MINI-REVIEW

Metalloproteinases and the modulation of GH signaling

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Abstract

The metzincin metalloproteinase, tumor necrosis factor-α-converting enzyme (TACE), also known as ADAM (a disintegrin and metalloproteinase) 17, has recently been identified as an important enzyme for cleavage of the GH receptor (GHR) and shedding of GH-binding protein (GHBP). Proteolysis can be induced by phorbol esters, platelet-derived growth factor and serum; it is dependent on protein kinase C and partially on MAP kinase pathways. Proteolysis occurs at the cell surface, leading to extracellular release of GHBP and intracellular GHR remnant accumulation. The GHR remnant is further processed by γ-secretase activity, possibly leading to biologically active products. TACE-dependent GHR proteolysis can be inhibited by GH as the dimerized GHR is resistant to cleavage. The cleavage site lies within a short juxtamembranous stem region that extends between the transmembrane helix and the globular dimerization domain of the GHR. GHR proteolysis leads to down-regulation of functional GHRs at the cell surface, and has complex secondary effects on GH action via GHBP and GHR remnant generation.


Introduction

Growth hormone (GH) signals through the GH receptor (GHR), a member of the cytokine receptor superfamily (Bazan 1989). The GHR is a 620 amino acid, integral membrane protein, consisting of a 246 residue extracellular domain involved in ligand binding and GHR dimerization, a single 24 amino acid transmembrane helix, and a 350 residue cytoplasmic domain involved in signal transduction (Leung et al. 1987). The GHR signals through the Jak-Stat, MAP kinase, phosphatidylinositol 3-kinase, and probably other pathways (Herrington & Carter-Su 2001, Frank & Messina 2002).

Binding of GH to the GHR results in ligand-induced GHR dimerization, with sequential binding of a first GHR to binding site 1 on GH, followed by recruitment of a second GHR to site 2 (Cunningham et al. 1991). This ternary complex between two GHRs and GH is then stabilized by direct interaction between the two GHR juxtamembrane domains. Dimerization causes a conformational change that initiates the signaling cascade. Figure 1 shows the crystallized extracellular GHR–GH–GHR complex (de Vos et al. 1992), with two distinct GHR domains: an amino-terminal binding domain and a more carboxy-terminal dimerization domain. An approximately ten amino acid ‘stem’ separates the dimerization domain from the cell membrane. This stem region was not visualized in the crystal.

The GH-binding protein (GHBP) and GHR proteolysis

Many integral membrane proteins are subjected to proteolytic cleavage near their membrane insertion, resulting in liberation of their extracellular domains into the pericellular space (Ehlers & Riordan 1991, Heaney & Golde 1996, Hooper et al. 1997, Müllberg et al. 2000) – a process known as ‘shedding.’ A list of integral membrane proteins undergoing this type of proteolysis has been published in tabular form; it includes cell adhesion molecules, leukocyte antigens, receptors and their ligands, ectoenzymes, viral membrane proteins, and other miscellaneous proteins (Hooper et al. 1997). The GHR is a prominent member of this class of transmembrane proteins; it generates substantial amounts of its ectodomain, known as GHBP, which can be measured in the circulation (for review see Baumann 2001). The biological importance of the GHBP, although still poorly understood, is underscored by the facts that (i) it is evolutionarily conserved among
vertebrates and (ii) it is generated by two separate mechanisms: proteolytic shedding in humans, rabbits and other species; and alternative GHR mRNA splicing in rodents (Baumann 2001). In rodents, a GHBP-specific exon (exon 8A) in the GHR gene encodes a hydrophilic sequence in lieu of the hydrophobic transmembrane domain of the GHR (encoded by exon 8), thereby permitting secretion of the alternative splice product (Edens et al. 1994, Zhou et al. 1994, 1996). Even attempts to abolish alternative splicing leading to the GHBP through inactivation of the exon 8A splice site or its polyadenylation signal fail to deplete GHBP production, which persists via a mechanism of readthrough into intron 7 (Zhou et al. 1997). Furthermore, the rodent GHR is not completely resistant to proteolysis (Guan et al. 2001), a property that represents yet another potential mechanism to generate GHBP. Thus, GHBP production is a consistent and probably important feature of GH/GHR biology. This review will focus on the proteolytic mechanism of GHBP generation, which operates in humans, rabbits and many other species. The protease responsible for GHR cleavage/GHBP generation has remained a mystery for many years, but has recently been identified.

Pharmacologically induced GHBP shedding

Trivedi & Daughaday (1988) showed that sulphhydryl inactivating agents, such as iodoacetamide and N-ethylmaleimide (NEM), could induce GHBP shedding from IM-9 cells, a human lymphoblast line expressing GHR. This observation led to the speculation that the membrane-proximate Cys241, which is unpaired, may be involved in GHR proteolysis. However, subsequent studies showed that substitution of Cys241 with alanine had no effect on GHBP shedding (Amit et al. 1999). NEM also has complex effects on intracellular membrane fusion and protein transport through the endoplasmic reticulum–Golgi system (Barr et al. 1997), but it is not clear whether or how this property might be linked to NEM’s effect on shedding. Thus, the mechanism by which iodoacetamide and NEM cause shedding remains poorly understood; it is not considered a physiological process.

Alele et al. (1988) demonstrated that GHBP shedding could be induced by the phorbol ester phorbol 12-myristate 13-acetate (PMA). This was accompanied by the proteolysis leading to the GHGP through inactivation of the exon 8A splice site or its polyadenylation signal fail to deplete GHBP production, which persists via a mechanism of readthrough into intron 7 (Zhou et al. 1997). Furthermore, the rodent GHR is not completely resistant to proteolysis (Guan et al. 2001), a property that represents yet another potential mechanism to generate GHBP. Thus, GHBP production is a consistent and probably important feature of GH/GHR biology. This review will focus on the proteolytic mechanism of GHBP generation, which operates in humans, rabbits and many other species. The protease responsible for GHR cleavage/GHBP generation has remained a mystery for many years, but has recently been identified.

Metzincins, a disintegrin and metalloproteinase (ADAM) and TNF-α-converting enzyme (TACE)

Metzincins constitute a large family of metalloproteinases, which are summarized in Table 1. Among the metzincins, ADAMs are especially relevant in this context as at least three members (ADAM 9, 10 and 17) are known plasma membrane-resident sheddases that can be activated by phorbol esters. ADAM 9 is involved in shedding of heparin-binding epidermal growth factor (EGF)-like growth factor (Izumi et al. 1994, 1996). ADAM 10 (also known as Kuzbanian in the fruit fly) is a critical component of the Notch pathway that is involved in Drosophila neurogenesis and in human sympathoadrenal cell development (Pan & Rubin 1997, Yavari et al. 1998). ADAM 17 (also known as TACE) is a well-characterized protease involved in cleaving soluble TNF-α as well as several other ectodomains from their membrane-bound precursors (Black et al. 1997, Moss et al. 1997). Figure 2 depicts the domain structure of TACE. The enzyme is synthesized as a precursor which includes a prodomain, a catalytic domain containing the Zn-binding site, a disintegrin domain, a cysteine-rich EGF/crambin domain, a transmembrane domain and a cytoplasmic domain (Black et al. 1997, Moss et al. 1997). The prodomain contains a cysteine that interacts with and neutralizes the Zn atom in the catalytic site, thereby maintaining the enzyme in an inactive zymogen form during its transit through the endoplasmic reticulum–Golgi apparatus. This prodomain is removed, probably by a furin-like enzyme, before fully active TACE is displayed at the cell surface. The catalytic domain of TACE has been crystallized, and the precise cleavage site in the TNF-α precursor has been identified (Maskos et al. 1998). Cleavage occurs in the 28 residue linear stem region extending between the cell surface and the globular TNF-α domain that forms a homotrimeric structure in the extracellular space (Maskos et al. 1998). Based on the important role of TACE as a sheddase for several cell surface proteins, we hypothesized that TACE may also serve as a GHBP sheddase. It is important to recognize that TACE has no simple specificity for a linear amino acid sequence. For example, several well-characterized TACE substrates show no consensus sequence for either recognition or cleavage (Table 2). Moreover, the binding affinity of TACE for synthetic cleavable peptides is rather low. For these reasons, it has been postulated that TACE proteolysis is a complex process depending on recognition of an epitope distant from the cleavage region and,
additionally, that spacial factors in the cell membrane, such as close apposition and proper relative orientation between cognate regions of the enzyme and substrate, are required for efficient catalysis (Maskos et al. 1998).

If TACE is considered a candidate for a GHBP sheddase, the question of co-expression of TACE and the GHR is pertinent. Both GHR and TACE are expressed ubiquitously, including in liver, an organ widely believed to be a major source of GHBP (Mercado et al. 1994, Maskos et al. 1998).

**TACE as a GHBP sheddase**

Based on the hypothesis that TACE may be involved in GHR proteolysis, we examined cells derived from the TACE knockout mouse. The TACE knockout mouse lacks the Zn-binding domain of TACE; it is totally devoid of TACE enzymatic activity and thus represents a functional TACE null mutant (Peschon et al. 1998a). It has a

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**Figure 1** Ribbon diagram of the extracellular structure of the dimerized GHR-GH complex. The outside of the cell membrane is indicated by the bubble surface at the bottom. One GHR on the left (green) and another on the right (blue) are shown together with bound GH (red). The compact ligand-binding domains as well as the dimerization domains (towards the bottom) are evident. The stem section between the cell membrane and the dimerization domain spans a linear sequence of approximately ten amino acids. Reprinted and adapted with permission from de Vos et al. 1992 (copyright 1992, American Association for the Advancement of Science; http://www.sciencemag.org).

**Table 1** The metzincin family: zinc-dependent metalloproteinases

| Astacins (e.g. bone morphogenetic protein 1) | Matrixins (e.g. collagenase) |
| Snake venom metalloproteinases | ADAMs (A Disintegrin And Metalloproteinase) |
| Serralysins (Bacterial proteases) |

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phenotype resembling the TGF-α knockout or EGF receptor knockout mouse, including open eyelids at birth, stunted and aberrant hair growth, and epithelial disorganization in multiple organs, including placenta, growth retardation, and pre-/perinatal mortality in 97% of animals (Peschon et al. 1998a). Interestingly, this phenotype does not result from deficiency of soluble TNF-α, but rather from failure to generate soluble TGF-α and EGF receptor ligands from their membrane-bound precursors due to lack of TACE activity. TNF-α knockout mice (or TNF receptor knockout mice) have no overt phenotype unless challenged with infectious agents (Pasparakis et al. 1996, Marino et al. 1997, Peschon et al. 1998b), whereas TGF-α- and EGF receptor-deficient mice resemble the TACE knockout mouse (Luetteke et al. 1993, Mann et al. 1993, Miettinen et al. 1995). Fibroblasts from TACE knockout mice (TACE null cells), transfected with the highly cleavable rabbit GHR, fail to generate any detected GHBP, either basally or after exposure to PMA (Zhang et al. 2000). Transfection of TACE null cells with TACE cDNA restores their ability to generate GHBP and GHR remnant, thereby strongly implicating TACE as an important GHBP sheddase (Zhang et al. 2000). The fact that the reconstituted shedding activity can be inhibited by IC3 further corroborates this conclusion (Zhang et al. 2000). Whether other metalloproteinases may also contribute to GHBP shedding in other cell systems remains to be determined.

We wish to emphasize that most of the experiments listed above have been performed with pharmacological agents (PMA, etc.) and relatively little is known about the dynamics of constitutive GHR proteolysis. PMA is considered an excellent surrogate for its physiological counterpart, diacyl-glycerol, in stimulating protein kinase C (PKC) pathways. We have shown that physiological agents, such as platelet-derived growth factor (PDGF) and calf serum, can also induce GHR proteolysis and GHBP shedding (Guan et al. 2001). Furthermore, constitutive GHR proteolysis is also blocked by metalloproteinase inhibitors (Alele et al. 1998, Zhang et al. 2000, 2001, Guan et al. 2001). Thus, we believe that physiological shedding is mediated by the same or similar mechanisms as PMA-induced shedding.

### Regulation of GHBP shedding

The regulation of TACE activity and GHR proteolysis is just beginning to be studied. Initial experiments showed that PKC inhibitors completely block GHBP shedding, whereas MEK inhibitors exert partial inhibition (Guan et al. 2001). These observations indicate that PKC pathways are critical for TACE activation, and that ERK-MAPK pathways also play a lesser role. The mechanistic link between PKC or MAPK activation and TACE activation remains to be elucidated. Whether or not native metalloproteinase inhibitors (TIMPs) are important in regulating GHR proteolysis remains to be determined. TIMP-3 is known to bind and inhibit TACE (Amour et al. 1998). Of interest, we have found that GH itself has an effect on GHR proteolysis. As indicated above, GH binding to the GHR induces GHR dimerization, and the dimerized GHR is much more resistant to proteolysis than the monomeric GHR (Zhang et al. 2001). Thus, GH

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Table 2 Linear sequences proteolytically cleaved by TACE

<table>
<thead>
<tr>
<th>Cleavage</th>
<th>Genbank accession No.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>…SPLAQVVRSSSR…</td>
<td>M10988</td>
</tr>
<tr>
<td>TGF-α</td>
<td>…HADLLAVVAASQ…</td>
<td>K03222</td>
</tr>
<tr>
<td>L-selectin</td>
<td>…CQKLDKSPSMIK…</td>
<td>BC020758</td>
</tr>
<tr>
<td>Interleukin-6 receptor</td>
<td>…TSLPVQ DSSSVP…</td>
<td>X12830</td>
</tr>
</tbody>
</table>

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Figure 2 Domain structure of TACE. The zinc active site is shown as ‘Zn’. EGF denotes the EGF-like domain, TM the transmembrane domain.
exerts a negative effect on GHR cleavage/GHBP shedding by promoting GHR dimerization.

Figure 3 summarizes the GHR cleavage events as presently understood. TACE, activated through PKC- and MAPK-dependent mechanisms, cleaves the (monomeric) GHR in the stem region at a presently unknown site (preliminary experiments have identified a murine GHR cleavage site nine residues amino-terminal to the first intramembranous amino acid (Wang et al. 2002)). This generates the soluble GHBP and a GHR remnant consisting of the transmembrane and intracellular domains. The GHBP is free to diffuse into the pericellular space and ultimately reaches the circulation. The fate of the remnant is as yet incompletely understood. However, very recent experiments have shown that the GHR remnant undergoes another, likely intramembranous, cleavage by a γ-secretase-like enzymatic activity (Cowan et al. 2002), akin to the presenilin cleavage of the amyloid precursor protein implicated in Alzheimer’s disease (Esler & Wolfe 2001), or to Notch remnant processing in Drosophila (Fortini 2001, Selkoe 2001). Whether the processed GHR remnants have biological activity in their own right (e.g. as transcription factors) remains to be determined. This is an intriguing possibility that has a precedent in Notch signaling (Brou et al. 2000).

As mentioned above, there is no known linear consensus sequence for TACE recognition or cleavage. This is also true for the GHR as a substrate. Some GHRs (e.g. rabbit, human) are highly sensitive to proteolytic shedding of GHBP, whereas others (e.g. mouse, rat) are very resistant. There is an about 70-fold difference between rabbit and mouse GHRs with respect to the amounts of GHBP generated in response to PMA (Guan et al. 2001). Indeed, in rodents in vivo, most if not all of the circulating GHBP is thought to be derived from alternative mRNA splicing rather than proteolysis (Sadeghi et al. 1990). Comparison of the amino acid sequences in the juxtamembrane stem reveals no clues for this difference. The structural requirements for GHR cleavability are poorly understood. Of interest, a recent study reported that the length of the extramembrane stem is critically important for cleavage as shortening by three residues abolished cleavability (Conte et al. 2002). This was shown to be due to stem length rather than sequence-specific parameters.
Impact of GHR proteolysis on GH signaling

Metalloproteinase activity has at least three consequences for GH action. First, GHR cleavage causes loss of functional GHRs from the cell surface, a process we call 'receptor decapitation'. This is one form of receptor downregulation; it has been directly demonstrated (Guan et al. 1997). Downregulation is probably important to keep the signaling system from being overstimulated. Secondly, two immediate cleavage products are generated: the GHBP and the GHR remnant. GHBP has its own complex activity, which includes inhibition of GH binding to GHRs through competition for ligand (Mannor et al. 1991) and inhibition of GHR signaling through unproductive GHR/GHBP heterodimer formation, analogous to what is seen with the truncated GHR variant (Ross et al. 1997). In vivo, the GHBP also has enhancing effects on GH action through prolongation of GH half-life and bioavailability (Baumann et al. 1987, Clark et al. 1996). Thirdly, the GH remnant is a potentially important modulator of GH action through as yet to be defined intracellular mechanisms. Further proteolytic processing of the GHR remnant clearly occurs (Cowan et al. 2002), and some of the products may possess bioactivities of their own, as suggested by precedents in similar systems (see above).

To be biologically relevant, GHR cleavage should be required to be subject to regulation. Initial studies of this issue have identified physiological regulatory factors in both a stimulatory (PDGF, serum) and inhibitory (GH) direction. The field of metalloproteinase regulation of GH action is still in its infancy, and additional insights are likely to accrue. The GHR/GHBP/GHR remnant trio is at present the most extensively studied metalloproteinase-dependent system in endocrinology; it should lead the way to discovery of other hormone receptor systems that are subject to modulation by metalloproteinases.

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