Ghrelin axis genes, peptides and receptors: Recent findings and future challenges

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Abstract

The ghrelin axis consists of the gene products of the ghrelin gene (*GHRL*), and their receptors, including the classical ghrelin receptor *GHSR*. While it is well-known that the ghrelin gene encodes the 28 amino acid ghrelin peptide hormone, it is now also clear that the locus encodes a range of other bioactive molecules, including novel peptides and non-coding RNAs. For many of these molecules, the physiological functions and cognate receptor(s) remain to be determined. Emerging research techniques, including proteogenomics, are likely to reveal further ghrelin axis-derived molecules. Studies of the role of ghrelin-axis genes, peptides and receptors, therefore, promises to be a fruitful area of basic and clinical research in years to come.

Keywords: ghrelin; alternative splicing; cryptein; receptor; peptide

Abbreviations: GHSR (growth hormone secretagogue receptor), GOAT (ghrelin *O*-acyltransferase), APT1 (acyl-protein thioesterase 1)

1.0 Introduction

In 1996 Howard *et al.* (Howard et al., 1996) described the growth hormone secretagogue receptor (GHSR), which is expressed in the pituitary and hypothalamus and mediates the growth hormone (GH)-releasing activities of synthetic peptide secretagogues and cyclic analogues (Howard et al., 1996). GHSR was shown to be a classical, 7-transmembrane domain, G protein-coupled orphan receptor, and its natural ligand was not known. Three years later, Kojima *et al.* (Kojima et al., 1999) isolated the natural GHSR ligand, ghrelin, from rat stomach. The ghrelin axis has now been described in a range of vertebrate species from teleost fish to humans (Kaiya et al., 2008). Although ghrelin was initially discovered as an endogenous GH releasing peptide, it soon became apparent that ghrelin has a wide range of different functions. Ghrelin is the most potent circulating orexigen, and plasma levels are elevated prior to meals and stimulates feeding (Wren et al., 2001). It also has roles in the regulation of metabolism, insulin and glucose balance, the immune system, cardiovascular system and has roles in sleep and memory.

Ghrelin is a 28 amino acid peptide, which is post-translationally cleaved by furin-like proteases from a larger (117 amino acid) preproghrelin protein. We have previously hypothesised that the ghrelin gene encodes a wide range of peptides, termed crypteins (meaning *to hide* in Greek) (Seim et al., 2009), and recent evidence supports this hypothesis. These hidden peptides, which are derived by alternative transcriptional splicing, proteolytic cleavage of larger precursor peptides and/or by other post-translational modifications, may have novel or altered functions compared to the wild-type ghrelin peptide. Although the human ghrelin gene was originally reported to consist of just four coding exons, recent evidence demonstrates that the human ghrelin gene locus is remarkably more complex in terms of transcriptional output, and it includes a large number of transcripts transcribed from both sense and antisense DNA strands (Seim et al., 2007; Seim et al., 2008).

There also appears to be considerable diversity in the ghrelin receptor. Alternative splicing of the *GHSR* gene generates the full-length transcript that encodes the active GHSR 1a and a truncated GHSR 1b isoform, the latter exhibits no calcium signalling in response to ghrelin treatment (Feighner et al., 1998; Howard et al., 1996), but may downregulate GHSR1a expression, acting as a dominant-negative mutant (Leung et al., 2007). There is also strong evidence for an unidentified, alternative ghrelin receptor(s) that could mediate some of the effects of ghrelin and its non-acylated form, desacyl ghrelin (Baldanzi et al., 2002; Bedendi et al., 2003; Broglio et al., 2004; Cassoni et al., 2001; Cassoni et al., 2004; Filigheddu et al., 2007; Gauna et al., 2005; Gauna et al., 2006; Kleinz et al., 2006; Martini et al., 2006; Muccioli et al., 2004; Sato et al., 2006; Thielemans et al., 2007; Thompson et al., 2004; Toshinai et al., 2006; Tsubota et al., 2005).

In this review, we will highlight what is known about human ghrelin-axis derived molecules, ghrelin receptors and RNA transcripts, many of which may play important roles in health and disease.

2.0 The ghrelin precursor, mature ghrelin peptide, GOAT and obestatin

The ghrelin gene contains four preproghrelin-coding exons (exon 1 to 4), and additional upstream exons have recently been reported (Seim et al., 2007). During preproghrelin processing, a 23 amino acid secretion-signal peptide is cleaved from the N-terminus of the 117 amino acid preprohormone, resulting in a 94 amino acid proghrelin peptide (Fig. 1). This proghrelin peptide is then further cleaved and gives rise to the 28 amino acid ghrelin peptide (amino acids 24–51, encoded by exon 1 and part of 2) and a 66 amino acid C-terminal propeptide, C-ghrelin (encoded by part of exon 2, plus exons 3 and 4 of the preproghrelin gene) (Pemberton et al., 2003). There are two major forms of the 28 amino acid mature form

of ghrelin. Following proteolyic cleavage from proghrelin, the ghrelin peptide can be post-translationally octanoylated (acylated) at its third residue, which is a serine, by ghrelin *O*-acyltransferase (GOAT) (Gutierrez et al., 2008; Yang et al., 2008a). This modified form is usually referred to as ghrelin in the literature. A non-octanoylated form of ghrelin (des-acyl ghrelin, des-ghrelin or unacylated ghrelin) circulates in the blood at higher levels than octanoylated ghrelin (Holmes et al., 2009; Patterson et al., 2005) and because it does not bind the GHSR 1a it was previously thought to be biologically inactive. As outlined in several articles in this issue, however, des-ghrelin is now known to have many functions in health and disease (Delhanty et al., 2010; Gauna et al., 2006; Gauna et al., 2007).

GOAT, the enzyme that octanoylates ghrelin, is a member from the membrane-bound *O*-acyl transferase (MBOAT) family of enzymes and is encoded by the *MBOAT4* gene and it is highly conserved in vertebrates (Gutierrez et al., 2008; Yang et al., 2008a). Using octanoyl CoA as a substrate, GOAT transfers the octanoate group to the third residue (serine) of ghrelin, forming an acyl ester (Zhao et al., 2010). The N-terminal 4 amino acids of ghrelin are likely to be the substrate recognition sites for the enzyme, and the first, third and fourth amino acids are required for octanoylation and are highly conserved (Ohgusu et al., 2009; Yang et al., 2008b). GOAT is a hydrophobic, membrane-bound enzyme with 8 membrane-spanning domains and it appears to be localised to the endoplasmic reticulum (Yang et al., 2008a). GOAT and ghrelin are co-expressed in cells in the stomach, and GOAT is also expressed in the pancreatic islets and to a lesser extent in other tissues (Gutierrez et al., 2008), including chondrocytes (Gomez et al., 2009). As ghrelin is uniquely modified by GOAT, the enzyme is believed to be an attractive and specific target for the modification of ghrelin octanoylation (Yang et al., 2008b) and GOAT could provide a target for the

development of drugs to prevent obesity, weight gain and insulin resistance (Barnett et al., 2010).

There is growing evidence that GOAT plays a role in the regulation of metabolism and it appears to play a role in lipid-sensing in the gut and linking energy intake with endocrine balance (Kirchner et al., 2009). GOAT knockout mice are unable to maintain adequate blood glucose levels in response to severe caloric restriction and weight loss and this effect is prevented when GH is co-administered (Zhao et al., 2010). While GOAT may play an important role in glucose balance during caloric restriction, it does not appear to play an important role in the storage of excess energy stores (Zhao et al., 2010). A ghrelin des-acylation enzyme (acyl-protein thioesterase 1, APT1) has recently been reported (Satou et al., 2010), suggesting that the levels of acylated ghrelin can be regulated by the bioavailability of GOAT and APT1. The role of ghrelin acylation in health and disease is extensively reviewed by Yi et al., in this Special issue.

The 66 amino acid C-terminal region of the prohormone, C-ghrelin (Pemberton et al., 2003), contains the 23 amino acid peptide obestatin (Zhang et al., 2005), which is encoded by part of exon 3. Obestatin, which is C-terminally amidated, was originally described as having opposite effects to ghrelin on food intake (Zhang et al., 2005), but it is now established that this is unlikely to be the case (Gourcerol et al., 2007b; Gourcerol et al., 2007a; Seoane et al., 2006). However, it is a multi-functional peptide hormone in its own right with several reports within the last five years describing functional roles for the obestatin peptide, in sleep, adipogenesis, pancreatic homeostasis and cancer (for review, see Seim *et al.* in this issue).

Cleavage of obestatin from C-ghrelin also generates an N-terminal and a C-terminal peptide, however, no functional studies involving these peptides have been reported.

2.1 Alternative exon usage may result in novel ghrelin gene-derived peptides

It is now clear that the human ghrelin gene harbours multiple first exons and is extensively spliced (Fig. 2). Until recently, however, the translation and/or function of the majority of these peptides was not appreciated (Seim et al., 2009). Prepro-des-Gln^{14}-ghrelin (Fig. 2) is a splice variant where an alternative splice site in exon 2 is employed, resulting in a 116 amino acid preproghrelin peptide (lacking one glutamine residue) that is likely to be processed and function in the same manner as the wildtype preproghrelin transcript (Hosoda et al., 2000; Hosoda et al., 2003). Another variant discovered by our laboratory, exon 3-deleted preproghrelin (Δ3 preproghrelin) (Fig. 2), encodes a 91 amino acid preprohormone, which lacks obestatin and, due to a change in reading frame, generates a novel 16 amino acid C-terminal sequence (Jeffery et al., 2003; Jeffery et al., 2005; Yeh et al., 2005). This sequence has a potential proteolytic cleavage site at its N-terminus. It is not yet known, however, if this novel C-terminal peptide (termed Δ3D) is released within tissues or circulates in the plasma, and the function of the Δ3 peptide is also not known. As the Δ3 preproghrelin splice variant would produce ghrelin, but not obestatin, this provides a potentially important mechanism for altering the balance between these molecules and, thereby, resulting in different physiological responses. This may be the case in cancers of the breast (Jeffery et al., 2002) and prostate (Yeh et al., 2005), where this exon 3-deleted preproghrelin splice variant is upregulated.

In 2007, Kineman and colleagues (Kineman et al., 2007) described a ghrelin gene transcript in the mouse that retained intron 1 of the gene. This splice variant, termed In2-ghrelin, is primarily expressed in the pituitary and hypothalamus and is regulated in response to

metabolic stress (Kineman et al., 2007). Gahete and colleagues recently reported that In2-ghrelin mRNA is present in humans and down-regulated in particular brain regions in Alzheimer's disease (Gahete et al., 2010b). In2-ghrelin encodes a 117 amino acid polypeptide that includes the signal peptide of preproghrelin, the first 12 amino acids of the 28 amino acid ghrelin peptide sequence, and a novel 81 amino acid, C-terminal peptide. The expression of In2-ghrelin parallels the expression of ghrelin *O*-acyltransferase (GOAT), suggesting that the peptide is likely to be acylated by GOAT (Gahete et al., 2010a). The 12 amino acid ghrelin-like region of In2-ghrelin is particularly interesting, as it contains the first 5 amino acids of ghrelin (GSSFL), which we previously termed G5-ghrelin (or G5) (Seim et al., 2009). This is the minimum sequence required for binding and stimulation of GHSR 1a *in vitro* (Bednarek et al., 2000) and for ghrelin acylation by GOAT (Yang et al., 2008b). The potential existence of endogenous, short ghrelin peptides warrants further investigation.

Several transcripts that do not contain exon 1 and, therefore, do not encode ghrelin, are also generated from transcription start sites within the alternative far upstream exon -1 and are spliced directly into exons 2, 3, and/or 4 (Seim et al., 2007) (Fig. 2). Exon -1 contains a putative signal peptide sequence and, therefore, the resulting putative peptides [which encode C-ghrelin only, obestatin only or Δ3D only (Fig. 2)] may be expressed quite independently of full-length preproghrelin itself (Seim et al., 2007). Differential regulation of transcription start site usage and alternative splicing in different physiological and/or pathophysiological states may partly provide an explanation for the lack of correlation between the expression levels of ghrelin and C-ghrelin or obestatin peptides.

2.2 Ghrelin antisense transcripts

We have reported the presence of a natural antisense gene, *GHRLOS*, on the opposite DNA strand of the ghrelin gene (*GHRL*) (Seim et al., 2007; Seim et al., 2008). This gene spans the promoter and untranslated regions of *GHRL*, overlaps with *GHRL*-adjacent genes and can be transcribed into a number of mRNA isoforms. *GHRLOS* encompasses several exons, is extensively spliced, is not well conserved across species and contains multiple stop codons - all hallmarks of potential non-coding (nc) RNA genes. It is highly expressed in several tissues (including the thymus, brain and testis) that are known sites of ncRNA expression (Mercer et al., 2008; Sasaki et al., 2007). It is currently not known if ghrelin antisense transcripts, derived from the *GHRLOS* gene, regulate ghrelin expression, other cellular functions, or play a role in disease. Such questions are now the focus of continuous research efforts. Antisense transcripts have been shown to influence gene silencing and tumour suppression and to represent markers for complex human diseases, among other roles (Taft et al., 2010).

3.0 Receptors in the ghrelin axis: more research questions than answers

Although the cognate ghrelin receptor, GHSR 1a, was identified more than 15 years ago [1], significant questions remain regarding the structure-function relationships of this receptor and its recognised truncated isoform, GHSR 1b. It is also unclear how many other GHSR-independent ghrelin receptors there are and the receptors for other ghrelin derived peptides, including the obestatin receptor, are unknown (Fig. 3).

GHSR 1a is a classical 7-transmembrane G protein-coupled receptor (GPCR) and a member of a broader ghrelin receptor family, which includes receptors for neurotensin and neuromedins, motilin and other GPCRs, including GPR39 (Holst et al., 2004). GHSR 1a is expressed in a wide variety of tissues and is established as the receptor through which some, but not all, of the recognised effects of ghrelin (including GH secretion, appetite regulation, insulin production, and cell proliferation) are mediated. The truncated, 5-transmembrane domain-spanning, GHSR 1b isoform is thought to be inactive, as it does not bind ghrelin and ghrelin does not activate signalling through this receptor (Feighner et al., 1998; Howard et al., 1996). It may, however, play a significant role in modulating other GPCRs, including GHSR 1a, through GPCR homo- and/or hetero-dimerisation (Chan et al., 2004; Chu et al., 2007; Leung et al., 2007; Takahashi et al., 2006). GPCR dimerisation is a well-recognised mechanism through which ligand recognition and receptor signalling can be modified (Dalrymple et al., 2008). GHSR 1a/1b heterodimerisation has been demonstrated in studies in sea bream (teleost fish), (Leung et al., 2007) and by interacting with GHSR 1a, GHSR 1b may attenuate the constitutive activation of phospholipase C by GHSR 1a (Chu et al., 2007; Leung et al., 2007). GHSR 1b may act as a dominant-negative regulator of GHSR 1a by reducing the cell surface expression of GHSR 1a and, therefore, reducing constitutive signalling (Leung et al., 2007).

GHSR 1b is over-expressed in lung cancer and dimerises with the neurotensin receptor, to form a new, functional receptor that promotes neuromedin U-induced cell proliferation (Takahashi et al., 2006). Although its function is not clear, the fact that it is differentially expressed compared to the GHSR 1a isoform in a number of tissues and physiological and pathophysiological states, including over-expression in many cancers, could reflect its potential importance (Barzon et al., 2005; Gnanapavan et al., 2002; Jeffery et al., 2002; Jeffery et al., 2005; Takahashi et al., 2006).

A large number of ghrelin gene-derived splice variants and post-translationally modified forms have been identified (Seim et al., 2009), and this reflects an unforeseen complexity in the physiology of the ghrelin axis. It also suggests that a number of receptors in the axis are yet to be discovered and, indeed, there is considerable evidence that alternative receptors for both ghrelin, and des-ghrelin and an unidentified obestatin receptor exist (Baldanzi et al., 2002; Bedendi et al., 2003; Broglio et al., 2004; Cassoni et al., 2001; Cassoni et al., 2004; Filigheddu et al., 2007; Gauna et al., 2005; Gauna et al., 2006; Kleinz et al., 2006; Martini et al., 2006; Muccioli et al., 2004; Sato et al., 2006; Thielemans et al., 2007; Thompson et al., 2004; Toshinai et al., 2006; Tsubota et al., 2005). Evidence for an alternative ghrelin receptor includes the fact that desacyl ghrelin is unable to bind to GHSR 1a, although this hormone is functional in a number of cellular systems. In addition, ghrelin and desacyl ghrelin stimulate cell signaling and a range of functions in cells that do not express GHSR 1a and can stimulate signaling in GHSR-knockout animal models (Delhanty et al., 2006; Granata et al., 2007). When the discovery of obestatin was originally reported, GPR39, a GPCR and a member of the small ghrelin receptor family, was thought to be the obestatin receptor (Zhang et al., 2005; Zhang et al., 2008). This has proven controversial, however, and numerous studies have shown that GPR39 is not the obestatin receptor, but it is a zinc (Zn^{2+}) activated receptor (Chartrel et al., 2007; Dong et al., 2009; Holst et al., 2004; Holst et al., 2007; Lauwers et al., 2006; Popovics et al., 2010).

4.0 Future studies on the ghrelin-GHSR axis

Current and emerging literature demonstrates considerable complexity within the ghrelin/ghrelin receptor axis. Multiple ghrelin gene products are translated, and post-translational modifications add to the potential functional diversity of peptides and receptors

in the ghrelin axis. The roles of many of these peptides are yet to be established. Clearly, a further dissection of the ghrelin axis peptidome in humans, as well as the mouse and other model systems, will be important in determining the exact role of each new ghrelin variant. The recent discovery of numerous ghrelin gene-derived peptides and the search for their cognate receptors, as well as further investigations into GHSR isoforms, will stimulate many new research initiatives that will lead to a better understanding of the role of the ghrelin axis in this second decade of ghrelin research.

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Figure captions

Figure 1. Schematic illustrating the structure of the ghrelin gene, ghrelin preprohormone mRNA and known processed peptides from preproghrelin.

Translation of ghrelin mRNA yields a 117 amino acid preprohormone precursor, which consists of a 23 amino acid signal peptide (SP), a 28 amino acid mature ghrelin peptide, and a 94 amino acid C-terminal peptide (termed C-ghrelin). Obestatin may be processed from C-ghrelin by proteolytic cleavage, or arise independently from distinct alternative, splice variants.

Figure 2. Overview of human ghrelin gene-derived transcripts and putative peptides. Ghrelin is shown in blue, obestatin in red, the Δ3D peptide in orange, the unique region of In2-ghrelin in green, the C-terminal peptide of In2c-ghrelin in purple. G5 denotes putative peptides that contain the first 5 amino acids of ghrelin (GSSFL) and harbour novel C-termini. Where applicable, other species where splice variants have been reported are indicated (mouse).

Figure 3. Overview of ghrelin gene-derived peptide biogenesis and receptors. Des-acylated (or desghrelin) is acylated by GOAT to form acylated ghrelin, whilst acylated ghrelin can be de-acylated by APT1. Ghrelin binds GHSR 1a and ghrelin and des-ghrelin are believed to also act through an unidentified alternative receptor. The 23 amino acid peptide obestatin and the 16 amino acid Δ3D peptide are shown as examples of other ghrelin gene-derived peptides and their receptors have not yet been identified.

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