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GPA2 and GPB5: Dispensable or indispensable monomers or subunits of a putative heterodimer?

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Abstract

Glycoprotein hormone alpha 2 (GPA2) and glycoprotein hormone beta 5 (GPB5) have been revealed because of their homologies with glycoprotein hormone (GPH) α and β subunits respectively. Because of these homologies, GPA2 and GPB5 have been claimed to form, like GPH subunits, a non-covalent heterodimer known as *Thyrostimulin*, because it was found to activate the thyroid-stimulating hormone receptor (TSHR) *in vitro* in vertebrates. However, the real biological function(s) of GPA2 and GPB5, and whether they form functional heterodimers under physiological conditions, remain uncertain. Transcriptional and histological evidence from various species shows that GPA2 and GPB5 are most often expressed in different cell types, thus not allowing the formation of a heterodimer. When GPA2 and GPB5 are synthesized in the same cells, a GPA2/GPB5 heterodimer can form but shows very low stability. Therefore, it is more likely to act as a local paracrine or autocrine signaling rather than systemic endocrine action. In invertebrates, the *gpa2* and *gpb5* genes are located at the same locus without any intervening genes. Notably, the Apocrita clade appears to have lost this locus, and consequently may have lost both *gpa2* and *gpb5* genes. Therefore, it is of particular interest to predict the physiological consequences resulting from the absence of GPA2 and GPB5 in this group.

Keywords: Apocrita, Endocrine evolution, Glycoprotein hormone, TSH receptor, Thyrostimulin.

1. Introduction

The *gpa2* and *gpb5* genes have been identified in most invertebrate and vertebrate species through their sequence homologies with those of the α and β glycoprotein hormone (GPH) subunits, respectively (Hsu *et al.*, 2002; Nakabayashi *et al.*, 2002). Therefore, GPA2 and GPB5 proteins belong to the cystine-knot growth factor family, like the subunits of vertebrate GPHs Luteinizing Hormone (LH), Follicle-Stimulating Hormone (FSH), Thyroid-

Stimulating Hormone (TSH), and Chorionic Gonadotropins (hCG, eCG) (Alvarez *et al.*, 2009; Schwarz, 2017). For the time being, the roles of GPA2 and GPB5, either alone or as GPA2/GPB heterodimers, are not clear (Cahoreau *et al.*, 2015; Dos Santos *et al.*, 2011; Querat, 2021). The GPA2/GPB5 heterodimer, when present, has been named "*Thyrostimulin*" in the princeps paper because of its TSH-like activity (Nakabayashi *et al.*, 2002). Occasionally, it has also been named "corticotroph-derived glycoprotein hormone" because of its production by the pituitary corticotroph cells as well as in the skin, testis, and retina (Okada *et al.*, 2006).

1.1. GPA2 and GPB5: Thyrostimulin subunits or independent factors?

Glycoprotein hormones (GPHs) consist of two subunits, α and β, which are polypeptide chains bearing oligosaccharide side-chains. They are co-synthesized in the endoplasmic reticulum, where they assist each other in folding to form the functional non-covalent heterodimers. Although non-covalently associated, the subunit-formed hormones (LH, FSH, TSH, CG) are stabilized by a β-subunit "seatbelt" embracing its α-partner (Figure 1). The formation of the seatbelt structure significantly enhances the stability of GPH α/β heterodimers (Lapthorn *et al.*, 1994).

Since 2002, a fifth member of the GPH family has been identified in the human pituitary gland and named *Thyrostimulin* because of its ability to activate the human TSH receptor (TSHR) (Vieira *et al.*, 2022; Tuncel M, 2017; Boutin *et al.*, 2016; de Lloyd *et al.*, 2010), leading to increased cAMP production, intracellular calcium concentration (Nagasaki *et al.*, 2006), and serum thyroxine levels (Okada *et al.*, 2006; Nakabayashi *et al.*, 2002). The *Thyrostimulin* subunits, GPA2 and GPB5, are chemically similar to each other but distinct from the conventional GPH α and β subunits. GPA2 shares 35% amino acid sequence identity with the common α subunit (GPA1), whereas GPB5 exhibits 27–32% homology with the β subunits of TSH, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and chorionic gonadotropin (CG) (Okajima *et al.*, 2008). The discovery of GPA2 and GPB5 as potential new GPH subunits, based on their structural similarity to other known glycoprotein hormone subunits (Hsu *et al.*, 2002), suggests an evolutionarily conserved signaling system that extends beyond the traditional gonadotropins and thyrotropins in vertebrates but is absent in invertebrates, where GPH is not present.

To form the heterodimeric structure of *Thyrostimulin*, both *gpa2* and *gpb5* genes must be co-expressed in the same cell, allowing their protein products, GPA2 and GPB5, to be co-synthesized and enter the endoplasmic reticulum together where they will take their 3-dimensional structure and get Post-Translation Modifications among which glycosylations (Bousfield et al., 2019). For gaining their 3D structure GPA2 and GPB5, like GPH subunits, act as chaperones to each other (Alvarez et al., 2009). Notably, for proper quaternary structure stabilization, GPHs require a structural motif known as the β-seatbelt (Figure 1). However, studies have shown that in all examined species, GPB5 lacks the region corresponding to the β-seatbelt found in classical GPH β-subunits (Figure 1) (Cahoreau *et al.*, 2015; Nakabayashi *et al.*, 2002). Indeed, the present analysis indicated that the GPA2/GPB5 heterodimer is considerably less stable than the GPH heterodimers during SDS-PAGE analyses (Alvarez *et al.*, 2009).

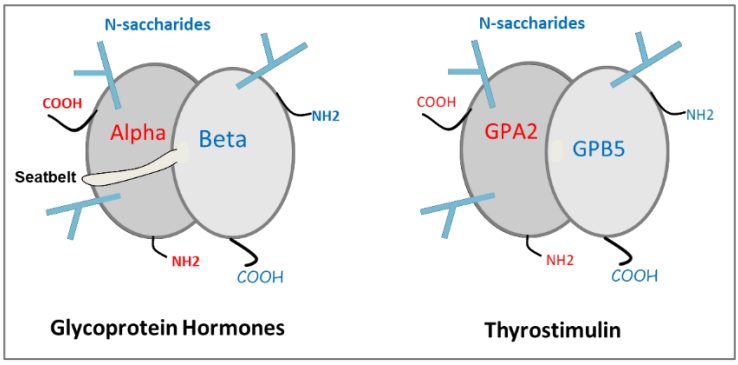

Figure 1. Structures of Glycoprotein Hormones and Thyrostimulin
The presence of a seatbelt in the β subunits of GPHs embracing their α counterpart considerably slows down the dissociation of the α / β dimers. The absence of such a seatbelt in GPB5 leads to a highly unstable GPA2/GPB5 heterodimer.

In addition, the number of oligosaccharide chains on GPA2 and GPB5 varies across species. In humans, GPA2 contains two N-linked glycosylation sites at Asn14 and Asn58, while GPB5 carries one at Asn63 (Ji *et al.*, 1997; Thotakura and Blithe, 1995). In contrast, in some species, such as insects and nematodes, GPA2 lacks oligosaccharides, and GPB5 is also devoid of them in mosquitoes and flies (Dos Santos *et al.*, 2009). Interestingly, Okajima et al. (2008) found that disruption of the N-glycosyl sites of both glycan chains on human GPA2 reduces *Thyrostimulin*'s ability to activate the TSH receptor, though it does not affect its cross-linking with GPB5, using divalent reagent (Okajima *et al.*, 2008). This contrasts with classical glycoprotein hormones (GPHs), where oligosaccharides are essential for proper heterodimer formation. For example, the absence of the glycan at Asn52 in GPA1 (α subunit) of hCG was shown to reduce dimerization with CGβ by up to 30% (Matzuk and Boime, 1988). These findings suggest that the removal of oligosaccharides from both GPA2 and GPB5 lowers their expression levels and highlights the critical role of glycans receptor activation (Okajima *et al.*, 2008). However, these experiments do not prove at all that GPA2 and GPB5 are actually dimerized to promote the observed bioactivity since the "dimerization" was assessed after bifunctional reagent action whereas the bioactivity was determined without such a modification thus possibly by the GPA2 and GPB5 monomers.

In many species where GPA2 and GPB5 have been identified, biological activity has only been demonstrated when the two glycoproteins are tethered, such as in the nematode *Caenorhabditis elegans* (Oishi *et al.*, 2009), the mosquito *Aedes aegypti* (Rocco and Paluzzi, 2020; Paluzzi *et al.*, 2014; Sellami *et al.*, 2011), the prawn *Macrobrachium rosenbergii* (Wahl *et al.*, 2022), the sea hare *Aplysia californica* (Heyland *et al.*, 2012), the amphioxus *Branchiostoma japonicum* (Wang *et al.*, 2018), the lamprey *Petromyzon marinus* (Hausken *et al.*, 2018), the catshark *Scyliorhinus canicula* (Jeanne *et al.*, 2024), as well as in the mouse (Heyland *et al.*, 2012) and rat (Nagasaki *et al.*, 2006). In most of these studies, GPA2 and GPB5 required artificial tethering using a bifunctional reagent to confirm heterodimer formation via SDS-PAGE. As a result, it remains uncertain whether GPA2 and GPB5 function naturally as heterodimers or independent monomers *in vivo*.

1.2. The issue of the endocrine or paracrine role of the GPA2/GPB5 heterodimer

Some studies suggest that *Thyrostimulin* may have co-evolved with and interacted with the ancestral glycoprotein hormone receptor (Hausken *et al.*, 2018; Brokken *et al.*, 2005; Hsu *et al.*, 2002). However, its exact mechanism of action and physiological functions remain

unclear. To date, heterodimeric *Thyrostimulin* has never been isolated from blood or tissues from any species to prove its existence. Instead, all studies rely only on recombinant *Thyrostimulin* to evaluate its binding affinity, receptor activation, and *in vivo/in vitro* effects (Wang *et al.*, 2018; Buechi and Bridgham, 2017; Okada *et al.*, 2006; Sudo *et al.*, 2005; Nakabayashi *et al.*, 2002).

Thyrostimulin has been proposed to exhibit several potential physiological or pathological roles, including cancer, immunity, and reproduction (Karponis and Ananth, 2017). Although it has been hypothesized to act as a pituitary endocrine hormone in lampreys (Hausken *et al.*, 2018; Sower *et al.*, 2015; Sower *et al.*, 2006), there is no clear evidence that this endocrine role is conserved in whale sharks or mammals (Dos Santos *et al.*, 2009; Nagasaki *et al.*, 2006). The initial report on *Thyrostimulin* proposed a paracrine mechanism of action, where the heterodimer is locally secreted within the pituitary and acts on stellate cells via their TSH receptors (TSHR) (Nakabayashi *et al.*, 2002). The presence of TSHR in both thyroidal and extrathyroidal tissues, along with the widespread expression of GPA2 and GPB5 across different tissues, supports the potential for paracrine signaling (Sun *et al.*, 2010; Okada *et al.*, 2006; Nakabayashi *et al.*, 2002). Furthermore, earlier studies suggest that GPA2 and GPB5 can only form heterodimers at supraphysiological concentrations in the circulation, reinforcing the idea that *Thyrostimulin* may act more as a short-range paracrine factor than a genuine endocrine hormone (Alvarez *et al.*, 2009).

Thyrostimulin has also been proposed to act via a paracrine mechanism in bone (Bassett *et al.*, 2015); GPA2 and GPB5 are expressed in neonatal bone but rapidly decline thereafter. Their mRNAs are found in both osteoblasts and osteoclasts at varying levels. In GPB5-deficient mice, increased bone mass and mineralization have been observed, attributed to enhanced osteoblast activity. However, *Thyrostimulin* does not activate major signaling pathways (Akt, ERK, or p38) or directly influence osteoblast function, despite its high affinity for TSHR (Bassett *et al.*, 2015). Another study has also shown that, in the rat ovary, *Thyrostimulin* can be secreted and act as a local paracrine factor that activates cAMP influx and the nuclear c-fos response in granulosa cells via the TSH receptor (TSHR). Moreover, *Thyrostimulin* significantly enhances cAMP production and c-fos gene expression in the presence of gonadotropins (Sun et *al.*, 2010).

In contrast, a study in *Drosophila* found that GPA2 and GPB5 are produced by the same neuroendocrine cell; however, these cells do not directly project to target organs, such as the hindgut, Malpighian tubules, or salivary glands, where their putative receptors are claimed to be expressed (Sellami *et al.,* 2011). This suggests that the GPA2 or GPB5 monomers or GPA2/GPB5 heterodimers do not act as neuroendocrine factors in this species. Based on the role of cAMP in antidiuretic activity in the insect hindgut, the GPA2/GPB5 heterodimers were proposed as an antidiuretic hormone, functionally similar to aldosterone or vasopressin in vertebrates (Sellami *et al.,* 2011). However, since their receptors are also expressed in other tissues, GPA2, GPB5, or GPA2/GPB5 may have additional functions beyond fluid balance regulation.

In the *Aedes aegypti* mosquito, GPA2 and GPB5 are co-expressed in neural cells but do not naturally form a heterodimer. A tethered GPA2/GPB5 construct was used to mimic dimerization, revealing that the so-fused GPA2 and GPB5 can activate the LGR1 receptor via the Gi/o signaling pathway (Rocco and Paluzzi, 2020). These findings suggest that heterodimerization would be a prerequisite for the biological activity of GPA2/GPB5. However, the absence of activity without artificial linking of GPA2 and GPB5 does not permit us to conclude that they form a stable heterodimer *in vivo*.

1.3. Absence of GPA2 and GPB5 in Apocrita

In invertebrates, recombinant GPA2 and GPB5 (or their putative GPA2/GPB5 heterodimer) have only been studied in a very limited number of species but have been claimed to play potential roles in development, minerals, water balance, and reproduction (Rocco and Paluzzi, 2020; Rocco and Paluzzi, 2016; Heyland *et al.*, 2012; Paluzzi *et al.*, 2014; Sudo *et al.*, 2005; Al-Dailami *et al.*, 2002a; Al-Dailami *et al.*, 2002b). In the quest to answer the question of the roles of GPA2 and GPB5, we found it interesting to consider a unique group lacking *gpa2* and *gpb5* genes.

We performed a BLAST search in UniProt and identified only a single protostome sequence for each gene: GPA2 (A0A0A1X2W0) from *Zeugodacus cucurbitae* (melon fruit fly) and GPB5 (A0A034WS09) from *Dacus dorsalis*. These sequences were then used as BLAST queries against the Hymenoptera Genome Database (Hymenoptera Mine v1.6) (Walsh *et al.*, 2022; Elsik *et al.*, 2018) using SequenceServer (Priyam *et al.*, 2019) to identify potential homologs.

BLAST analysis detected GPA2 and GPB5 sequences only in Neodiprion (Tenthredinoidea) and *Cephus cinctus* (Cephoidea), both belonging to Symphyta, within the Hymenoptera Genome Database. No homologs were found in Apocrita, a much larger suborder within Hymenoptera that includes approximately 100,000 described species (bees, wasps, ants, etc.) (Figure 2), suggesting that these proteins may be evolutionarily restricted to this clade. In summary, the *gpa2* and *gpb5* genes appear to be present across all Bilateria, including Orussidae, but absent in Apocrita (the sister group of Orussidae, as shown in Figure 3).

GPA2

# Similar sequences	Query coverage (%)	Total score	E value	Identity (%)
1. gnl|Ccin1v2|XP_015608408.1	77	325	4.58×10^{-37}	65.6%
2. gnl|iyNeoLeco1.1|XP_046590270.1	93	155	8.04×10^{-12}	32.1%
3. gnl|iyNeoVirg1.1|XP_046608824.1	86	153	1.62×10^{-11}	32.7%
4. gnl|iyNeoFabr1.1|XP_046417468.1	86	150	3.90×10^{-11}	31.7%
5. gnl|iyNeoPine1.1|XP_046472622.1	37	113	4.93×10^{-6}	44.2%

GPB5

# Similar sequences	Query coverage (%)	Total score	E value	Identity (%)
1. gnl|Ccin1v2|XP_015608427.1	82	331	1.15×10^{-38}	59.8%
2. gnl|iyNeoPine1.1|XP_046473037.1	92	120	6.53×10^{-7}	30.4%
3. gnl|iyNeoLeco1.1|XP_046590384.1	92	120	6.73×10^{-7}	30.4%
4. gnl|iyNeoFabr1.1|XP_046416969.1	92	113	6.89×10^{-6}	30.4%
5. gnl|iyNeoVirg1.1|XP_046610650.1	92	113	7.11×10^{-6}	30.4%

Figure 2. GPA2 and GPB5 detected in HymenopteraMine

Ccin: Cephus cinctus; iyNeoPine: Neodiprion pinetum; iyNeoLeco: Neodiprion lecontei; iyNeoFabri: Neodiprion fabricii; iyNeoVirg: Neodiprion virginianus

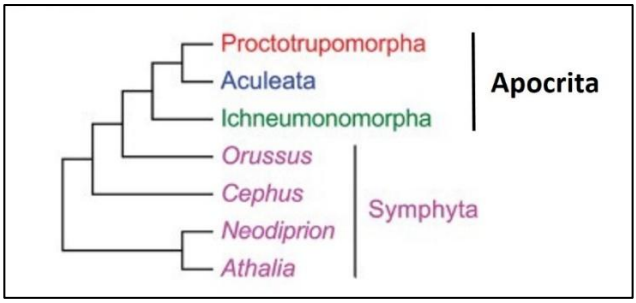

Figure 3. Evolution of Hymenoptera

gpa2 and gpb5 are found in the genomes of the Symphyta, which form a paraphyletic group, whereas Ichneumonomorpha + Aculeata + Proctotrupomorpha form the monophyletic group Apocrita. (Adapted from Robertson et al., 2018 and Tvedte et al., 2017).

Cephoidea represents the closest extant lineage to Orussoidae and Apocrita (Peters et al., 2017). Members of the Cephoidea have retained a number of ancestral insect genes that were subsequently lost in Apocrita, including several membrane chemoreceptors (Robertson et al., 2018). It is therefore likely that the *gpa2* and *gpb5* genes were also lost during evolution, at some point between the divergence of Cephoidea and Apocrita. Using a bioinformatics approach, Hauser et al. screened the recently sequenced genome of the parasitic wasp *Nasonia vitripennis* (Apocrita) and demonstrated that the glycoprotein hormone GPA2/GPB5 is absent in this species (Hauser *et al.*, 2010). The conservation of these genes has been shown by numerous studies to play crucial roles in the development and/or physiology of most metazoans (Okada et al., 2006; Macdonald et al., 2005). This raises the intriguing question of how Apocrita could have adapted and survived in the absence of these genes.

As discussed above, GPA2 and GPB5 are generally believed to form the *Thyrostimulin* heterodimer, which exhibits thyroid-stimulating hormone (TSH)-like activity, although the TSH receptor (TSHR) itself is only present in vertebrates (Okada *et al.*, 2006; Hsu *et al.*, 2002; Nakabayashi *et al.*, 2002). In invertebrates, which lack TSHR, these subunits have been implicated in a variety of physiological processes (Bassett *et al.*, 2015; Alvarez *et al.*, 2009; Okada *et al.*, 2006; Nakabayashi *et al.*, 2002). Identifying a unique physiological feature in Apocrita associated with the absence of GPA2 and GPB5 would be of considerable interest, albeit unlikely. Therefore, we focus on exploring subtle evolutionary or physiological differences between Apocrita and other hymenopterans that might account for the loss of GPA2 and GPB5 in the former and their retention in the latter.

Morphology: The main external morphological difference between Symphyta and Apocrita is that the former taxon does not have a "wasp waist" or "petiole" in contrast to the latter. As for now, no relationship between the occurrence of wasp waist and the loss of GPA2 and GPB5 in wasps, ants, and bees can be evidenced.

Sex determination: A prominent feature of Hymenoptera species is their haplodiploid sex-determination system: females are diploid, and males are haploid (they develop parthenogenetically from unfertilized eggs). Haplodiploid species have significantly lower chromosome numbers than diploid species (Blackmon *et al.*, 2015). It is thus tempting to hypothesize that GPA2 and GPB5, in most other Bilateria, play a role in the control of chromosome segregation during meiosis or in the early steps of sex determination during development. Sperm production starting from the haploid state of Apocrit males could produce haploid spermatozoa, with only mitotic divisions. However, meiotic reduction must

occur in diploid females except if the germ cell line remains haploid. If not, it is interesting to point out that the number of centrioles in haploid and diploid cells in the wasp *Anisopteromalus calandrae* is the same (Uzbekov *et al.*, 2024). Haplodiploid sex determination also exists in many arachnids (some mites and ticks) and has evolved multiple times in Acari. Therefore, the loss of GPA2 and GPB5 does not appear to be specifically related to haplodiploid sex determination.

Social behavior and odorant receptors: A most interesting feature of Apocrita is their eusocial behavior. In Hymenoptera, the correlation between the number of odorant receptors and eusociality is a matter of discussion (Gautam *et al.*, 2024). All the known species of eusocial ants, termites, bees, and wasps comprise only about two percent of the one million known insect species. Nevertheless, eusociality exists in non-apocrita hymenoptera species as well as non-hymenopteran species (including the mammal mole-rat) (Wilson and Nowak, 2014) and inversely, several Hymenopteran species are non-eusocial. Therefore, the specificity of the absence of *gpa2* and *gpb5* genes in Hymenoptera cannot be responsible for their social structure. Likewise, there is no correlation between the extent of odorant receptor repertoire and the presence of *gpa2* and *gpb5* genes.

Digestive tract: The digestive systems of Hymenoptera are similar to those of their ancestors. However, the development of parasitoid habits might be a cause of the simplification of the digestive system in the Apocrits compared to Tenthredinoids (Uzbekov *et al.*, 2024). Evolutionary trends toward parasitism led to changes in midgut enzyme compartmentalization and the loss of midgut caeca (replaced in function by the anterior midgut). This simplification might have arisen through losing a locus to which GPA2 and GPB5 belonged and, maybe, without any role for the products of these genes in the digestive system (Terra and Ferreira, 2020). Nevertheless, a possible role for GPA2 and GPB5 in the digestive tract has been previously proposed in the mosquito *Aedes aegypti* (Paluzzi *et al.*, 2014), suggesting a functional role in the digestive tract in insects except in the Apocrita.

Parasitism: Symphyta are the most basal Hymenoptera, with Orussoidea as the sister group of Apocrita (Robertson *et al.*, 2018; Peters *et al.*, 2017, Tvedte *et al.*, 2017). From an ancestral form that parasitized insect larvae in wood, Hymenoptera have since diversified greatly in both parasitic strategies and host range, even incorporating viruses to manipulate their hosts (Polaszek and Vilhemsen, 2023). Because most Apocrita are parasitic, it has been hypothesized that they may compensate for the loss of these genes by relying on their hosts. However, the absence of these genes is also found in non-parasitic species, making it difficult to determine the specific role of GPA2/GPB5 in parasitism.

1.4. Conclusion and Perspectives

Understanding the evolutionary origin of unique features found in the wasps, ants, and bees, such as the 'wasp waist' (petiole) absent from most Orussidae, or other distinct characteristics, could be informative for the search for a possible relationship with the loss of *gpa2* and *gpb5* genes in Apocrita. At the present stage, it cannot be concluded from the available data what the indispensable role of GPA2 and GPB5 is (in Symphita and other non-Apocrita Bilateria) and whether they act independently or as a paracrine heterodimer. The absence of a clear phenotype in *gpb5*$^{-}$ mice does not support a prominent role for GPB5 or a putative GPA2/GPB5 heterodimer during development. The reason for the outstanding conservation of these two molecules during the evolution of all Bilateria, except Apocrita, remains elusive. The obtention and analyses of *gpa2*$^{-}$ and *gpa2*$^{-}$/*gpb5*$^{-}$ mice would of very high interest to decipher whether GPA2 and GPB5 play similar roles, or independent complementary roles or act as heterodimers. Although, GPA2 and GPB5 are present in all Bilateria except Apocrita, this latter taxon eventually gave rise to the most successful highly-organized insect

societies.

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Conflict of interests

The authors affirm that they have no competing interests to disclose.

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