSTR3 is very similar to that of SSTR2. Both human and rat forms rapidly internalize following agonist stimulation in various transfected cell lines (Hukovic et al. 1996; Roosterman et al. 1997; Roth et al. 1997b; Cescato et al. 2006). The receptor is phosphorylated at the C-terminus, a critical determinant for agonist-induced internalization (Roth et al. 1997a; Tulipano et al. 2004). Internalization follows a clathrin-mediated pathway, a property dependent on the recruitment of β-arrestin (Kreuzer et al. 2001; Tulipano et al. 2004). Desensitization of the receptor follows a slow recovery rate, as demonstrated by its effector coupling to adenylate cyclase (Roosterman et al. 1997; Roth et al. 1997b). This could be explained by the high avidity of β-arrestin binding to the receptor, however; both proteins are found colocalized in intracellular endocytic compartments for relatively short time periods (Kreuzer et al. 2001; Tulipano et al. 2004). Given that the receptor is more prone to degradation unlike SSTR2, it is more probably that sequestration to lysosomes dictates its slow recovery (Tulipano et al. 2004).

The final receptor in the SRIF1 class, SSTR5, undergoes differential regulation in a species-specific manner. For instance, human SSTR5 is rapidly internalized following activation with either SST-14 or SST-28 when expressed in CHO-K1 cells (Hukovic et al. 1996, 1998; Cescato et al. 2006). Desensitization has also been observed as demonstrated by a reduced ability to couple to adenylate cyclase following prestimulation, a property highly dependent on the structural domains

present at the C-terminus (Hukovic et al. 1998). The loss of cell surface receptors for rat SSTR5 is rather moderate compared to its human counterpart, as a rapid recycling rate has been described for this difference (Stroh et al. 2000). Similar to human SSTR5, the rat homolog also undergoes agonist-regulated desensitization (Roosterman et al. 1997; Roth et al. 1997b; Stroh et al. 2000). More recently, the interaction of β-arrestin with SSTR5 has been described (Tulipano et al. 2004; Grant et al. 2008b), and although rat SSTR5 can be categorized as a class A receptor as determined by its transient association with β-arrestin (Tulipano et al. 2004), its human homolog does not show any interaction (Grant et al., 2008b). The species-related differences in the regulation of human and rat SSTR5 may in part be explained by their differential association with β-arrestin.

As previously mentioned, the SRIF2 class of SSTRs (SSTR1 and SSTR4) is generally resistant to internalization but not desensitization by agonist. For instance, when transfected in CHO-K1 cells, rat SSTR1 is quickly phosphorylated but slowly sequestered within cells (Liu and Schonbrunn 2001). Similarly, activation of endogenously expressed SSTR1 in both neurons of the hippocampus and cortex of rat did not cause its internalization (Stroh et al. 2000). The upregulation of SSTR binding sites in GH4C1 cells was attributed to the presence of SSTR1, as these cells predominantly express this subtype (Presky and Schonbrunn 1988). A similar occurrence was observed for hSSTR1 when expressed in CHO-K1 cells, where upregulation rather than downregulation predominates followed prolonged agonist exposure (Hukovic et al. 1996). Further examination revealed that the upregulation of hSSTR1 was not dependent on *de novo* synthesis, but rather on dephosphorylation of amino acid residues present at the C-terminus and the recruitment of pools of intracellular receptor (Hukovic et al. 1999). However, when hSSTR1 was expressed in COS-7 cells, only a small fraction of receptor-bound ligand was internalized with the majority of receptors remaining within or just beneath the cell membrane (Nouel et al. 1997).

Contrary to human SSTR1, hSSTR4 does show moderate levels of internalization when expressed in CHO-K1 cells; however compared to hSSTR1, prolonged treatment with agonist does induce its upregulation (Hukovic et al. 1996). The low level of internalized hSSTR4 observed following agonist stimulation was attributed to a rapid recycling rate (Smalley et al. 2001). Species-related differences have been documented between the regulation of human and rat SSTR4 homologues. For instance, rat SSTR4 does not internalize when transfected in either HEK-293 or rat insulinoma cells following agonist-activation (Smalley et al. 2001). Interestingly, internalization is apparent when part of the C-terminal portion of the receptor is removed, suggesting a negative-regulatory motif involved in controlling the internalization of rat SSTR4 (Roosterman et al. 1997; Roth et al. 1997b). Further investigation using rat SSTR4 mutants, revealed threonine 331 as the residue most accountable for inhibiting internalization (Kreienkamp et al. 1998). Taken together, the in vitro analysis of rat SSTR4 is in good agreement with in vivo results, as intracerebroventricular administration of SST-14 does not promote its sequestration (Schreff et al. 2000). Despite the variability in the trafficking of SSTR1 and SSTR4,

it is clear that neither of them depend on interaction with β-arrestin for internalization (Tulipano et al. 2004).

14 Dimerization of SSTRs

Physical evidence for the dimerization of SSTRs was first introduced in 2000 by Rocheville et al. using a combination of pharmacological, biochemical, and biophysical techniques (Rocheville et al. 2000a; Rocheville et al. 2000b). In these studies, it was determined that human SSTR5 dimers could be stabilized following agonist treatment in a dose-dependent fashion. Furthermore, using a functional complementation technique with a signaling deficient variant of SSTR5, receptor-activation could be restored when SSTR1 was introduced, presumably due to heterodimerization (Rocheville et al. 2000b). Heterodimerization was suggested to be a specific process, as signaling by the SSTR5 variant could not be reconstituted by SSTR4 expression. Human SSTR1 is known to be resistant to agonist-mediated internalization; however, in cells coexpressing both SSTR1 and SSTR5, internalization could be observed (Rocheville et al. 2000b). In a related study, the homo- and heterodimerization of SSTR1 and SSTR5 were specifically shown in live cells using a combination of RET techniques (Patel et al. 2002). In these studies, although SSTR5 was demonstrated to form both homo- and heterodimers with SSTR1 in an agonist-regulated fashion, SSTR1 remained as a monomer when expressed alone despite its activation with agonist. This was the first study which demonstrated using RET techniques that not all GPCRs require dimerization to function, as several other groups have since shown (Gripentrog et al. 2003; Meyer et al. 2006; Whorton et al. 2007). In a follow-up study, the heterodimerization of human SSTR1 and SSTR5 was shown as being subtype specific, that is, the interaction was preferentially regulated by the ligand-binding of SSTR5 and not SSTR1 (Grant et al. 2004b). This intriguing observation that human SSTR1 is incapable of forming homo- or heterodimers in either an active or inactive state (Patel et al. 2002; Grant et al. 2004b), appears to correlate with its resistance to internalize and upregulate on prolonged agonist treatment (Hukovic et al. 1996, 1999). Swapping the carboxyl-terminal tails of SSTR1 with that of SSTR5, reconstitutes the ability of this receptor to internalize and dimerize following stimulation (Grant et al. 2004b). The importance of the carboxyl-terminal tail in GPCR dimerization has been demonstrated earlier: on investigation of the δ-opioid receptor-trafficking (Cvejic and Devi 1997), in the masking of an ER retention motif on heterodimerization of the γ-aminobutyric acid receptor-subtypes ($GABA_BR$) $GABA_BR1$ and $GABA_BR2$ (White et al. 1998; Kuner et al. 1999), and in generation of the μ- and δ-opioid receptor heterodimer (Fan et al. 2005). Similarly, in line with the results of Fan et al. the heterodimerization between μ- and δ-opioid receptors could also be modulated by uncoupling of G-protein from the receptors (Law et al. 2005). Given that the carboxyl-terminal tails of GPCRs are important for G-protein coupling, a mechanism for the heterodimerization of this receptor pair can be described.

Furthermore, a detailed account on the heterodimerization of the adenosine A_{2A} and the dopamine D_2 receptors was shown to occur between the carboxyl-terminal tail of A_{2A} and the third IL of D_2 (Canals et al. 2003). More specifically, the interaction was dependent on arginine-rich residues in the IL of the D_2 receptor with either two aspartate residues or a phosphorylated serine residue in the carboxyl-terminal portion of A_{2A} (Ciruela et al. 2004).

The inhibition of adenylate cyclase and cAMP synthesis, a typical hallmark of SSTR activation, was shown as being more efficient following formation of SSTR1/SSTR5 heterodimers (Grant et al. 2004b). More specifically, an approximate 50-fold increase in signaling efficiency was seen with the drug octreotide (SMS 201-995) in cells coexpressing both SSTR1 and SSTR5 compared to SSTR5 alone (Grant et al. 2004b), despite its absence in affinity to SSTR1 (Patel 1999). However, although the signaling efficiency was increased, the actual efficacy was decreased - suggesting that the formation of the heterodimer results in a new receptor with distinct signaling characteristics (Grant et al. 2004b). This alteration in maximum coupling efficacy could have functional implications, as human prolactinomas show poor responses to octreotide treatment. These tumors originate from the pituitary and hypersecrete the hormone prolactin. Coincidently, prolactinomas predominantly express SSTR1 and SSTR5 (Shimon et al. 1997a; Jaquet et al. 1999). In cultured studies of human excised prolactinomas, tumors that displayed increased expression of SSTR1 responded poorly to treatment with octreotide in controlling prolactin release, as opposed to those showing lower expression levels regardless of SSTR5 expression (Jaquet et al. 1999). Interestingly, both SSTR1 and SSTR5 are highly coexpressed in β-cells of the human pancreas (Kumar et al. 1999), suggesting a possible role for heterodimerization in the control of insulin secretion.

Many tumors often express SSTR2, especially those of neuroendocrine origin (Lamberts et al. 2002; de Herder et al. 2003), which makes this receptor subtype an appropriate target to investigate. Using both coimmunoprecipition and RET techniques, it was determined that hSSTR2 exists at the cell surface as a preformed homodimer (Grant et al. 2004a; Duran-Prado et al. 2007). Surprisingly, treatment with agonist causes it to dissociate into monomers (Grant et al. 2004b; Duran-Prado et al. 2007). Although ligand-induced dissociation has been reported in the regulation of other GPCR combinations (Cvejic and Devi 1997; Gines et al. 2000; Cheng and Miller 2001; Pfeiffer et al. 2001; Latif et al. 2002; Berglund et al. 2003; Law et al. 2005), few have shown functional relevance for their occurrence. In the report by Cvejic and Devi, dissociation of δ-opioid receptor dimers was reported essential for proper receptor-internalization. However, regulated dimerization and not dissociation of the platelet activating factor receptor and the thyrotropin-releasing hormone receptor, was shown to increase internalization (Perron et al. 2003; Song and Hinkle 2005). Further investigation on the dissociation of SSTR2 dimers, like the δ-opioid receptor, led us to conclude its importance in receptor-internalization, as crosslinking SSTR2 dimers to prevent dissociation, drastically impaired its internalization rate (Grant et al. 2004a). Interestingly, in the report by Duran-Prado et al. dissociation of porcine SSTR2 dimers was also determined to be a feature occurring prior to its

internalization (Duran-Prado et al. 2007), possibly suggesting a common characteristic for this subtype amongst all species.

Two other members of the SSTR family were shown to dimerize in the laboratory of S. Schulz, namely SSTR2 and SSTR3 (Pfeiffer et al. 2001). In their investigations, rodent SSTR2 and SSTR3 were demonstrated to form constitutive homodimers and heterodimers when coexpressed in HEK 293 cells. Interestingly, in cells coexpressing SSTR2 and SSTR3, the SSTR3-selective agonist L-796,778 displayed marked reductions in binding affinity, suggesting negative cooperativity of SSTR2 on SSTR3. Furthermore, GTP binding, inhibition of adenylate cyclase, and phosphorylation of ERK1/2 by the heterodimer reflected the characteristics of SSTR2 when expressed alone in the same cells. However, unlike SSTR2, the SSTR2/SSTR3 heterodimer displayed a strong resistance to agonist-induced desensitization (Pfeiffer et al. 2001). The physiological relevance of these observations remains unclear; however, both receptors colocalize in tissues such as the pancreas, the anterior lobe of the pituitary (Pfeiffer et al. 2001), and in medullablastoma tumoural cells (Cervera et al. 2002). The SSTR2-mediated inactivation of SSTR3 may explain the absence of SSTR3 binding sites in the cerebellum of developing rats, as mRNA levels for both SSTR2 and SSTR3 are highly expressed in early development (Viollet et al. 1997).

In a recent report by Grant et al. SSTR2 and SSTR5 were demonstrated to physically interact - a property that was regulated by the binding of agonist (Grant et al., 2008a). Interestingly, heterodimerization was not modulated by treatment with the endogenous pan-agonist SST-14, but instead was induced by a selective agonist for SSTR2 and not SSTR5 (Grant et al., 2008a). This is contrary to regulation of the hSSTR1/hSSTR5 heterodimer, where treatment with SST-14 enhances its formation (Rocheville et al. 2000b; Patel et al. 2002; Grant et al. 2004b). Although concurrent stimulation had been shown as a requirement in the stabilization of heterodimers between members of other family A GPCR subfamilies (Gines et al. 2000; Mellado et al. 2001; Yoshioka et al. 2002; Rodriguez-Frade et al. 2004; Kearn et al. 2005; Jiang et al. 2006; Pello et al. 2008), several heteromeric interactions were found to be equally fostered following activation of just one of the receptor protomers (Rocheville et al. 2000a; McGraw et al. 2006; Baragli et al. 2007). The SSTR2/SSTR5 heterodimer demonstrated an approximate 10-fold greater efficiency for G-protein coupling and an enhanced ability to activate MAPK (Grant et al., 2008a). More importantly, the heterodimer conferred an extended growth inhibitory response that was related to an increased induction of the cyclin-dependent kinase inhibitor $p27^{Kip1}$.

An interesting observation that was also addressed in the Grant et al. study was that heterodimerization altered the sequestration of β-arrestin and recycling of SSTR2 (Grant et al., 2008a). GPCRs often require the interaction of β-arrestins to internalize following their stimulation. This process is typically promoted by phosphorylation of the carboxyl-terminal portion of the receptor by a G-protein coupled receptor kinase (GRK). β-arrestins are responsible for the recruitment of several factors implicated in the internalization machinery including clathrin, AP-2, and ARF6, to name a few (Claing et al. 2002; Pierce et al. 2002; Lefkowitz and Shenoy 2005). There are two main types of β-arrestin mediated internalization, class A and

class B, that are primarily dependent on the avidity of β-arrestin to the receptor. Class A GPCRs form transient interactions with β-arrestin during internalization, whilst class B GPCRs form stable interactions during and following their sequestration. The avidity of β-arrestin to the receptor has direct effects on receptor recycling rates, as class A GPCRs recycle back to the cell surface more efficiently than class B, which is often sorted to the lysosomal compartment for degradation. Of the SSTRs investigated, only SSTR2 and SSTR3 were shown to form stable interactions with β-arrestin, indicative of a class B-dependent subtype (Tulipano et al. 2004). Heterodimerization of SSTR2 and SSTR5 as induced by activation with a SSTR2-selective agonist, caused a transient interaction of β-arrestin with SSTR2 resulting in a rapid recycling rate, indicative of a class A GPCR (Grant et al., 2008a). Similarly, selective activation of SSTR2 and not concurrent stimulation of both SSTR2 and SSTR5, had been shown to reduce desensitization and increase the recycling rate of SSTR2 in AtT-20 cells - the murine anterior pituitary-derived cell line that endogenously expresses SSTR2 and SSTR5 (Sharif et al. 2007).

SST-analogs are frequently administered as first line treatment in acromegaly, caused by GH hypersecreting pituitary adenomas to regulate endocrine function (Tichomirowa et al. 2005). Over 90% of patients on SST-analogs show decreases in circulating GH levels, while approximately 70% of those achieve biochemical normalization. As previously mentioned, SST-analog therapy frequently results in tumor shrinkage in roughly 50% of patients (Lamberts et al. 2002; Bevan 2005; Melmed et al. 2005; Ferrante et al. 2006; Zatelli et al. 2006; Resmini et al. 2007). It is known that GH secreting pituitary adenomas seldomundergodesensitization to treatment, as acromegalic patients rarely show any signs of tachyphylaxis despite years of SST-analog therapy. Interestingly, this property is specific to tumors of the pituitary, as neither islet cell nor most other NETs share this feature; prolonged administration usually results in desensitization and relapse, as symptoms invariably return (Lamberts et al. 1996; de Herder et al. 2003; Hofland and Lamberts 2003). Incidentally, the two SSTRs predominantly expressed in GH hypersecreting pituitary adenomas are SSTR2 and SSTR5 (Jaquet et al. 2000; Park et al. 2004), therefore, heterodimerization of these two receptors - as induced by treatment with SST-analogs - which share higher affinities for SSTR2, could account for this behavior.

SSTRs have not only been shown to form dimers within their family but also with other related members, such as the dopamine and opioid receptor families. For instance, when expressed in CHO-K1 cells, human SSTR5 and human dopamine (D2R) could be triggered to heterodimerize when activated by either a dopamine- or a SST-related agonist (Rocheville et al. 2000a). Furthermore, heterodimerization provided positive cooperativity to SST binding, a property that was also related to enhanced receptor-signaling. Immunohistochemical analysis made evident the possibility of identifying these heterodimers under normal physiological conditions, as colocalization of both SSTR5 and D2R were shown in a subset of neurons from both the cortex and striatum of the rodent (Rocheville et al. 2000a). Recently, a physical interaction between human SSTR2 and D2R was documented and shown to be regulated by agonist-binding (Baragli et al. 2007). Interestingly, unlike the SSTR5/D2R heterodimer, positive cooperativity was a property observed for D2R, as the binding affinity of dopamine was markedly enhanced by agonist-bound SSTR2 (Baragli et al. 2007).

In addition, combined treatment of SST-14 with either dopamine or the D2R agonist quinpirole improved signaling efficiency. There have been several indications suggesting a functional linkage between the somatostatinergic and dopaminergic systems. For instance, dopamine enhances SSTR-mediated inhibition of adenylate cyclase in rat striatum and the hippocampus (Rodriguez-Sanchez et al. 1997). Additionally, SSTR2 has been shown to mediate striatal dopamine release (Hathway et al. 1999). Although SSTRs are the primary targets in the medical treatment of acromegaly caused by growth-hormone hypersecreting pituitary adenomas, the dopamine agonist, cabergoline, provides effective control in 29–39% of patients (Abs et al. 1998; Cozzi et al. 1998). Incidentally, combination treatment of SST and dopamine agonists has been shown to be more effective than the activation of SST-analogs alone (Marzullo et al. 1999). Incidentally, heterodimers between D2R and SSTR2 were observed in cultured rat striatal neurons (Baragli et al. 2007). The recent development of chimeric molecules that target both SSTR2 and D2R attest to these findings and suggest an interaction between both receptors to account for their behavior (Saveanu et al., 2002, 2006, 2008; Jaquet et al. 2005).

Finally, the SST-analog octreotide, has been observed to behave as an antagonist in morphine-dependent individuals (Maurer et al. 1982) and patients undergoing morphine withdrawal have presented with reduced vomiting following octreotide administration (Bell et al. 1999). Since both receptors, SSTR2 and the μ-opioid receptor (μOR), have been shown to be colocalized in neurons of the locus coeruleus (Pfeiffer et al. 2002), a region of the brain implicated in opioid dependency and withdrawal (Gold et al. 2003), it is not unreasonable to assume that heteromeric interactions may exist. Indeed, when expressed in HEK-293 cells, heterodimerization between SSTR2 and the μOR could be demonstrated (Pfeiffer et al. 2002). Although it was determined that ligand binding profiles of either the SST-analog L-779,976 or the μOR agonist DAMGO were unaltered by heterodimerization, receptor regulations such as phosphorylation, desensitization, and internalization were affected. For instance, binding of either L-779,976 or DAMGO to the heterodimer resulted in cross-phosphorylation of each receptor-subtype (Pfeiffer et al. 2002). Furthermore, this form of heterologous desensitization translated into a loss of coupling to adenylate cyclase and a diminished MAPK signaling response. Interestingly, cointernalization of SSTR2 and μOR was only observed following stimulation of SSTR2 and not by activation of the μOR agonist DAMGO (Pfeiffer et al. 2002). These results implicate SSTR agonists in the stabilization of this heterodimer. A similar finding was reported for the SSTR2/SSTR3 heterodimer; however, in this case, activation of SSTR2 resulted in its selective-internalization while SSTR3 was maintained at the cell surface (Pfeiffer et al. 2001).

15 Conclusions

The history of SST has come a long way, from its initial discovery as a hypothalamic regulator of GH secretion from the anterior pituitary, to its role in the antiproliferation of tumor growth. Intense investigation surrounds the development of

new SST-analogs with the capability of either targeting a wider distribution of SSTRs - as is the situation for SOM-230 - or chimeric molecules, that target in addition to SSTRs, dopamine receptors,and coined dopastatins. Dopastatins are an exiting new class of NET regulators that are currently under clinical investigation (Ipsen, Paris, France). In addition, the application of radiolabeled SST-analogs in PRRT has shown promise in the treatment of patients with inoperable or metastasized NETs. Whatever the ligand, it is the receptor that is the target and with the understanding of GPCR dimerization, a new dimension in SSTR drug discovery may unfold. We have come a long way since the development of the first SST-analog over 30 years ago, and yet the achievements in the field have proven unrelenting.

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Ghrelin: From Gene to Physiological Function

Masayasu Kojima and Kenji Kangawa

Abstract The discovery of ghrelin has elucidated the role of the stomach as an important organ in the regulation of growth hormone release and energy homeostasis. Ghrelin is orexigenic; it is secreted from the stomach and circulates in the blood stream under fasting conditions, indicating that it transmits a hunger signal from the periphery to the central nervous system. Ghrelin is a peptide hormone, in which serine 3 (threonine 3 in frogs) is modified by an *n*-octanoic acid; this modification is essential for ghrelin's activity. Recently the enzymes responsible for the processing from the ghrelin precursor to active *n*-octanoyl-modified ghrelin have been identified. This review surveys the processing pathway from ghrelin gene to mature ghrelin peptide and summarizes our knowledge of the regulatory mechanism of ghrelin secretion and function.

1 Introduction

Ten years have passed since the discovery of ghrelin, a peptide hormone originally isolated from rat stomach (Kojima et al. 1999). The name "ghrelin" derives from "ghre", a word root for "grow" in Proto-Indo-European languages, to indicate its ability to stimulate GH release from the pituitary gland.

The characteristic structure of ghrelin is an acyl-modification at the third amino acid - serine (threonine in the bullfrog and edible frog) (Kojima et al. 2008) (Fig. 1). Serine 3 (or threonine 3 in frogs) is modified with a medium chain fatty acid, typically *n*-octanoic acid. Interestingly, this modification is essential for ghrelin's biological activity.

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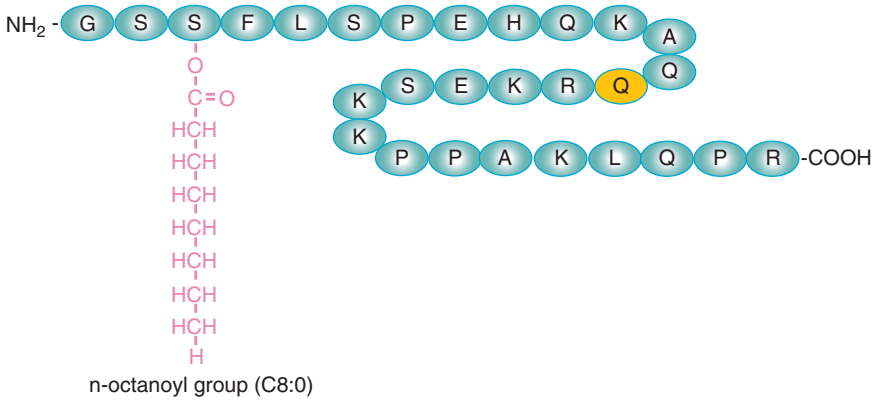

Fig. 1 Structures of rat ghrelins. Rat ghrelin is a 28-amino-acid peptide, in which Ser3 is modified by a fatty acid, primarily *n*-octanoic acid. This modification is essential for ghrelin's activity

Two main physiological functions of ghrelin are (1) releasing growth hormone (GH) and (2) stimulating appetite (Kojima and Kangawa 2005). Ghrelin also exhibits cardiovascular effects (Nagaya et al. 2001), mediates increases in gastric movement and gastric acid secretion (Masuda et al. 2000), suppresses sympathetic nerve output (Nagaya et al. 2004; Yasuda et al. 2003; Mano et al. 2009), and contributes to the regulation of glucose metabolism (Thorens and Larsen 2004; van der Lely et al. 2004).

Ghrelin is produced in gastrointestinal organs in response to hunger and starvation (Cummings et al. 2004; Toshinai et al. 2001; Tschop et al. 2001), circulates in the blood (Hosoda et al. 2000a), and serves as a peripheral signal advising the central nervous system to stimulate feeding (Nakazato et al. 2001; Tschop et al. 2000; Wren et al. 2001a, b). A hunger signal by ghrelin seems to be transmitted through the vagus nerve, since vagotomy ablates the orexigenic effect of ghrelin (Date et al. 2002a; le Roux et al. 2005).

This review surveys the processing pathway from ghrelin gene to mature ghrelin peptide and summarizes our knowledge of the regulatory mechanism of ghrelin secretion and function.

2 Genomic Structure of Ghrelin

Figure 2 shows the processing steps from the ghrelin gene to the active ghrelin peptide.

The human ghrelin gene is localized on the chromosome 3p25-26, while the human ghrelin receptor gene has also been identified on chromosome 3, at position q26-27 (Smith et al. 1997).

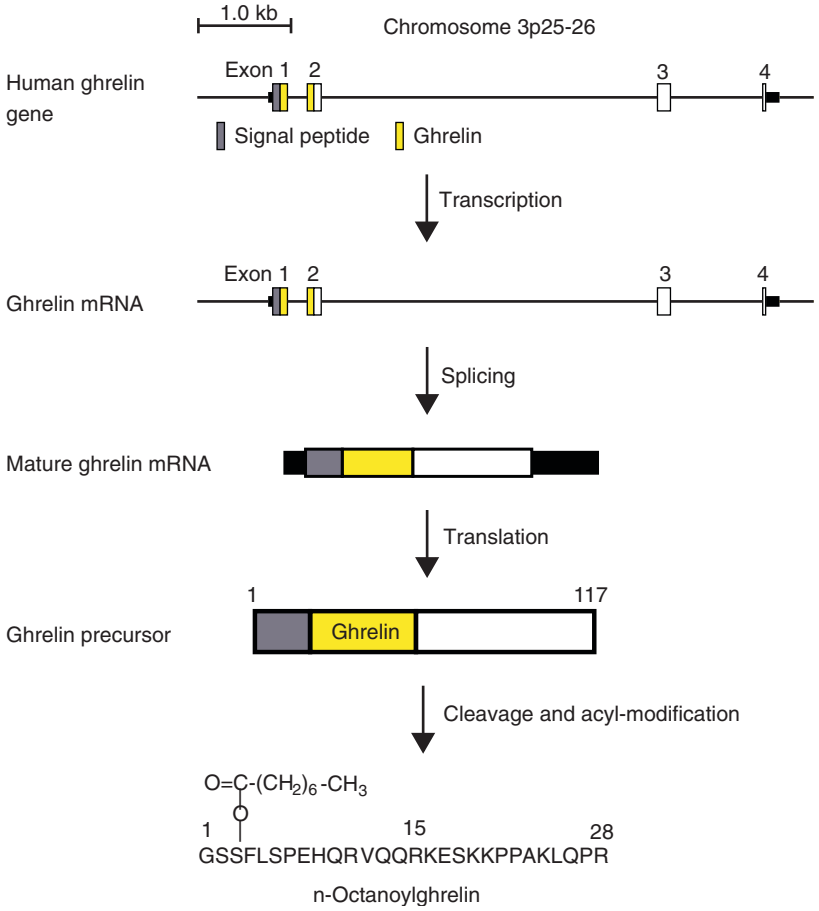

Fig. 2 From the human ghrelin gene to an active *n*-octanoyl modified ghrelin. The human ghrelin gene comprises four exons. This mRNA is translated into a 117-amino acid ghrelin precursor. Protease cleavage and acyl-modification of the ghrelin precursor result in the production of a 28-amino-acid-long active acyl-modified ghrelin peptide

The human ghrelin gene, like the mouse gene, comprises four exons (Kanamoto 2004). Another short exon that contains only 20 bp, which encode part of the 5′-untranslated region, is transcribed as a minor component of ghrelin mRNA (Tanaka et al. 2001).

The twenty-eight amino acids of the functional ghrelin peptide are encoded in exons 1 and 2. In the rat and mouse ghrelin genes, the codon for Gln14 (CAG) is used as an alternative splicing signal to generate two different ghrelin mRNAs (Hosoda et al. 2000b). One mRNA encodes the ghrelin precursor, and another encodes a des-Gln14-ghrelin precursor. Des-Gln14-ghrelin is identical to ghrelin, except for the deletion of Gln14.

3 Promoter and Expression of Ghrelin Gene

The 5′-flanking region of the human ghrelin gene contains a TATA box-like sequence (TATATAA; −585 to −579), as well as putative binding sites for several transcription factors, such as AP2, basic helix-loop-helix (bHLH), PEA-3, Myb, NF-IL6, hepatocyte nuclear factor-5, and NF-(kappa)B, and half-sites for estrogen and glucocorticoid response elements (Kanamoto et al. 2004; Tanaka et al. 2001; Kishimoto et al. 2003). However, neither mutation nor deletion of the TATA box-like element decreased the promoter activity, suggesting that this element is not used. There was neither a typical GC nor a CAAT box.

Studies of ghrelin promoter activity in TT cells, a human medullary carcinoma cell line, revealed the presence of activating sequences within −1509 to −1110 and −349 to −193 in the 5′-flanking region of the ghrelin gene (Kanamoto et al. 2004). In another report by Nakai et al. using TT cells, a significant level of promoter activity was observed in the 1225–1107 bp upstream region of the translation initiation site and a sequence-specific protein boundto the promoter region of −1129 to −1100 (Nakai et al. 2004). Furthermore, in a study by Kishimoto et al. using ECC10 cells, a human stomach-derived cell line, indicated that −2000 to −605 in the 5′-flanking region of the ghrelin gene contains an activating sequence (Kishimoto et al. 2003). These results suggest that ghrelin gene expression may be cell-type specific.

In the 5′-flanking region of the ghrelin gene, several E-box consensus sequences exist (Kanamoto et al. 2004). Destruction or site-directed mutagenesis of these sites decreased the promoter activity in TT cells, implicating them in promoter activation. Upstream stimulatory factors (USF), a member of the bHLH-LZ family of transcription factors, bind to these E-box elements and may thus regulate human ghrelin gene expression. A recent report also indicates that KLF4 (Krüppel-like factor 4) positively regulates human ghrelin gene expression by binding to a KLF-responsive element, which is situated between −1228 and −1105 (Lee et al. 2009).

It is well known that fasting increases ghrelin mRNA expression; however, transcription factor(s) that link fasting and ghrelin gene expression has/have not yet been identified.

4 Processing of Ghrelin Precursor to Des-Acyl Ghrelin

The amino-acid sequences of mammalian ghrelin precursors are well conserved (Kojima et al. 1999). In mammalian ghrelin precursors, the 28-amino-acid active ghrelin sequence immediately follows the signal peptide (Fig. 3). The cleavage site for the signal peptide is the same in all mammalian ghrelins. Although propeptides are usually processed at dibasic amino acid sites by prohormone convertases, the C-terminus of the mammalian ghrelin peptide is processed at an uncommon Pro-Arg recognition site.

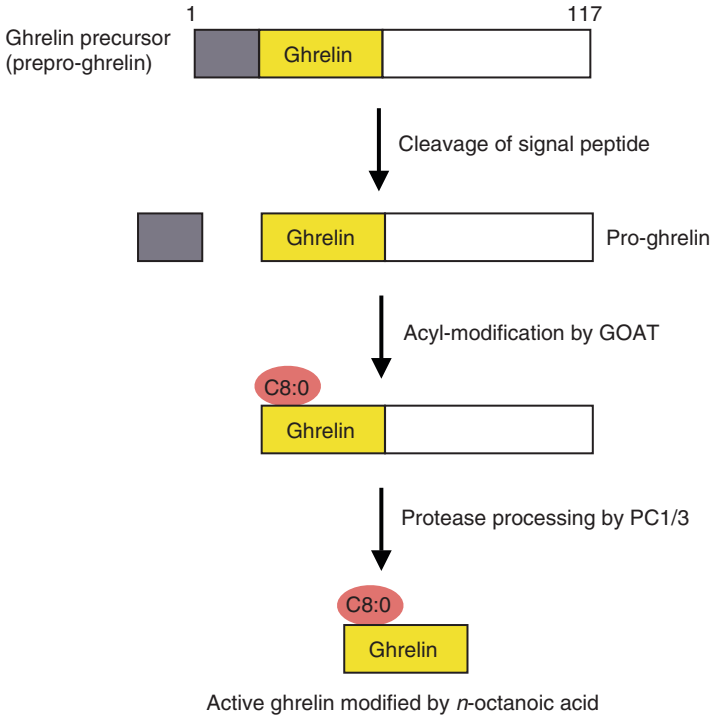

Fig. 3 Proposed processing steps of ghrelin. After removing the signal peptide, ghrelin precursor is modified with an acyl acid (mainly *n*-octanoic acid) by GOAT and cleaved by a processing protease PC1/3 to produce active ghrelin

In contrast to mammalian ghrelins, most of the C-terminal processing sites of nonmammalian ghrelin are dibasic amino acids, Arg-Arg, a typical protease processing signal (Kojima et al. 1999). Interestingly, the amino acid sequences of the C-terminal processing sites in fish ghrelins are Gly-Arg-Arg, a typical processing signal for the C-terminal amide conversion (Steiner 1998). Indeed, the C-terminal amino acids in fish ghrelins are amidated; however, the amide structure is not necessary for the activity of ghrelin (Kaiya et al. 2003, 2008). The N-terminal processing sites of nonmammalian ghrelin are the same as mammalian ghrelins, in which the cleavage site is located just after the signal peptide.

Zhu et al. have identified the processing proteases that participate in the processing of proghrelin to 28-amino acid ghrelin peptide (Zhu et al. 2006). Using knockout mice of prohormone convertases, PC1/3, PC2, and PC5/6A, they showed that proghrelin was processed in all but the PC1/3 knockout mouse. They extracted the peptide fraction from the stomachs of prohormone convertase knockout mice and examined the molecular weight of ghrelin by western blotting. They found that in the PC1/3 knockout mouse the molecular weight of ghrelin was approximately 11 kDa while the ghrelins in PC2 and PC5/6A knockout mice were both

approximately 3.4 kDa. These results suggest that proghrelin is not processed in the stomach of the PC1/3 knockout mouse. Moreover, with immunohistochemical studies, PC1/3 and ghrelin were shown to colocalize in the same cells of the stomach. Thus, PC1/3 is likely a processing protease responsible for proghrelin processing in the stomach.

In pancreas, ghrelin was found to be colocalized with glucagon in islet α cells or is contained in ε cells, a newly reported pancreatic cell type, and not colocalized with insulin in β cells (Date et al. 2002b; Heller et al. 2005; Prado et al. 2004). PC1/3 is expressed mainly in β cells for proinsulin processing. However, it is reported that some but not all ghrelin positive cells were also positive for PC1/3 or PC2 (Walia et al. 2009). These results suggest that in pancreas, proghrelin is not processed exclusively by PC1/3 like gastric proghrelin, but that PC2 also plays some part in the processing of pancreatic proghrelin.

5 Acyl-Modification of Ghrelin by Ghrelin *O*-Acyltransferase (GOAT)

Ghrelin is the first and only known case of a peptide hormone modified by a fatty acid, and this acyl-modification of ghrelin is essential for its activity (Kojima et al. 1999; Matsumoto et al. 2001). However, the enzyme that catalyzes this acyl transfer to ghrelin had not been identified until February 2008 when two independent groups reported the identification of GOAT (Yang et al. 2008a; Gutierrez et al. 2008; Gardiner and Bloom 2008).

The group led by Brown and Goldstein from The University of Texas Southwestern Medical Center searched through the genome database and picked up several orphan acyltransferases, that is the function of the enzymes had not been clarified (Yang et al. 2008a). They expressed both ghrelin and putative enzyme cDNAs in a cell culture system and examined the production of acyl-modified ghrelin. They finally identified MBOAT4 (Membrane-Bound *O*-Acyltransferase) as the enzyme that acyl-modified the ghrelin peptide.

Another group from Eli-Lilly also picked up several candidate clones of acyltransferases in the genome database and suppressed their expression by siRNA in TT cells, a thyroid medulla carcinoma cell line in which acyl-modified ghrelin has been endogenously expressed (Gutierrez et al. 2008). They also identified MBOAT4, or GOAT, as the enzyme responsible for ghrelin acyl-modification. The group created MBOAT4 knockout mice and confirmed that ghrelin was not acyl-modified in the knockout mice.

Thus, MBOAT4 is the acyltransferase that catalyzes transfer *O*-acyl acid to the hydroxyl group of the third serine in ghrelin. GOAT is a membrane-bound enzyme with multiple transmembrane domains. GOAT is a member of an acyltransferase family that comprises at least 16 acyltransferase enzymes. Among them, only GOAT shows ability to acyl-modify ghrelin. Like the existence of ghrelin in all vertebrate species, GOAT is also found in mammals, birds, and fish. The distribution

of GOAT is similar to that of ghrelin: GOAT is predominantly found in gastrointestinal organs – in particular in the stomach (Yang et al. 2008a; Gutierrez et al. 2008; Sakata et al. 2009).

6 Substrate Specificity of GOAT

GOAT specifically modifies the third amino acid serine and does not modify other serine residues in human ghrelin. When the serine residues at the second, sixth, and 18th amino acids in ghrelin were replaced by alanine, the third serine residue was still correctly modified by *n*-octanoic acid (Yang et al. 2008a). However, when the third serine was replaced by alanine, GOAT did not modify it. The third amino acid of frog ghrelin is threonine and modified by *n*-octanoic acid as with Ser3 ghrelins (Kaiya et al. 2001). When the third amino acid serine of rat ghrelin was replaced to threonine, GOAT modified the replaced threonine by *n*-octanoic acid (Yang et al. 2008a). Thus, the position of serine or threonine as the third amino acid is important for *n*-octanoyl modification of ghrelin, and GOAT modifies not only serine but also threonine residues.

Interestingly, when the first amino acid glycine or the fourth amino acid phenylalanine was replaced by alanine, the third serine was not modified by an acyl acid (Yang et al. 2008b). Replacements of Ser2, Leu5, Ser6, or Pro7 by Ala did not have an effect on the acyl modification of ghrelin. In addition, N-terminal addition of two amino acids (Ser-Ala) significantly suppressed the acyl-modification of the third serine residue of ghrelin. These results indicate that the three amino acids, Gly1, Ser3, and Phe4, are important for the acyl-modification reaction.

Substrate specificity of acyl acids that GOAT uses for acyl-modification of ghrelin is broad: from acetic acid (C2:0) to tetradecanoic acid (C16:0) (Gutierrez et al. 2008). However, the main molecular form of gastric ghrelin is modified by *n*-octanoic acid and other acyl-modified forms of ghrelin are only found at low levels (Hosoda et al. 2000b, 2003). These results indicate that the content and concentration of acyl acid in the stomach may determine the acyl-modification form of ghrelin.

In addition, the acyl acid that is used for ghrelin modification is not a free but a CoA-conjugated form. The ghrelin modification reaction does not occur with free acyl acid or a mixture of free acyl acid and CoA (Ohgusu et al. 2009).

The five amino acid peptide derived from the ghrelin N-terminal peptide (GSSFL-NH_2, GSAFL-NH_2, GSSFL-COOH) inhibits *n*-octanoyl modification of ghrelin (Yang et al. 2008b). Moreover, the five amino acid peptide with *n*-octanoyl modification, GSS(C8:0)FL-NH_2, also exerts GOAT inhibiting activity. In particular, the peptide in which the third amino acid serine was replaced to (S)-2,3-diaminopropionic acid (Dap) and modified by *n*-octanoic acid potently inhibits the acyl-modification activity of GOAT. [Dap]octanoyl-ghrelin (1-5)-NH_2 also showed potent GOAT inhibiting activity.

The optimal pH of GOAT is 7–8 (Ohgusu et al. 2009). Because the optimal pH of the processing protease PC1/3 is rather acidic and the pH value in the secretary granules is also acidic (pH 5–6), the acyl-modification of ghrelin should occur before protease processing. In other words, ghrelin seems to be modified in the forms of preproghrelin and proghrelin. In fact, proghrelin has been shown to be modified by *n*-octanoic acid (Zhu et al. 2006). Thus, acyl-modification precedes protease cleavage.

7 *n*-Octanoyl Ghrelin Is the Major Form of Gastric Ghrelin

After proghrelin processing by PC and GOAT, the main molecular form of ghrelin in the stomach is a 28-amino-acid peptide, in which serine 3 (Ser3) is *n*-octanoylated (Kojima et al. 1999; Hosoda et al. 2000a).

In rat stomach, a second type of ghrelin peptide was purified and identified as des-Gln14-ghrelin (Hosoda et al. 2000b). With the exception of the deletion of Gln14, des-Gln14-ghrelin is identical to ghrelin, retaining the *n*-octanoic acid modification. Des-Gln14-ghrelin has similar potency of activities as that of ghrelin. Thus, although two types of *n*-octanoyl-modified ghrelin peptides are produced in the rat stomach – ghrelin and des-Gln14-ghrelin – des-Gln14-ghrelin is only present in low amounts, indicating that ghrelin is the major active form.

8 Des-Acyl Ghrelin

The nonacylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in both the stomach and blood (Hosoda et al. 2000a). In blood, des-acyl ghrelin circulates in amounts far greater than acylated ghrelin. Des-acyl ghrelin does not replace radiolabeled ghrelin at the binding sites of acylated ghrelin in the hypothalamus and pituitary gland and shows no GH-releasing or other endocrine activities in rats. Moreover, des-acyl ghrelin shares a number of nonendocrine actions with active acyl-modified ghrelin, including stimulation of increased food intake, modulation of cell proliferation, and minor effects on adipogenesis (Cassoni et al. 2004; Sato et al. 2006; Muccioli et al. 2004; Granata et al. 2006, 2007). Zhang et al. created transgenic mice that overexpress des-acyl ghrelin under the control of fatty acid-binding protein-4 (FABP4) promoter and observed impairment of white adipose tissues and altered glucose tolerance and insulin sensitivity (Zhang et al. 2008). Toshinai et al. reported that ICV administration of des-acyl ghrelin stimulated food intake in GHS-R deficient mice, but not orexin-deficient mice (Toshinai et al. 2006). These results indicate that des-acyl ghrelin stimulates food intake not through the GHS-R, the active ghrelin receptor, but by activating the orexin neuron.

One question is whether there is a specific receptor for des-acyl ghrelin. Baldanzi and coworkers have suggested the existence of another ghrelin receptor in the

cardiovascular system (Baldanzi et al. 2002). They showed that ghrelin and des-acyl ghrelin both recognize common high-affinity binding sites on H9c2 cardiomyocytes, which do not express the ghrelin receptor. However, BLAST searches of the human genome using ghrelin receptor cDNA as a search sequence have not revealed any ghrelin receptor homologs. Further study is required to determine whether des-acyl ghrelin is biologically active and binds to an as-yet-unidentified receptor.

9 Multiple Forms of Ghrelin in Human Stomach

Several minor forms of human ghrelin peptides have been isolated from human stomach (Hosoda et al. 2003). These can be classified into four groups according to the type of acylation observed at Ser3: nonacylated, octanoylated (C8:0), decanoylated (C10:0), and decenoylated (C10:1). All peptides identified thus far are either 27 or 28 amino acids in length. The 27-amino-acid forms of ghrelin lack C-terminal Arg28, which may be removed by carboxypeptidase digestion. As in the rat, the major active form of human ghrelin is a 28-amino-acid peptide with an octanoylated Ser3. Synthetic octanoylated and decanoylated ghrelins stimulate increases in intracellular Ca^{2+} in GHS-R-expressing cells and stimulate GH release to a similar degree in rats.

10 Molecular Forms of Ghrelin in Jejunum, Pancreas and Hypothalamus

Gastric ghrelin was the first identified octanoyl-modified peptide. Previously, it was not known whether ghrelin is also modified by *n*-octanoic acid in tissues other than the stomach.

The molecular forms of ghrelin in the jejunum, pancreas, and hypothalamus have been investigated more recently (Date et al. 2000, 2002b; Sato et al. 2005). Peptide extracts from these tissues were analyzed and characterized by RP-HPLC and ghrelin-specific RIA. Two ghrelin forms were identified in these tissues, *n*-octanoyl ghrelin and des-acyl ghrelin, as seen in gastric tissue. These results indicate that acyl-modification is not specific to stomach tissue, but occurs in tissues in which the ghrelin gene is expressed and ghrelin peptide produced.

11 Obestatin, a Ghrelin Precursor-Derived Peptide?

In November 2005, Zhang and colleagues from Stanford University reported a novel peptide hormone “obestatin” from the Latin “obedere”, meaning to devour, and “statin”, meaning suppression, because it suppressed food intake (Zhang et al.

2005). Intriguingly, obestatin is processed from the ghrelin precursor; in other words, these two peptide hormones with opposing action on food intake, orexigenic ghrelin, and anorectic obestatin, are derived from the same hormone precursor. They proposed that no obvious phenotypes in ghrelin knockout mice were due to the lack of both ghrelin and obestatin. Moreover, obestatin is reported to be the endogenous ligand for GPR39, an orphan GPCR that shows amino-acid sequence homology in ghrelin, motilin, neurotensin, and neuromedin U receptors (McKee et al. 1997).

However, several subsequent reports raised objections to obestatin in its proposed action and the matched receptor. Several independent research groups reported that obestatin does not bind and activate GPR39 (Dong et al. 2009). In addition, the original group which first reported obestatin could not replicate its observation that obestatin bound to GPR39 and stimulated cAMP and SRE (serum-responsive element) responses. Moreover, several groups observed that injection of obestatin does not suppress food intake (Chartrel et al. 2007; Iglesias et al. 2007; Gourcerol et al. 2007; Gourcerol and Tache 2007; Yamamoto et al. 2007). The original group reported later that an exact 15 min delay – not 0 or30 min – in food replacement after obestatin injection is essential to demonstrate the food suppressive effect of obestatin (Chartrel et al. 2007).

From the comparison of the amino acid sequence, it is true that the sequences of mammalian obestatins are well conserved. However, in nonmammalian species obestatin parts are not conserved, while ghrelin parts are well conserved. Moreover, the original paper on obestatin reported that the C-terminal amide structure of obestatin is essential to bind and activate GPR39; however, the precursor parts that seem to contain nonmammalian obestatins lack the Gly residue for the amide formation. Thus, nonmammalian obestatins, if they were contained in the stomach, did not have a C-terminal amide structure. Furthermore, the general processing sites for the prohormone convertases, such as Arg-Arg or Lys-Arg, were not found in the nonmammalian obestatin parts. In addition, if both ghrelin and obestatin are processed from the same ghrelin precursor protein, the amount and secretion of both ghrelin and obestatin should be of similar level and manner. However, the plasma content of ghrelin is much higher than that of obestatin and after fasting, plasma obestatin concentration did not change while ghrelin concentration increased (Zhang et al. 2005). Thus, it is likely that obestatin is not an endogenous hormone derived from a proper processing of the ghrelin precursor, but a digested peptide fragment produced by a nonspecific protease digestion.

12 Regulation of Ghrelin Secretion

It is still not clear what factors are involved in the regulation of ghrelin secretion. The most important factor that regulates ghrelin secretion is feeding: plasma ghrelin concentration is increased when fasting and decreased after food intake (Tschop et al. 2001; Cummings et al. 2001). Blood glucose level may be

critical as oral or intravenous administration of glucose decreases plasma ghrelin concentration (McCowen et al. 2002; Shiiya et al. 2002). Plasma ghrelin concentration is sensitive, however, to the makeup of a meal—it is decreased by a high lipid meal and increased by a low protein one (Erdmann et al. 2003; Greenman et al. 2004). Moreover, mechanical distention of the stomach does not induce ghrelin release.

Plasma ghrelin concentration is low in obese people and high in lean people (Hansen et al. 2002; Haqq et al. 2003a; Cummings et al. 2002a). Related to this fact, plasma ghrelin level is highly increased in anorexia nervosa patients and returns to normal levels upon weight gain and recovery from the disease (Ariyasu et al. 2001; Cuntz et al. 2002; Otto et al. 2001; Soriano-Guillen et al. 2004).

Patients with gastric bypass lose their weight and their ghrelin levels decrease (Cummings et al. 2002a; Geloneze et al. 2003). Changes in ghrelin concentration associated with food intake are diminished in these patients, confirming that the stomach is the main site of ghrelin production. Plasma ghrelin concentration also decreases in patients with short bowel syndrome, probably due to the loss of ghrelin-producing tissues (Krsek et al. 2003).

Exogenous treatment with somatostatin and its analogs, such as octreotide, as well as infusion of urocortin-1, a potent anorexigenic peptide, suppresses plasma ghrelin concentration (Haqq et al. 2003b; Davis et al. 2004; Norrelund et al. 2002). However, administration of leptin does not modify ghrelin levels (Chan et al. 2004).

Exogenous GH decreases stomach ghrelin mRNA expression and plasma ghrelin concentration, but does not affect stomach ghrelin stores (Qi et al. 2003). These results suggest that pituitary GH exhibits feedback regulation on stomach ghrelin production.

In contrast to ghrelin-suppressing factors, any factor that stimulates ghrelin production and secretion has not been fully understood. Interesting factors that stimulate ghrelin secretion are the amino acid tryptophan and zinc oxide (ZnO) (Zhang et al. 2007; Yin et al. 2008).

Dietary tryptophan increased the expression level of ghrelin mRNA in gastric fundus and duodenum in weaning pigs (Zhang et al. 2007). Plasma ghrelin level was increased more in weaning pigs with a 0.19% and 0.26% tryptophan diet than those with a 0.12% tryptophan diet. Thus, tryptophan stimulates both ghrelin mRNA expression and ghrelin secretion.

When pigs were pair-fed the same amount of food as the control pigs, the group supplied with ZnO showed increased plasma ghrelin concentration (Yin et al. 2008). Messenger RNA level of gastric ghrelin concentration had not been changed by dietary ZnO. Moreover, addition of ZnO to primary gastric mucosal cells in vitro stimulated ghrelin release into the culture medium without any change of ghrelin mRNA expression level. Thus, ZnO is a ghrelin secretagogue, although the molecular mechanism of ZnO on ghrelin secretion is not clear.

Regular daily feeding may establish ghrelin secretion rhythm from the stomach (Sugino et al. 2002a, b). Sugino et al. designed schedules of restricted feeding in sheep: daily feeding twice for each 1 h. They found that plasma ghrelin concentration was significantly increased 1 h before the meal and sharply decreased after feeding.

When the restricted feeding was four times a day, four peaks of ghrelin concentration were observed. Moreover, in fed ad libitum condition no obvious ghrelin peak was observed. Preprandial ghrelin surges were also observed in scheduled feeding humans.

13 Abnormal Ghrelin Secretion in Prader–Willi Syndrome

PWS is a complex genetic disorder characterized by mild mental retardation, hyperphagia, short stature, muscular hypotonia, and distinctive behavioral features (Nicholls and Knepper 2001). Excessive appetite in PWS causes progressive severe obesity, which in turn leads to an increase in cardiovascular morbidity and mortality. The PWS genotype is characterized by a loss of one or more paternal genes in region q11-13 on chromosome 15. It has been suggested that this genetic alteration leads to dysfunction of several hypothalamic areas, including appetite regulatory regions. Moreover, GH deficiency is common in PWS.

High plasma ghrelin concentration is observed in Prader–Willi syndrome (PWS) patients (DelParigi et al. 2002; Cummings et al. 2002b). The mean plasma concentration of ghrelin was higher by 3-to-4-fold in PWS than in a reference population. Thus, ghrelin may be responsible, at least in part, for the hyperphagia seen in PWS. However, Feigerlova et al. report that high ghrelin concentration was observed even during the first year of life and precedes the onset of obesity (Feigerlova et al. 2008). This result indicates that genetic abnormality affects the production of ghrelin. It is however not clear, what mechanism underlies the increased ghrelin levels in these patients. Imprinting of paternal genes in region q11-13 on chromosome 15 may induce the production of excessive amounts of transcription factors that increase ghrelin expression or, alternatively, a loss of a transcription inhibitory factor that normally suppresses ghrelin expression. In this context, it is noteworthy that the deletion of a small nucleolar RNA Snord116 region, which is the comparable region of imprinted chromosome 15q11.2 in PWS, causes high circulating ghrelin concentration, a similar phenotype of PWS (Ding et al. 2008). Elucidation of the precise mechanism by which ghrelin gene expression is regulated may reveal the genetic cause of hyperphagia in PWS.

14 Pathway of Ghrelin Signal: From Peripheral Tissues to Central Nervous System

Circulating ghrelin stimulates hypothalamic neurons and stimulates food intake. In general, circulating peptides do not pass the blood-brain barrier. In fact, the rate at which circulating ghrelin passes the barrier has been shown to be very low. Thus, peripheral ghrelin should activate the appropriate hypothalamic regions via an indirect pathway.

The detection of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (Date et al. 2002a; Sakata et al. 2003; Zhang et al. 2004). Moreover, the observation that ICV administration of ghrelin induces c-Fos in the dorsomotor nucleus of the vagus and stimulates gastric-acid secretion indicates that ghrelin activates the vagus system (Date et al. 2001).

Vagotomy inhibits the ability of ghrelin to stimulate food intake and GH release (Date et al. 2002a). A similar effect was also observed when capsaicin, a specific afferent neurotoxin, was applied to vagus nerve terminals to induce sensory denervation. However, the basal level of ghrelin concentration is not affected after vagotomy. On the other hand, fasting-induced elevation of plasma ghrelin is completely abolished by subdiaphragmatic vagotomy or atropine treatment (Williams et al. 2003).

Moreover, peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus (Date et al. 2006). Bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in the hindbrain that express dopamine beta-hydroxylase (an NA synthetic enzyme), abolished ghrelin-induced feeding. Thus, the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin. These results indicate that the response of ghrelin to fasting is transmitted through vagal afferent transmission.

15 Ghrelin Stimulates Appetite in the Hypothalamus

Recent identification of appetite-regulating humoral factors has established regulatory mechanisms not only in the central nervous system, but also mechanisms mediated by factors secreted from peripheral tissues (Badman and Flier 2005). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain, while ghrelin, produced in the stomach, is an appetite-stimulating factor that transmits hunger signals to the brain. Ghrelin, thus, is functionally a natural antagonist to leptin (Fig 4).

The hypothalamic arcuate nucleus is the main site of ghrelin's activity in the central nervous system (Fig. 5). The arcuate nucleus is also a target of leptin, an appetite-suppressing hormone, and NPY and AgRP, which are both appetite-stimulating peptides (Wren and Bloom 2007; Flier 2004). NPY and AgRP are produced in the same population of neurons in the arcuate nucleus, and their appetite-stimulating effects are inhibited directly by leptin. At least part of the orexigenic effect of ghrelin is mediated by upregulating the genes encoding these potent appetite stimulants (Fig. 6).

ICV injection of ghrelin induces Fos expression in NPY-expressing neurons and increases the amount of NPY mRNA in the arcuate nucleus (Nakazato et al. 2001; Shintani et al. 2001). Moreover, ICV ghrelin injection increases the AgRP mRNA level in the hypothalamus. An antagonist of NPY receptor 1 blocks the appetite-stimulating

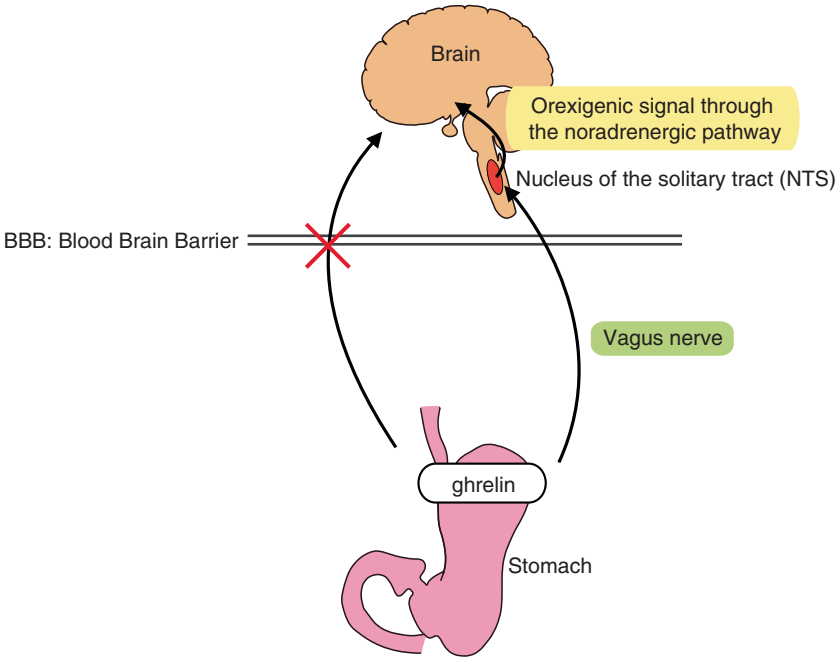

Fig. 4 Signal transduction of ghrelin from the stomach to the central nervous system. Most of the peptide hormones cannot directly pass through the blood brain barrier. Ghrelin secreted from the stomach stimulates the vagus nerve and transmits a hunger signal to the nucleus of the solitary tract in the medulla. The signal is transmitted to the hypothalamic appetite regulatory region through the noradrenergic pathway

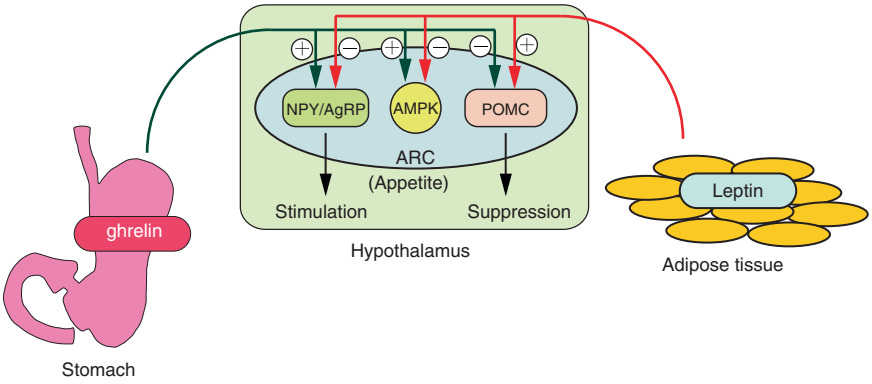

Fig. 5 Hypothalamic appetite regulation by ghrelin and leptin. The arcuate nucleus (ARC) of the hypothalamus is the main target of ghrelin and leptin. Ghrelin is a peripheral orexigenic signal secreted from the stomach, whereas leptin is a peripheral anorexigenic signal secreted from adipose tissue. The effects of ghrelin are opposite to those of leptin. In the ARC, ghrelin stimulates NPY/AgRP neuron and suppresses POMC neuron. On the other hand, leptin suppresses NPY/AgRP neuron and stimulates POMC neuron. Moreover, ghrelin increases AMPK activity in the hypothalamus, whereas leptin decreases AMPK activity

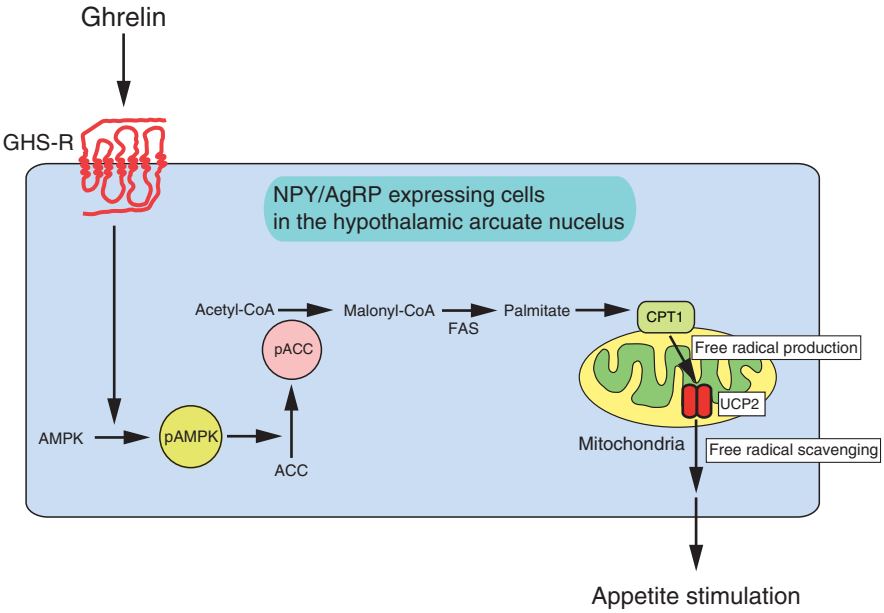

Fig. 6 Intracellular action of ghrelin in NPY/AgRP cells in the hypothalamic arcuate nucleus. In NPY/AgRP cells in the hypothalamic arcuate nucleus, ghrelin stimulates AMPK and regulates fatty acid metabolism, leading to CPT1 activation. Ghrelin's effects on mitochondrial respiration are dependent on the function of UCP2, and hence result in an increase of food intake

effects of ghrelin. ICV injections of an AgRP inhibitor, anti-NPY IgG, and anti-AgRP IgG inhibit the appetite-stimulating effects of ghrelin. Intravenous injection of ghrelin also stimulates NPY/AgRP neurons in the hypothalamus. Immunohistochemical analysis indicated that ghrelin neuron fibers directly contact NPY/AgRP neurons (Cowley et al. 2003). These results indicate that ghrelin exerts its feeding activity by stimulating NPY/AgRP neurons in the hypothalamus to promote the production and secretion of NPY and AgRP peptides. Studies with knockout mice of NPY, AgRP or both confirm these results. Although deletion of either NPY or AgRP caused modest or no effect on the orexigenic action of ghrelin, the double knockout mice lacked the action of ghrelin completely (Chen et al. 2004).

16 Molecular Mechanism of Appetite Stimulation by Ghrelin

Recently, AMP-activated protein kinase (AMPK) has been shown to be involved in hypothalamic appetite regulation (Xue and Kahn 2006; Minokoshi et al. 2004). Injection of 5-amino-4-imidazole carboxamide riboside (AICAR), an activator of AMPK, significantly increases food intake. Administration of ghrelin in vivo increases AMPK activity in the hypothalamus (Andersson et al. 2004). By contrast, injection of leptin decreases hypothalamic AMPK activity (Minokoshi et al. 2004).

Lipid metabolism in the hypothalamus is thought to be important for appetite regulation (Lopez et al. 2008). After binding of ghrelin, the ghrelin receptor activates hypothalamic AMP-K, and the activated AMP-K then suppresses lipid synthesis (Kola et al. 2005). In these results, malonyl-CoA level in the hypothalamus decreases and activity of carnitine palmitoyltransferase 1 (CPT1) is increased. Activated CPT1 then accelerates lipid transport into mitochondria to catabolize lipids.

UCP2 in the hypothalamus may be the key protein for appetite stimulation by ghrelin, because ghrelin regulates mitochondrial oxidation in the hypothalamic cells through UCP2 (Andrews et al. 2008). Ninety percent of UCP2 expressing cells in the hypothalamus also express the ghrelin receptor. By using isolated hypothalamic synaptosomes, ghrelin increased oxygen consumption and total respiration. Ghrelin administration increased the number of mitochondria and UCP2 expression in NPY/AgRP expressing cells of the hypothalamic arcuate nucleus.

It has been known that the orexigenic effect by ghrelin is exerted through NPY and NPY mRNA level in the arcuate nucleus is increased by ghrelin. However, in UCP2-deficient mice, ghrelin administration did not increase NPY level and the number of c-Fos protein positive cells in the arcuate nucleus NPY/AgRP neurons was decreased.

Moreover, in the UCP2-deficient mice, AMPK activity was not changed by ghrelin administration. AICAR stimulates mitochondrial respiration in the hypothalamus and increases appetite, whereas UCP2-deficient mice did not show any effects by AICAR. In contrast, AMPK inhibitor, compound C, suppressed appetite stimulation by ghrelin; however, in UCP2-deficient mice, appetite suppression by compound C was not observed. These results suggest that AMPK activation by ghrelin is upstream of UCP2. In other words, ghrelin stimulates UCP2 through AMPK.

By lipid consumption in the hypothalamic mitochondria, free radical production was increased. The produced free radicals are scavenged by UCP2. In UCP2-deficient mice, ghrelin increased free radical production as compared to wild type.

Taken together, these results suggest that lipid metabolism and mitochondrial free radical production in the hypothalamic neurons are key factors for the regulation of appetite.

17 Epilogue

Since its discovery in 1999, intensive research has been performed on ghrelin, including its molecular mechanism of appetite regulation and the processing pathway for ghrelin maturation, etc. Clinical application of ghrelin is now in phase II to target the diseases anorexia nervosa and cachexia due to chronic diseases. In the near future, ghrelin can hopefully be used for treatment of these diseases.

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Posttranslational Processing of Progastrin

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Abstract Gastrin and cholecystokinin (CCK) are homologous hormones with important functions in the brain and the gut. Gastrin is the main regulator of gastric acid secretion and gastric mucosal growth, whereas cholecystokinin regulates gall bladder emptying, pancreatic enzyme secretion and besides acts as a major neurotransmitter in the central and peripheral nervous systems. The tissue-specific expression of the hormones is regulated at the transcriptional level, but the posttranslational phase is also decisive and is highly complex in order to ensure accurate maturation of the prohormones in a cell specific manner. Despite the structural similarities of gastrin and CCK, there are decisive differences in the posttranslational processing and secretion schemes, suggesting that specific features in the processing may have evolved to serve specific purposes. For instance, CCK peptides circulate in low picomolar concentrations, whereas the cellular expression of gastrin is expressed at higher levels, and accordingly gastrin circulates in 10–20-fold higher concentrations. Both common cancers and the less frequent neuroendocrine tumors express the gastrin gene and prohormone. But the posttranslational processing progastrin is often greatly disturbed in neoplastic cells.

The posttranslational phase of the biogenesis of gastrin and the various progastrin products in gastrin gene-expressing tissues is now reviewed here. In addition, the individual contributions of the processing enzymes are discussed, as are structural features of progastrin that are involved in the precursor activation process. Thus, the review describes how the processing depends on the cell-specific expression of the processing enzymes and kinetics in the secretory pathway.

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1 Introduction

Gastrin and cholecystokinin (CCK) were originally discovered as hormones secreted from the antral part of the stomach (G-cells) or the small intestine (I-cells), regulating gastric acid secretion and gall bladder emptying, respectively (Edkins 1905; Ivy and Oldberg 1928). Decades later when the amino acid sequences of the hormones were identified, it was discovered that they are structurally homologous and hence constitute a hormone family with an identical bioactive site. Later, the identification of a likely common ancestor established the evolutionary relationship of the peptides (Johnsen and Rehfeld 1990). The structural relationship is also reflected in their sharing of a receptor. Thus, while the CCK-A receptor binds only tyrosine-sulfated CCK peptides, the CCK-B receptor binds both gastrin and CCK peptides. Consequently, the concentrations of circulating peptides are decisive for the physiological response and normally gastrin circulates in 10–20-fold higher concentrations than CCK (Rehfeld 1998a, b).

As for most peptides hormones, cloning and sequencing of the gastrin and CCK cDNAs revealed the structures of larger prohormones that need posttranslational processing for activation. Since then, generation of specific assays of prohormone derivatives has made it possible to study cellular expression in detail. This has led to a far more complex picture of expression and function. Both hormones are expressed in multitude of tissues that typically display differences in their posttranslational processing. For instance, it is now clear that in addition to the cellular synthesis in the gut, a highly processed form, CCK-8 is the most abundant neuropeptide in the brain. Similarly, the gastrin gene is to a limited extent also expressed in certain neurons, the pituitary, the fetal pancreas and in the spermatozoes, all displaying differences in posttranslational processing efficiency. In addition, the gastrin gene is expressed in certain cancers, where growth promoting actions of less processed progastrin fragments have been proposed (Dockray et al. 2005; Ferrand and Wang 2006; Rehfeld and van Solinge 1994; Rengifo-Cam and Singh 2004; Seva et al. 1994; Watson et al. 2006). Although so far there has not been structural evidence of receptors that bind precursorforms of gastrin, the discussion demonstrates the importance of understanding the mechanisms involved in cellular activation of prohormones and their possible regulation. The aim of this chapter is to describe the processing events in the biosynthesis of gastrin, highlighting structural determinants in progastrin. For details of the physiological functions of the gastrin family of peptides, we refer to recent reviews on this subject (Dockray 1999; Friis-Hansen 2007; Rehfeld 1998c).

Because the posttranslational processing of peptide hormone precursors is often extensive and complicated, the literature often uses confusing or even misleading nomenclature for the resulting peptides. In the discussion of progastrin processing, we will use the nomenclature recently proposed (Rehfeld et al. 2004).

2 The Structure of Progastrin

The unifying sequence of gastrin/CCK family members is a carboxyamidated pentapeptide at the C-terminus of the bioactive peptides (Fig. 1). The four last residues are sufficient for receptor binding, but among the mammalian peptides, the position of the tyrosyl residue and its O-sulfation determines receptor binding (Dufresne et al. 2006). Both peptides are initially synthesised as propeptides that display a similar overall organisation. Hence, the prepropeptide consists of a signal peptide, an N-terminal flanking 'spacer' peptide (NTFP), a bioactive domain, and a C-terminal flanking peptide (CTFP) (Fig. 2a). Although the primary sequences of the prohormones are well conserved among mammalian species (Johnsen 1998), only the C-terminal part that includes the receptor binding sites displays significant homology between the hormones (Fig. 2b).

Both progastrin and proCCK undergo extensive posttranslational maturation processes prior to release from secretory granules in the regulated secretory pathway. These include endo- and exoproteolysis as well as side-chain modifications. As expected, posttranslational processing in the homologous region display many unifying features, whereas particular processing of the NTFP varies. Because our understanding of progastrin activation is much better than that of proCCK, we focus on the specific characteristics of progastrin processing. First, however, we describe the common processing features of progastrin and proCCK.

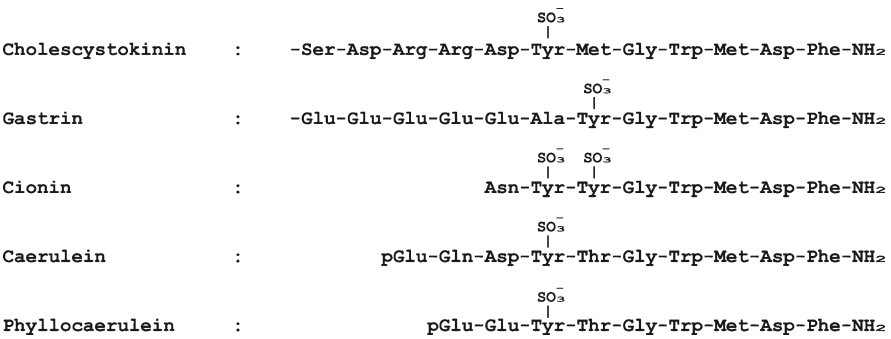

Fig. 1 Structures of the bioactive forms of the members of the gastrin/CCK peptide family. Gastrin and CCK are the only mammalian members of the family, whereas cionin is an assumed phylogenic ancestor. Caerulein and phyllocaerulein are peptides isolated from frog skin. The C-terminus is highly conserved, whereas the position of the sulfated tyrosine(s) defines the gastrins and the CCKs. All peptides are C-terminally amidated

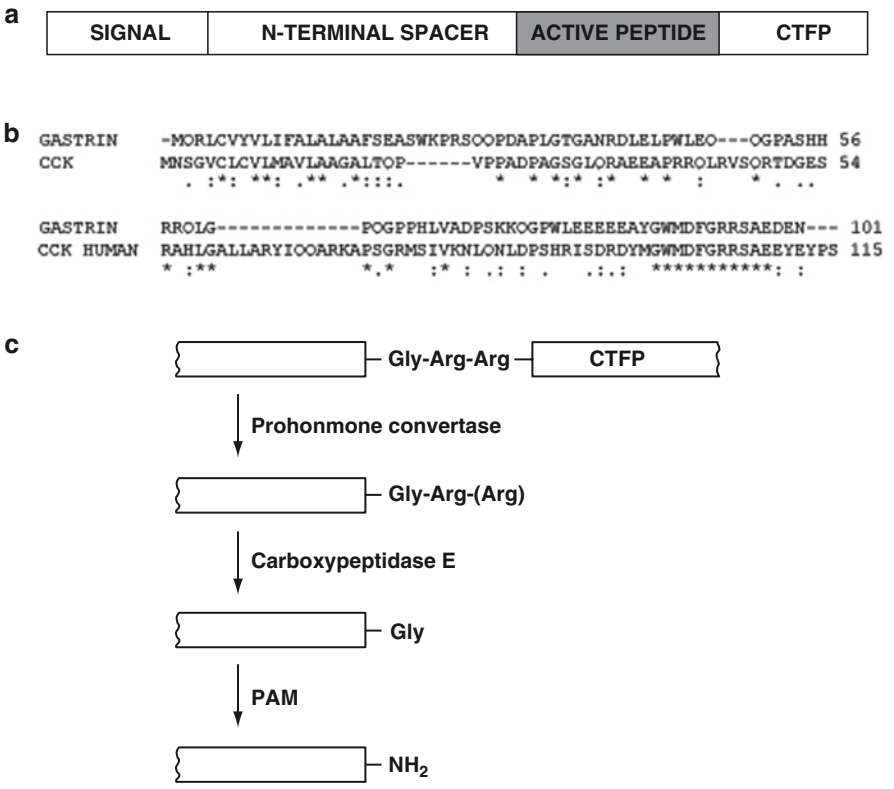

Fig. 2 Panel **a** shows the common organization of progastrin and proCCK. The active peptides are flanked both N- and C-terminally by fragments that are cleaved by prohormone convertases prior to activation. Panel **b** shows an alignment of human progastrin and proCCK. The homology is limited to the C-terminus including the active site and the CTFP. Panel **c** shows the common activation reactions of progastrin and proCCK in the C-terminal end of the prohormones. PAM denotes the peptidyl-glycine alfa-amidating monooxygenase

3 Intracellular Transport and Posttranslational Processing Events

Posttranslational processing begins in the endoplasmic reticulum/Golgi, where partial serine phosphorylation in the CTFP is mediated by an as yet unidentified casein kinase (Dockray et al. 1987; Varro et al. 1988). The significance of this modification is unclear but has been proposed to be regulatory of the endoproteolytic removal of the CTFP (Varro et al. 1988). However, mutation studies that substitute the serine with an alanine showed no changes in the production of gastrin under steady-state conditions, suggesting that serine phosphorylation is not a prerequisite for C-terminal processing of progastrin (unpublished results). Similar results were found with substitutions in the CTFP of proCCK (Vishnuvardhan and

Beinfeld 2000). Before reaching the secretory granules, progastrin and proCCK are partly sulfated on a single or double tyrosyl residues in the *trans* Golgi network by the tyrosylprotein sulfotransferases (TPST) (Moore 2003). The stoichiometri of tyrosine sulfation varies not only between the hormones, but also in a species, tissue and cell specific manner. Thus, proCCK derived peptides are usually reported as highly sulfated peptides (Mutt 1980), whereas the sulfation stoichiometri of progastrin derived peptides vary between unsulfated to almost completely sulfated, depending on the cells of expression (Andersen 1984, 1985; Andersen et al. 1985; Brand et al. 1984a; Gregory et al. 1966; Rehfeld and Larsson 1981). The reason for these differences could be structural variations around the sulfation site, where proCCK have an acidic residue N-terminally of the tyrosyl (Fig. 1), which is thought to facilitate sulfation over the alanyl residue found in the same position in progastrin (Huttner 1988; Niehrs et al. 1994). Indeed, when the alanyl residue is substituted for an aspartyl and expressed in a heterologous system that produces partially sulfated wild type gastrin (Bundgaard 2002), the progastrin mutant becomes completely sulfated (Bundgaard et al. 1995). Hence, the variability in tyrosine sulfation of progastrin may be a result of differences in TPST concentration, type or capacity or caused by changes in secretion kinetics in different situations.

In contrast to seryl phosphorylation, tyrosyl sulfation plays a pivotal role in peptide activity. In the case of CCK, tyrosine sulfation is a prerequisite for binding the CCK-A receptor (Huang et al. 1989; Mutt 1980), but less important for binding to the CCK-B or gastrin receptor. Gastrin binds exclusively the CCK-B receptor, and the tyrosine sulfation stoichiometri of gastrin has only negligible effects on receptor binding [(Huang et al. 1989) and own unpublished results].

Following tyrosine sulfation and exit from the *trans* Golgi network, the propeptides enter immature granules of the regulated secretory pathway - a secretory pathway that is specific to endocrine and neuroendocrine cells (Kelly 1985). These organelles maintain the important role of completing peptide activation while simultaneously undergoing maturation in order to release its cargo only at requested time. Improper sorting will lead to unregulated secretion of immature peptide products through the constitutive secretory pathway, and it follows that the sorting of peptides to the regulated pathway is of utmost importance for the posttranslational activation of precursors. Selection of cargo to the immature secretory granules is thought to rely on aggregation of propeptides or by specific interactions of prohormone domains acting as 'sorting signals' with granule membrane anchored molecules functioning as 'sorting receptors' [reviewed in (Arvan and Castle 1998; Dannies 2001; Dikeakos and Reudelhuber 2007)]. Although the identification of both sorting signals and sorting receptors has been reported for several peptides secreted by the regulated secretory pathway, the precise mechanism is still unclear.

Within the maturing secretory granules, both progastrin and proCCK undergo extensive endo- and exo-proteolysis, of which the endoproteolytic cleavage and C-terminal trimming between the bioactive domain and the CTFPs particularly display common features (Fig. 2c). The CTFP is first separated from the bioactive domain by endoproteolytic cleavage at the C-terminal side of double arginines by specific prohormone convertases [for reviews, see (Müller and Lindberg 1999;

Zhou et al. 1999)]. The endoproteases involved in these reactions belong to a family of subtilisin-like serine proteases of which PC1/3, PC2, and PC5/6 are all present in many endocrine and neuroendocrine tissues and involved in endoproteolysis of a number of peptide hormones, including progastrin and proCCK. Post-cleavage residual basic residues are next removed by the carboxypeptidase E (Fricker 1988), leading to what is frequently, but somewhat misleadingly, referred to as glycine-extended peptides, here denoted progastrin-gly and proCCK-gly. These peptides are targets of the peptidyl-glycine alfa-amidating monooxygenase (PAM) that utilises the glycyl residue as an amide donor to alfa-amidate the carboxyl group of the C-terminus of the peptide (Prigge et al. 2000), a prerequisite for CCK-A and CCK-B receptor binding of both gastrin and CCK (Morley et al. 1965).

In the following sections the specific characteristics of progastrin processing will be described.

4 Processing Characteristics of Progastrin

As mentioned above, the human gastrin gene is expressed in a variety of tissues under both normal and pathological conditions. However, the processing of progastrin differs markedly within the sites of expression. Figure 3 shows the fragments derived from human progastrin, that have been isolated and identified in various tissues. In the main site of expression – the G-cells of the antrum and proximal duodenum – about 95% of progastrin is processed to partially sulfated, bioactive forms, mainly gastrin-17 (85%) and gastrin-34 (10%) (Brand et al. 1984b; Hilsted and Rehfeld 1987a; Jensen et al. 1989). Although gastrin-17 is the predominant product of these cells, gastrin-34 is the major component in circulation due to major differences in metabolic clearance (Walsh et al. 1976). Another site of eutopic expression in fetal life is the pancreas, which mainly produces gastrin-17 in completely sulfated form (Brand et al. 1984a, b; Larsson et al. 1976). Other sites of expression include the ileum (Friis-Hansen and Rehfeld 1994), the colon (Lüttichau et al. 1993), certain neurons (Rehfeld 1991; Uvnäs-Wallensten et al. 1977), the pituitary (Larsson and Rehfeld 1981; Rehfeld 1978; Rehfeld and Larsson 1981), spermatozoes (Schalling et al. 1990) and a variety of cancers (Rehfeld and van Solinge 1994). However, in most cases, progastrin remains incompletely processed at the bioactive side with the notable exception of pancreatic adenocarcinomas, where the CCK-B receptor and its ligand, carboxyamidated gastrin, are co-expressed (Goetze et al. 2000).

The wide distribution of expression and the differences in processing has spurred studies of the posttranslational phase of gastrin expression to investigate potential value as a diagnostic tool and involvement of gastrin and progastrin derived products in cancer progression (Rehfeld and Bardram 1991). To examine the posttranslational phase of progastrin maturation, several laboratories have developed specific radioimmunoassays against structures of progastrin and its processing products to analyse the processing in the cells of expression. Studies have shown, that besides

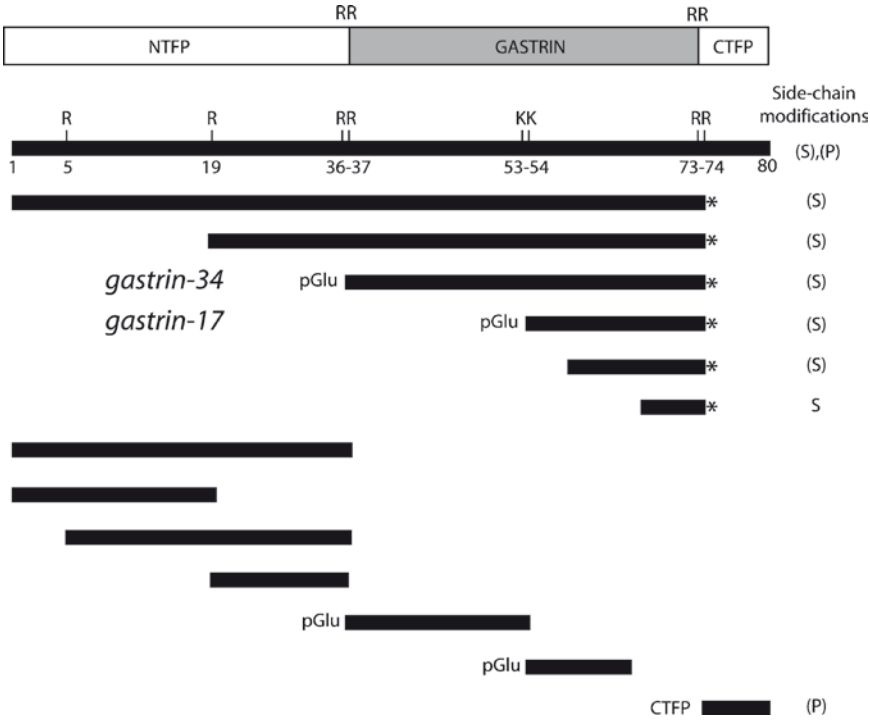

Fig. 3 Overview of all reported processing fragments of human progastrin and their side-chain modifications. (P) and (S) denote serine phosphate and tyrosine sulfate, respectively; the asterisk refers to either progastrin-gly or the carboxyamidated gastrins. The top half shows all fragments containing the bioactive sequence, the lower fragments lacking known biological activity. PGlu denotes pyroglutamyl residues

gastrin-17 and gastrin-34, human G-cells produce low amounts of gastrin-71 (Rehfeld and Johnsen 1994), gastrin-52 (Rehfeld et al. 1991), gastrin-14 (Gregory et al. 1979; Rehfeld and Stadil 1973) and gastrin-6 (Gregory et al. 1983; Rehfeld et al. 1995). Based on structural analyses, the two latter fragments are not products of direct processing by members of the prohormone convertase family, but presumably generated by aminopeptidase trimming of gastrin-17 in the case of gastrin-14, or specific endoproteolysis by an as yet unidentified post-polyglutamyl directed protease followed by trimming in the case of gastrin-6 (Rehfeld et al. 1995).

Although most progastrin is processed to the bioactive gastrin; antral G-cells do produce, store, and secrete the immediate precursor, progastrin-gly. Thus, in the human antrum, the progastrin-gly concentration is about 1% of that of gastrin (Jensen et al. 1989), but in other species higher concentrations have been reported (Hilsted and Rehfeld 1987b; Sugano et al. 1985). Interestingly, the progastrin-gly forms tend to be less processed by the prohormone convertases. Thus, in the human antrum, progastrin-71-gly and progastrin-34-gly constitute by far most of the progastrin-gly forms (Hilsted and Rehfeld 1987b).

To analyse the occurrence of fragments originating from the NTFP, assays directed against the N-terminus of progastrin and sequences at the C-terminal side of R_{19} have been established. Using such assays to monitor the purification, progastrin(1-80), progastrin(1-35), progastrin(1-19), progastrin(6-35), progastrin(20-35), progastrin(20-36) and gastrin-71 (Rehfeld and Johnsen 1994) have been identified. This demonstrates that the NTFP undergoes extensive processing just as the C-terminal part of progastrin does. Analysis of the occurrence of N-terminal fragments in the antral G-cells using the N-terminal specific assay showed that the most abundant peptides, including the N-terminal sequence of progastrin, were progastrin(1-35) and progastrin(1-19) (Goetze et al. 2006). Moreover, the G-cells only contain half the concentrations of NTFP associated peptides compared to C-terminally processed peptides whereas NTFP associated peptides circulate in higher concentrations than gastrins. Because the difference could not be ascribed to differences in clearance from the bloodstream, the results suggest that NTFP-derived peptides are secreted from the G-cells at a higher rate than the amidated gastrins, although food intake does stimulate the secretion of NTFP-derived peptides as it does gastrins (Goetze et al. 2006). The biological role of the NTFP is unclear, but was thought to be important in the biosynthesis of gastrin, possibly for the intracellular transport to the secretory granules. To investigate this, a series of N-terminal truncation mutants were expressed heterologously in a beta-cell line. Analysis showed, that production of bioactive gastrin was unaltered even when the 30 most N-terminal residues of the NTFP was truncated, suggesting that the NTFP is not necessary for correct intracellular transport (Bundgaard et al. 2004). Thus, the function of the NTFP is at present unclear.

Following the identification and cloning of the prohormone convertases and other processing enzymes, mouse models lacking the specific activity have been generated to study the functions on peptide hormone biosynthesis. Using the specific assays, the individual contributions of processing enzymes to progastin processing have been examined. Thus, analysis of antral gastrin in the *fat/fat* mouse, which has severely reduced carboxypeptidase E activity (Naggert et al. 1995), showed that although the antral G-cells contained almost normal amounts of bioactive gastrin, large amounts of progastrin-gly peptides accumulated in the cells as a result of diminished processing efficiency (Lacourse et al. 1997). Knockout mice of PAM are embryonic lethal (Czyzyk et al. 2005), but analysis of antrum from heterozygous mice showed unaltered amounts of amidated gastrins compared to wild type mice (Pintar and Bundgaard, unpublished results). This suggests that C-terminal amidation by PAM is not the rate-limiting step in normal progastrin processing. Similarly, gastrin biosynthesis was studied in mouse strains lacking prohormone convertase 2 (PC2) and 7B2. 7B2 is a molecular chaperone for PC2 necessary for the activation of the enzyme in the regulated secretory pathway (Müller and Lindberg 1999), so both mouse strains are models lacking PC2 activity. Lack of PC2 activity resulted in a reduction of proteolytical processing of progastrin at $K_{53}K_{54}$ to about half of normal in the PC2, and less than one third in the 7B2 knockout (Rehfeld et al. 2002). The cellular concentrations of progastrin processing intermediates and amidated gastrins were unaltered, suggesting that PC2 is not

involved in cleavage around the C-terminal, bioactive site. Recently, a PC1/3 deficient mouse was also developed (Zhu et al. 2002), and analysis of antral processing of progastrin in this model showed a completely different pattern (Rehfeld et al. 2008). Here, the concentrations of progastrins were three times elevated and the production of amidated gastrin reduced to 25% of normal. Taken together, knockout mouse studies have shown that PC1/3 and PC2 are the major contributors to progastrin cleavage, but that other proteases contribute to a smaller extent.

Another method of investigating progastrin processing has utilized heterologous expression systems. These were first intended to examine the tissue-specific processing, and cell lines originating from pituitary cells and pancreatic beta-cell lines (Bundgaard et al. 1996) were used. Expression in cell lines generally results in incomplete processing, much as observed during pathological hypersecretion, and did not lead to major advances in the understanding of biosynthesis. However, expression of mutated progastrins enabled examination of specific questions related to the posttranslational processing. For instance, labelling experiments had suggested that serine phosphorylation of the CTFP had regulatory functions in the processing pathway, but expression of progastrin having the serine exchanged with an alanine showed unaltered processing. The unusual tyrosine sulfation stoichiometri of gastrin has also been utilized to examine the general requirements for tyrosine sulfation (Bundgaard et al. 1997) - studies that linked disclosed tyrosine sulfation directly to the processing of gastrin-34 to gastrin-17 by PC2 (Bundgaard et al. 1995).

A domain-like structure of progastrin has been elucidated by other mutagenesis experiments. In a search for structures of progastrin that are involved in the sorting process of progastrin to the regulated secretory pathway, truncations and point mutations were analysed for changes in secretion pathway. Two sorting signals were found consisting of either the dibasic residues that constitute the prohormone convertase cleavage sites, or a stretch of negatively charged residues, that work in synergy to direct progastrin to granules that are responsive to secretagogue (Bundgaard et al. 2004). Two models of prohormone sorting to the regulated secretory granules have been proposed. One proposes that sorting occurs in the selection of cargo in the trans Golgi network to the immature secretory granules (sorting for entry), whereas another model suggests sorting occurs within the immature secretory during maturation by retention of selected cargo (Arvan and Castle 1998). The synergetic effect of the two sorting domains in progastrin strongly suggests that sorting of progastrin occurs by sequential action of both mechanisms. This was supported by detailed analysis of variations in posttranslational processing and secretion of progastrin and mutants carrying substitutions in the acidic sorting signal (Bundgaard and Rehfeld 2008). It was shown, that amidated gastrins and progastrin-gly peptides differed in secretion suggesting that progastrin-gly peptides were secreted in a more constitutive-like manner than the amidated gastrins. In other words, processing to the bioactive gastrins can be affected by intracellular transport and is not merely dependent on the amounts of processing enzyme present. The exact mechanism of sorting is not understood, but presumably involves both direct interactions with other proteins or membrane and peptide aggregation within the secretory granules.

5 A Model for the Biosynthesis of Progastrin

Based on the studies on biosynthesis, a model for the posttranslational activation of progastrin can be proposed (Fig. 4). During transport from the ER to the TGN, side-chain modifications like seryl phosphorylation and tyrosine sulfation occurs, and endoproteolysis by PC1/3 at $R_{36}R_{37}$ is initiated. N-terminally derived peptides liberated hereby escape the sorting process to the immature secretory granules and are secreted via constitutive secretory vesicles (CSV). Progastrin entering the immature secretory granules next undergo further endoproteolytic cleavages at $R_{36}R_{37}$ and $R_{73}R_{74}$ by PC1/3 and at $K_{53}K_{54}$ by PC2, which is activated in this compartment (Müller and Lindberg 1999). During condensation of the immature granules, progastrins are

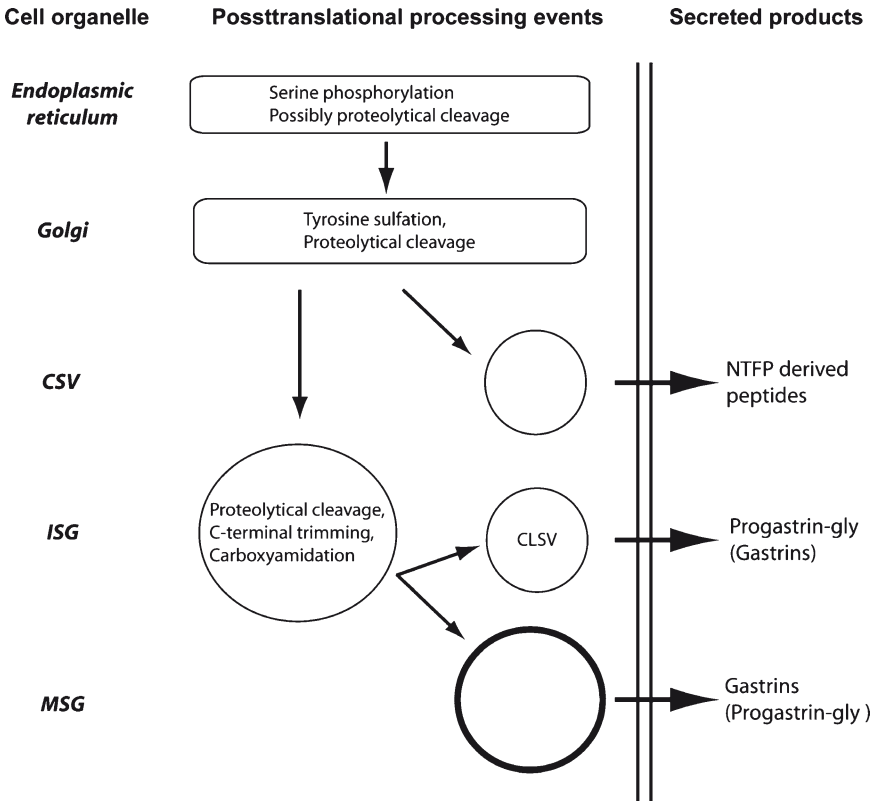

Fig. 4 A model of the relationship between progastrin processing, intracellular transport and secretion of processing products. Secretion is mediated by three types of secretory vesicles, the constitutive secretory vesicles (CSV), the constitutive-like secretory vesicles (CLSV), and the mature secretory vesicles (MSG). The latter vesicles are shown in a fat circle to indicate that this granule is responsive to external stimuli. As a result of differences in contact with the posttranslational processing machinery, different mixtures of progastrin derivatives are secreted from the different classes of vesicles

C-terminally trimmed and carboxyamidated. But during condensation, progastrin derivatives and some amidated gastrin undergo secretion via constitutive-like secretory granules. Thus, when granules are mature, they mainly contain gastrins that can be secreted in response to external stimulation.

The model suggests, that the incomplete processing of progastrin observed in situations with hypersecretion may not only be a result of saturation of the processing enzymes, but could be a result of defects in formation of the mature granules.

6 Conclusions

Although gastrin and CCK are evolutionary related, only the sequences around bioactive sites are evolutionary conserved. Accordingly, the biosynthetic processes vary in agreement with the functional requirements. Thus, proCCK processing differs in the brain and the gut as a result of different requirements for a classical peptide hormone and as a neurotransmitter. The differences in processing appear to be governed by the expression of prohormone convertases. In the case of gastrin, a single site produces the majority of gastrin, but a large variety of progastrin derived peptides are also produced, particularly under pathological conditions. These variations might also be caused by differences in prohormone convertase expression, but evidence suggests that secretion kinetics may affect progastrin processing.

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VIP and PACAP

Jan Fahrenkrug

Abstract Vasoactive intestinal polypeptide (VIP) is derived from a 170 amino acid precursor which in addition is processed to preproVIP 22-79, PHI, preproVIP 111-122 and preproVIP 156-170. All preproVIP-derived peptides have been shown in normal tissue and VIP-producing cell lines and elevated quantities occur in plasma and tumour tissues from patients with VIP-producing tumours. In some tissues the dibasic cleavage site after PHI is uncleaved resulting in a C-terminally extended form, PHV. PHI and VIP are present in a 1:1 molar ratio in large dense core vesicles and released in roughly equimolar amounts. Carboxyamidation of VIP and PHI is not critical and glycine-extended forms of both peptides have been demonstrated. Pituitary adenylate cyclase activating polypeptide (PACAP) is derived from a 170 amino acid long precursor, which gives rise to PACAP 38, PACAP 27 and PACAP related peptide (PRP). All peptides are present in tissue, the dominating form being PACAP 38. Prohormone convertase (PC) 1 and 2 seem to be involved in the processing of PACAP, except in the testes and ovary, where the PACAP precursor is substrate for PC4.

1 Introduction

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are structurally related peptides, which are both derived from larger precursor molecules (preproVIP and preproPACAP, respectively). By proteolytic processing, a number of preproVIP- and preproPACAP-derived peptides are generated, some of which are biologically active. In this chapter the present knowledge on the expression of preproVIP-derived peptides and preproPACAP-derived peptides, their secretion and their biosynthesis, including the role of prohormone convertases (PCs), is briefly reviewed.

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2 Discovery of VIP

VIP is one of the earliest discovered neuropeptides, originally isolated from porcine ileum (Said and Mutt 1970a). The peptide has derived its name from the profound and long lasting vasodilatory action seen upon systemic administration (Said and Mutt 1970b). By subsequent amino acid sequence analysis, porcine VIP was found to be a highly basic single chain linear polypeptide, which contains 28 amino acid residues in its sequence and has a C-terminal asparagine amide (Mutt and Said 1974). VIP of mammalian origin has a remarkable constant structure, since the sequences of cow, man, sheep, goat, dog, rabbit and rat are shown to be identical to that of the porcine peptide. Guinea pig VIP, however, differs in four amino acid residues from VIP in the other mammalian species. VIP was originally considered to be a gut hormone, but the peptide is now a well-established neurotransmitter in both the central nervous system and in peripheral autonomic nerves involved in the control of smooth muscle tone motility, blood flow and secretion in the digestive tract, respiratory tract and urogenital tract (Fahrenkrug 1993).

3 Structure of the VIP Precursor

VIP is coded by a single copy gene, consisting of seven introns and six exons, five of which have coding sequences; the human VIP gene is located on chromosome 6q24 (Tsukada et al. 1985; Linder et al. 1987) and the rat gene on chromosome 1p11 (Lamperti et al. 1991). The mRNA coding for rat and for human VIP is translated into a 170 amino acid precursor peptide, preproVIP (Fig. 1a) (Itoh et al. 1983; Nishizawa et al. 1985). The precursor contains, besides VIP, another biologically active peptide, PHI, in its sequence. PHI, which stands for peptide with N-terminal histidine and C-terminal isoleucine amide, was isolated in 1980 by means of a chemical method to detect C-terminal amides (Tatemoto and Mutt 1981).

The human counterpart has a C-terminal methionine and is designated PHM. PHI is structurally related to VIP and shares many of its biological actions, although in most systems less potent than VIP. Processing of the VIP precursor can follow an alternative pathway in which the dibasic cleavage site after PHI is uncleaved, resulting in a C-terminally extended form, PHV, which is found to be just as potent as VIP in relaxing smooth muscle activity (Palle et al. 1992; Yiangou et al. 1987). In the precursor, VIP as well as PHI are flanked C-terminally by the amino acids Gly–Lys–Arg, which constitute a putative proteolysis and amidation site. N-terminally, the PHI sequence is only flanked by Arg, another potential cleavage site, while the VIP sequence N-terminally is flanked by a typical dibasic cleavage sequence Lys–Arg. Processing of the VIP precursor could thus result in a number of cleavage products: preproVIP 22-79 (N-terminal flanking peptide), PHI, preproVIP 111-122 (bridging peptide or intervening sequence), PHV (PHI plus preproVIP 111-122), VIP itself and preproVIP 156-170 (C-terminal flanking peptide).

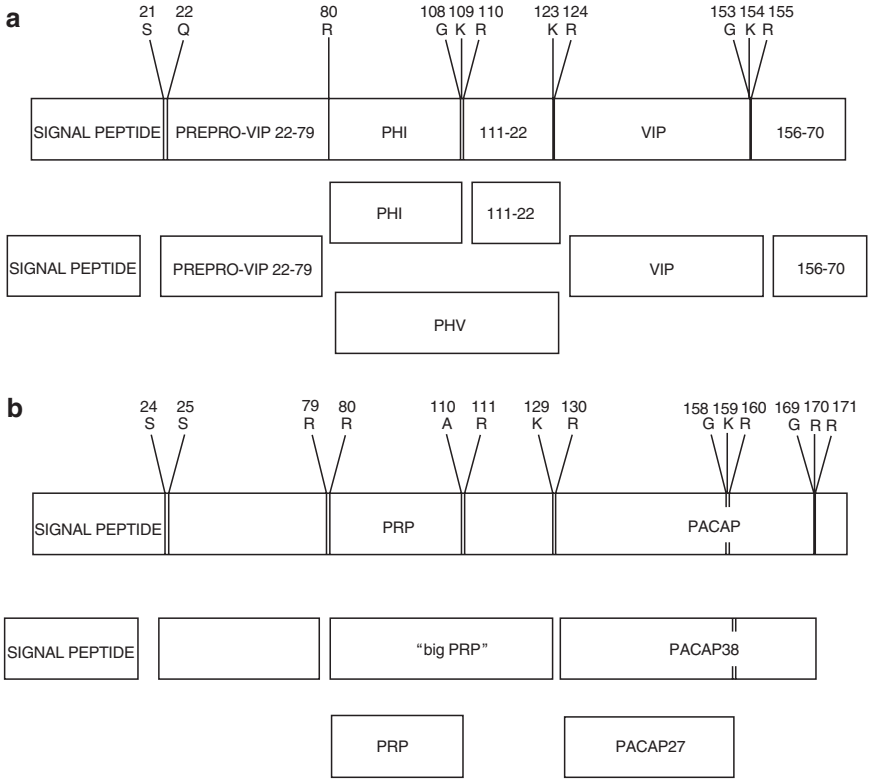

Fig. 1 Schematic representation of the structure of rat preproVIP (**a**) and rat preproPACAP (**b**) and their processing products. Amino acid residues at posttranslational processing sites are shown (*A* alanine; *G* glycine; *K* lysine; *Q* glutamine; *R* arginine; *S* serine). Abbreviations: *PACAP* pituitary adenylate cyclase activating polypeptide; *PHI* peptide with N-terminal histidine and C-terminal isoleucine amide; *PHV* peptide with N-terminal histidine and C-terminal valine; *PRP* PACAP related peptide; *VIP* vasoactive intestinal polypeptide

4 Co-Existence and Co-Secretion of VIP and PHI

The expression of the two structurally related peptides, VIP and PHI, derived from the same precursor, has been examined by radioimmunochemical measurements with region specific antibodies. Radioimmunological analysis of tissue extracts revealed a widespread and parallel distribution of the two peptides (Buhl et al. 1996; Christofides et al. 1984; Fahrenkrug et al. 1985; Lundberg et al. 1984a; Mikkelsen and Fahrenkrug 1994; Palle et al. 1989; Yiangou et al. 1985). The levels of VIP and PHI were approximately tenfold higher in the digestive tract than in the urogenital tract and endocrine system. In the digestive tract the concentration of the two peptides increased anally being maximal in the colon. In most tissues the VIP levels were higher than the corresponding PHI concentrations, and in general the

ratios between the two peptides varied considerably which could be explained by differences in posttranslational processing or by different rates of metabolism of the two peptides in various tissues. By immunohistochemistry the two peptides were found to be co-localised in neuronal cell bodies and nerve fibres in the central nervous system, the gastrointestinal tract, the pancreas, the reproductive and respiratory systems and autonomic ganglia (Buhl et al. 1996; Fahrenkrug et al. 1985; Holst et al. 1987; Lundberg et al. 1984a; Mikkelsen and Fahrenkrug 1994; Palle et al. 1989). The concentrations of VIP and PHI in tissue extracts reflect the sum of the peptides present in cell bodies, nerve fibres and terminals. VIP has been shown to be present in large dense core vesicles of nerve terminals (Agoston et al. 1985) and to answer the question whether PHI is co-packed with VIP in these storage granules Agoston et al. (1989) performed subcellular fractionation combined with chromatography and radioimmunoassay. PHI and VIP were found to be present in a close 1:1 molar ratio in the storage vesicles, which indicates that they were initially charged with the VIP precursor and that posttranslational proteolytic modification occurs within the vesicle (Agoston et al. 1989). In accordance, during activation of the VIP-containing nerves in the gastrointestinal tract and exocrine glands, VIP and PHI are co-released with an almost identical secretion profile and in roughly equimolar amounts (Fahrenkrug 1987; Holst et al. 1987; Lundberg et al. 1984b).

The functional significance of the co-release of VIP and PHI is not fully understood. PHI has a spectrum of actions which is similar to that of VIP, but the potency of administered PHI is, however, in most systems, orders of magnitude lower than those of VIP (Inoue et al. 1988; Lundberg et al. 1984a; Moriarty et al. 1984; Palle et al. 1989; Suzuki et al. 1991; Tapia-Arancibia and Reichlin 1985; Tornøe et al. 2001; Tse et al. 2002). PHI is thus considered a VIP agonist for VIP receptors, and there is presently little evidence for the existence of distinctive receptors selective for this peptide in mammals.

5 Glycine-Extended VIP

Like many other peptide hormones or neuropeptides, VIP and PHI are C-terminally carboxyamidated. It has, however, been shown that unlike most bioactive peptides, carboxyamidation of VIP is not critical to the biological activity (Fahrenkrug et al. 1989). In the VIP precursor, VIP as well as PHI are followed by a glycine residue and a pair of basic amino acids. Biosynthetic processing includes removal of the two basic amino acid residues and cleavage of the amide donor, glycine, by a specific amidation enzyme. The intermediate preceding precursors are glycine-extended VIP or glycine-extended PHI. Fahrenkrug developed radioimmunoassay specific to glycine-extended VIP and showed that this intermediate biosynthetic precursor of VIP occurs in high concentration in rat tissue. The identity of the peptide was substantiated by cation exchange HPLC. The ratio of glycine-extended VIP to amidated VIP was shown to vary considerably in rat tissues from 3% in the brain, to 62% in the small intestine (Fahrenkrug 1991). It is likely that this intermediate VIP form plays a

physiological role, since glycine-extended VIP has the same biological activity (inhibition of smooth muscle activity) as the fully processed and amidated VIP (Fahrenkrug et al. 1989). Similarly it has been shown that glycine-extended PHI is expressed in rat tissue (Cauvin et al. 1989a, b) and that the peptide is biologically active (Cauvin et al. 1990). It remains, however, to be clarified if glycine-extended VIP and PHI are secreted.

6 Expression of Other VIP Precursor-Derived Peptides

In later studies, further information on the molecular forms expressed in tissue was provided (Fahrenkrug and Emson 1989; Bredkjær et al. 1991, 1997; Buhl et al. 1995, 1996; Ottesen et al. 1995) using immunochemical assays with antisera detecting well-defined regions of the VIP precursor in combination with HPLC In the rat and human brains, the rat and human gastrointestinal tracts and the human male and female urogenital tracts all the putative preproVIP-derived peptides: preproVIP 22-79, PHI (PHM), preproVIP 111-122, VIP and preproVIP 156-170 were expressed, but not in equimolar amounts (Fig. 2b). A processing pathway in favour of the PHV was indicated by significantly higher PHV concentrations compared to PHM observed in the human cervix, myometrium, ovary and gastric antrum (Fig. 2a, c) (Bredkjær et al. 1991, 1997). Why a proportion of the VIP precursor follows an alternative processing pathway in which the dibasic conversion sites after PHM is uncleaved, remains to be clarified; but the finding is an example of differential processing of a single precursor. So far limited information of the processing enzymes involved in the tryptic cleavage of preproVIP during biosynthesis is available. In PC2 deficient mice the VIP levels in the adrenal, hypothalamus, cerebral cortex, the remaining brain, ileum and duodenum were not significantly altered compared to wild type animals (Miller et al. 2003). The differential expression of PHM is interesting, since the extended form, PHV, is more potent than PHM in relaxing smooth muscle activity of the stomach and the female genital tract (Yiangou et al. 1987; Palle et al. 1992). No significant biological effect has so far been attributed to preproVIP 22-79, preproVIP 111-122 and preproVIP 156-170 (Bredkjær et al. 1997).

As mentioned, measurements of absolute and relative concentrations of preproVIP-derived peptides in tissues represent a mixture of what is present in cell bodies, fibres and terminals. Neurotransmitter peptides are synthesised in the soma and further processed during transport of the axon to the nerve terminals. To get further information on the biosynthetic processing of VIP in rats, Nilsson and Fahrenkrug (1995) examined VIP precursor-derived peptides in peripheral parasympathetic neurons by radioimmunoassay, HPLC and gel filtration. They examined the sphenopalatine ganglion, which has a large number of VIP-containing neuronal cell bodies and their proximal axons, and one of its terminal projection areas, the nasal mucosa, where VIP immunoreactivity is confined to nerve terminals surrounding glandular acini and blood vessels (Nilsson and Fahrenkrug 1995).

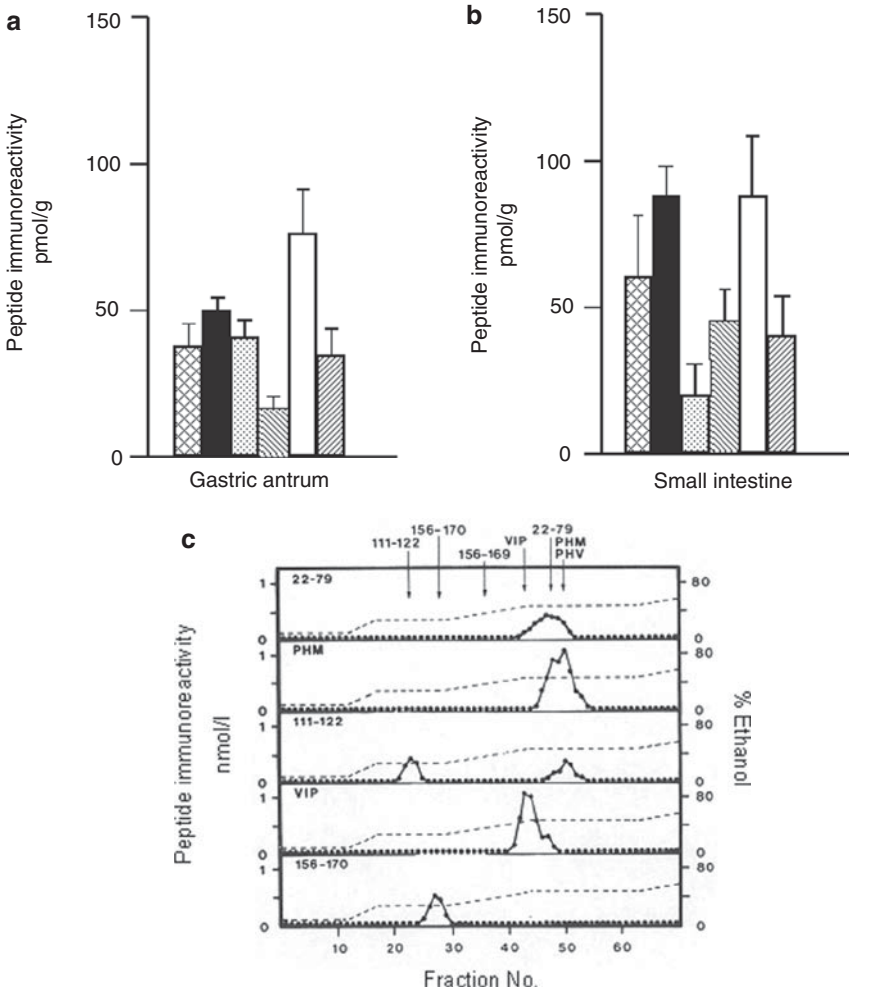

Fig. 2 Concentrations of preproVIP 22-79 (▨), PHM (■), PHV (▨), preproVIP 111-122 (▨),VIP (□) and preproVIP 156-170 (▨) in extracts of human gastric antrum (**a**) and small intestine (**b**) from eight subjects. Values (pmol g^{-1} wet weight) are given as means ± SEM. Reverse-phase HPLC chromatograms of extracts from human gastric antrum are shown in (**c**). Aliquots of identical fractions were assayed by radioimmunoassay for the peptides indicated. *Arrows* indicate elution position of the synthetic peptides preproVIP 22-79, PHM, PHV, preproVIP 111-122, VIP, preproVIP 156-170 and preproVIP 156-169. Ordinate values of immunoreactivities are based on assay standards of the peptides. The ethanol gradient is indicated by the *dashed line*

In the sphenopalatine ganglion, high concentrations of all preproVIP-derived peptides were present. In addition larger fragments representing incompletely processed preproVIP were demonstrated, but these fragments were not further characterised. In the nasal mucosa, on the other hand, amidated VIP was clearly

the most prevalent peptide, and the fully processed VIP constituted the major part of VIP immunoreactivity. The findings indicate that substantial processing of prepro VIP takes place already in the soma and proximal axon, but that further processing might occur during axonal transport of the secretory vesicles to enable the preferential accumulation of amidated VIP in the terminals.

7 PreproVIP-Derived Peptides in Patients with VIP-Producing Tumours

High concentrations of VIP are present in the plasma and tumour tissue of patients with VIP-producing tumours (VIPomas), and most likely VIP is responsible for the secretory diarrhoea seen in these patients (Bloom and Polak 1982). Elevated quantities of preproVIP 22-79, PHM, PHV, preproVIP 111-122, VIP and preproVIP 156-170 have been shown in extracts of tumours of different origins such as neuroblastomas, ganglioneuromas, ganglioneuroblastomas, pancreatic carcinomas, and pheochromocytomas as compared with normal tissue. The peptides were present in non-equimolar amounts and the relative proportion of the various peptides differed markedly from tumour to tumour (Rønnov-Jensen et al. 1991). The contribution of the extended form, PHV, showed considerable variation, but amounted up to 70% of the total PHM/PHV immunoreactivity in some of the tumours. It was also found that unlike in normal tissue, a fraction of the C-terminal fragment preproVIP 156-170 was having its C-terminal lysine residue removed. All the peptide sequences produced in the tumour tissue were secreted as evidenced by their presence in plasma in elevated concentrations. The plasma levels of preproVIP 22-79, preproVIP 111-122 and PHV exceeded those of the remaining preproVIP-derived peptides, suggesting that determination of these peptides in a patient with VIP-secreting tumours may be better markers than VIP itself. The findings are most likely explained by differences in clearance half-lives of the various peptides in the circulation.

In the VIP-producing neuroblastoma cell line NB1, all the preproVIP-derived peptides have been demonstrated to be expressed and secreted, and a two to fivefold induction of the various peptides in the medium could be produced by retinoic acid (Georg et al. 1994). Furthermore, most peptides eluted as their corresponding synthetic peptide; however, a fraction of the VIP immunoreactive material eluted earlier than synthetic VIP, indicating larger molecular forms. In addition, not all VIP and PHM were found to be amidated.

8 Discovery of PACAP

PACAP was isolated from extracts of ovine hypothalamus in 1989 on the basis of its ability to stimulate cAMP formation in rat pituitary cells (Miyata et al. 1989). Characterisation of the peptide revealed that it comprises 38 amino acid residues

and is C-terminally amidated (Miyata et al. 1989). Later another peptide with similar ability to stimulate adenylate cyclase activity was isolated, and this peptide corresponded to the N-terminal 27 amino acid sequence of PACAP 38 (Miyata et al. 1990). PACAP 27 is also amidated in its C-terminal end. PACAP 27 shares a 68% amino acid homology with VIP, and both PACAP forms are members of the VIP/glucagon/growth hormone-releasing factor/secretin superfamily of structurally related peptides. The amino acid sequence of PACAP is identical in all mammals, and in other species only few amino acids are substituted suggesting that PACAP is highly conserved and has remained almost unchanged during an evolutionary period of ca. 700 million years (Arimura 1998). PACAP can interact with three subtypes of receptors of which two are shared with VIP. PACAP is widely distributed in the brain and peripheral organs, and since its discovery, numerous studies have provided evidence that PACAP is a pleiotropic substance having a broad spectrum of biological functions. Thus, the peptide can act as a hormone, neurohormone, autocrine/paracrine substance, neurotransmitter, neuromodulator, neurotrophic factor and immunomodulator (Vaudry et al. 2000).

9 Structure of PACAP Precursor

The human PACAP gene was cloned in 1992 and mapped to the P11 region of chromosome 18 (Hosoya et al. 1993), while the rat PACAP gene is located on chromosome 9q37 (Cai et al. 1995). The human PACAP gene is composed of five exons and four introns. The mature PACAP 38 is encoded by exon 5, the PACAP related peptide (PRP) by exon 4, while exon 1 is untranslated. Translation of the mRNA sequence produced from the PACAP gene yields a 176 amino acid long precursor, prepro PACAP, including the signal peptide (Fig. 1b). Primary structure of the precursor reveals the existence of seven mono or dibasic residues, which could be cleaved by the various PCs. There is no evidence of the existence of an additional exon, which encodes only for PACAP 27, ruling out the possibility that PACAP 27 could be encoded by mRNAs generated by alternative splicing mechanisms. The precursor PACAP 38 is followed by a Gly–Lys–Arg sequence for proteolytic processing and amidation. Mature PACAP 38 also contains paired basic amino acid residues, Lys 29, Arg 30, preceded by Gly 28 which is the possible site for posttranslational proteolytic processing and amidation to generate PACAP 27, and thus both peptides are derived from the same precursor. PRP is a 29 amino acid long peptide (amino acids 82–110 in preproPACAP), which corresponds to PHI/PHM in the VIP precursor. The primary sequence of PRP, which shows 22% similarity to PACAP 27, is not as well preserved between the species as that of PACAP. It has been assumed that intragenomic duplication of a VIP/PACAP ancestor sequence has occurred before duplication of the whole ancestor gene (Ohkubo et al. 1992).

10 Expression of PACAP Precursor-Derived Peptides

Using specific radioimmunoassay for PACAP 38, PACAP 27 and PRP combined with high-pressure liquid chromatography and gel chromatography it has been shown that all three PACAP precursor-derived peptides are detectable in tissue extracts from various brain regions, the gastrointestinal tract, the urogenital tract and testes (Fig. 3)

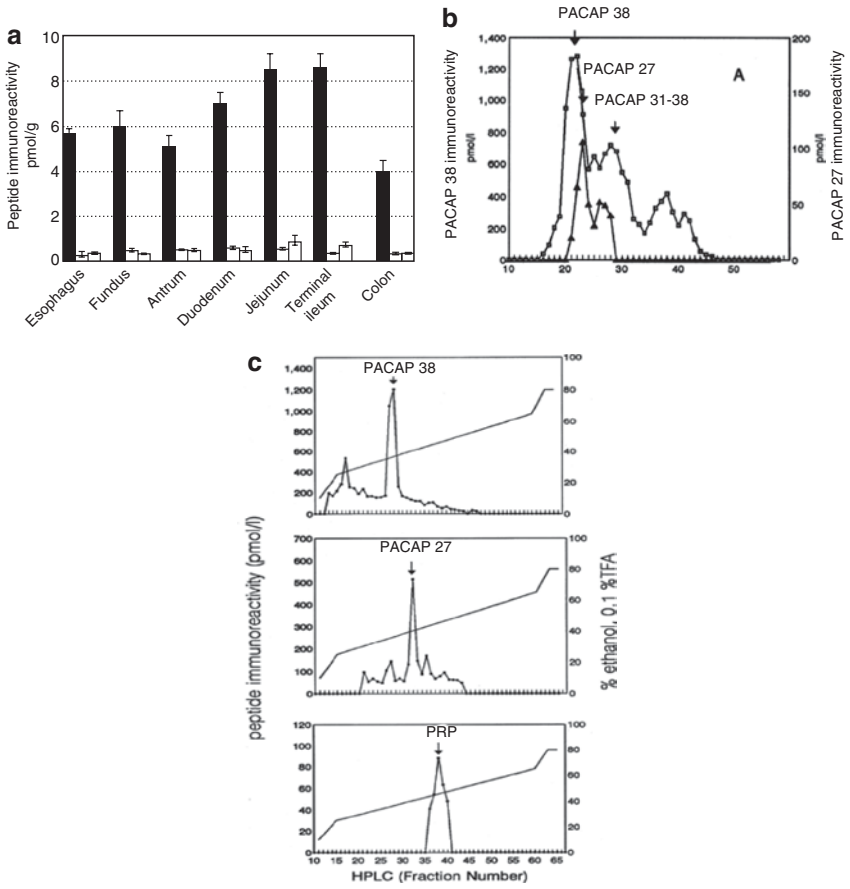

Fig. 3 Concentrations of PACAP 38 (■), PACAP 27 (□) and PRP (▨) in extracts of gastrointestinal tract of five rats (**a**). Values (pmol g^{-1} wet weight) are given as means ± SEM. Size chromatography of extracts from rat gastric fundus analysed for PACAP 38 (□) and PACAP 27 (▲) immunoreactivity is shown in (**b**). The major peak corresponds to the synthetic PACAP 1–38 and PACAP 1–27, respectively. A minor fraction elutes in a position corresponding to synthetic PACAP 31–38 (indicated by *arrows*). (**c**) Reverse-phase HPLC chromatograms of tissue extracts from the rat gastric fundus. Peptide immunoreactivities for PACAP 38 (*upper*), PACAP 27 (*middle*) and PRP (*lower panel*) were measured by radioimmunoassays. *Arrows* indicate the elution positions of the three synthetic peptides. Analysis was performed on the same tissue extracts in separate runs and the amount of tissue applied was not identical

(Fahrenkrug and Hannibal 1996, 1998; Hannibal et al. 1995, 1998; Hannibal and Fahrenkrug 1995). Surprisingly the not fully processed form of PACAP, PACAP 38, is the dominating peptide in all tissues examined (Fahrenkrug and Hannibal 1996, 1998; Ghatei et al. 1993; Hannibal et al. 1995, 1998; Hannibal and Fahrenkrug 1995). The tissue concentrations of PACAP 27 and PRP amount to only 7–20 and 1–10% of that of PACAP 38, respectively (Fig. 3a) (Fahrenkrug and Hannibal 1996, 1998). By immunohistochemistry, a complete co-localization of PRP and PACAP has been demonstrated in all PACAP containing cells examined so far (Hannibal et al. 1995; Mikkelsen et al. 1995). Whether this co-existence is of functional significance and whether PRP, PACAP 27 and PACAP 38 are co-released or co-secreted remain to be solved. The effects of PACAP 38 and PACAP 27 are well described and are in most biological systems identical (Vaudry et al. 2000). Our knowledge of PRP's biological actions is, however, limited. A few studies have demonstrated that PRP has similar effects as PACAP but displays lower potency, while the extended form of PRP, named big PRP, the existence of which is questionable, has limited biological actions (Kitada et al. 1992; Wray et al. 1995).

Processing of the PACAP precursor has been studied in Chinese hamster ovary cells, transfected with the human PACAP cDNA (Okazaki et al. 1992). Characterisation of the various peptides secreted in the incubation medium by HPLC combined with radioimmunoassay detection confirmed that processing of the PACAP precursor actually yields the formation of PACAP 38, PACAP 27 and PRP. The possible role of the PCs has been examined in rat pituitary PH4C1 cells, which were co-transfected with the human PACAP cDNA and either rat PC1 or PC2 cDNA. Extracts of these cells were analysed by reverse-phase HPLC for proPACAP, PACAP 38 and/or PACAP 27 by radioimmunoassays using antibodies with different specificity and subsequently bioassayed for the ability to stimulate adenylate cyclase. The cells transfected with PACAP cDNA alone yielded PACAP-like immunoreactivity corresponding to molecular weights of approximately 15–20 kDa without bioactivity. Co-transfection of these cells with PC1 or PC2 generated PACAP immunoreactivity, which co-eluted with synthetic PACAP 38 and PACAP 27. The HPLC fractions containing the PACAP immunoreactivity co-eluting as synthetic PACAP 38 and 27 showed marked bioactivity (Li et al. 1999). On the other hand, in the testes, where PACAP is particularly abundant, PC4 can process a PACAP precursor to generate both PACAP 38 and 27 (Li et al. 1998). These findings indicate that the testicular PACAP precursor is a substrate for PC4. Accordingly, studies using PC4 knockout mice revealed that neither the testis nor the ovary expressed PACAP 38 or PACAP 27 (Li et al. 2000). The levels of PACAP mRNA in the testis and ovary of homozygote PC4 deficient mice were, however, considerably elevated compared with those of the wild type and heterozygote animals. Interestingly, male PC4 deficient mice exhibit severely impaired fertility although spermatogenesis appeared to be normal, while the female PC4-null mice exhibited delayed folliculogenesis in the ovary (Mbikay et al. 1997). Whether absence of PACAP could explain this reproductive dysfunction remains to be clarified.

Interestingly, elevated concentrations of the three peptides derived from the PACAP precursor are found in the majority of VIP-producing tumours, while in non-VIP-secreting endocrine tumours, the PACAP and PRP concentrations were not elevated (Fahrenkrug et al. 1995). Chromatographic and electrophoretic studies showed that the PACAP immunoreactivities in tumour extracts eluted corresponding to synthetic PACAP 38, PACAP 27 and PRP, indicating that the tumours are able to fully process the PACAP precursor to the mature peptides. Like in normal tissue, PACAP 38 was the dominating peptide in all tumour cases. Whether the VIPoma patients also have elevated PACAP 38 concentrations in plasma remain to be clarified. It has, however, been shown difficult to accurately measure circulating PACAP 38 because it is tightly bound to ceruloplasmin (Tams et al. 1999).

11 Conclusion

All preproVIP-derived peptides occur in normal tissue and VIP-producing cell lines and elevated quantities occur in plasma and tumour tissues from patients with VIP-producing tumours. In some tissues the dibasic cleavage site after PHI is uncleaved resulting in a C-terminally extended form, PHV, which is more biologically active than PHI. PHI and VIP are present in a 1:1 molar ratio in large dense core vesicles and released in roughly equimolar amounts. Carboxyamidation of VIP and PHI is not critical for biological activity and glycine-extended forms of both peptides occur in normal tissue. All preproPACAP-derived peptides are present in tissue - the dominating form being PACAP 38. PC1 and PC2 seem to be involved in the processing of PACAP, except in the testes and ovary, where the PACAP precursor is substrate for PC4.

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