Mutations in the *Drosophila* ortholog of the vertebrate Golgi pH regulator (GPHR) protein disturb endoplasmic reticulum and Golgi organization and affect systemic growth

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Summary

Sorting of secretory cargo and retrieval of components of the biosynthetic pathway occur in organelles such as the Golgi apparatus, the endoplasmic reticulum and the endosomes. In order to perform their functions in protein sorting, these organelles require a weakly acidified lumen. In vitro data have shown that Golgi luminal pH is in part regulated by an anion channel called Golgi pH Regulator (GPHR). Mammalian cells carrying a mutated GPHR version present an increased luminal pH leading to delayed protein transport, impaired glycosylation and Golgi disorganization. Using *Drosophila* as a model system, we present here the first phenotypic consequences, at the organism level, of a complete lack of GPHR function. We show that, although all individuals carrying complete loss-of-function mutations in the *dGPHR* gene can go through embryonic development, most of them die at late larval stages. The *dGPHR* mutations are, however, sublethal and can therefore generate escapers that are smaller than controls. Using cellular and molecular readouts, we demonstrate that the effects of *dGPHR* mutation on larval growth are not due to Insulin signaling pathway impairment and can be rescued by providing dGPHR in only some of the larval tissues. We reveal that, although functionally exchangeable, the invertebrate and vertebrate GPHRs display not completely overlapping sub-cellular localization. Whereas the mammalian GPHR is a Golgi-only associated protein whose inactivation disturbs the Golgi apparatus, our data suggest that dGPHR is expressed in both the ER and the Golgi and that dGPHR mutant flies have defects in both organelles that lead to a defective secretory pathway.

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Key words: GPHR, *Drosophila*, Golgi, Growth control, Endoplasmic reticulum

Introduction

The main function of the Golgi apparatus is to sort molecules that are transported through this organelle en route to the plasma membrane, the extracellular medium and the endosomal/lysosomal compartments. As for each individual organelle, the function of the Golgi apparatus depends on the establishment and stringent maintenance of a distinct pH (Casey et al., 2010). In mammalian cells, luminal acidification of the Golgi apparatus is essential for its function and to maintain cellular homeostasis (Weisz, 2003). Indeed, when the acidic luminal pH is artificially alkalized, the trafficking, processing and glycosylation of cargo proteins and lipids are impaired. As a result, some proteins become misrouted and the morphological integrity of the Golgi is compromised (Axelsson et al., 2001; Rivinoja et al., 2006). Consequently, mutations that alter Golgi luminal pH have been shown to perturb cell metabolism leading to congenital diseases and cancer (Weisz, 2003). The acidic pH of organelles, including the Golgi, is regulated by a balance between the proton pump V-ATPase, which is the sole proton delivery source, and the counterion channel GPHR (Moriyama and Nelson, 1989; Nishi and Forgac, 2002; Schapiro and Grinstein, 2000; Wu et al., 2001). This voltage dependent anion channel dissipate the membrane potential formed by the proton influx allowing proton pump to transfer more protons into the Golgi lumen and therefore facilitating Golgi luminal acidification and hence Golgi function. Cells in which GPHR is inactive show elevated luminal pH of the Golgi but not of the endosomal/lysosomal compartments (Maeda et al., 2008). Using a keratinocyte-specific GPHR-knockout mice model, Medea and collaborators have recently shown that GPHR is essential for the homeostasis of the epidermis including the formation of lamellar bodies and for establishing its barrier function (Tarutani et al., 2012).

*Drosophila* has recently been established as a good alternative model system to study the Golgi apparatus that shares many morphological and functional similarities with the mammalian one (Kondylis and Rabouille, 2009; Kondylis et al., 2007; Sisson...
et al., 2000). Drosophila is also very suitable to study the anatomical and physiological consequences associated with gene inactivation at the organism level. We present here the molecular and phenotypic characterization of null loss-of-function alleles of the Drosophila ortholog of the mammalian GPHR anion channel. We show that the complete inactivation of dGPHR is not always associated with fly lethality but dramatically impairs its developmental growth independently of the insulin signaling (IS) pathway. We also demonstrate that although dGPHR is indeed the functional ortholog of the human GPHR, its subcellular localization and function differ from the mammalian protein. Whereas mammalian GPHR is a Golgi-only protein required for pH maintenance, the Drosophila ortholog co-localized with markers of both endoplasmic reticulum and Golgi and is required for correct organization of both organelles.

Materials and Methods

Drosophila melanogaster strains and maintenance

The following strains were used in this work: dGPHRLMedFRT1, dGPHR::HA, dGPHRk34, c601-Gal4 (BL#30844), Mef2-Gal4 (BL#72390), daughterless-Gal4 (BL#5460), UAS-Gut:GFP (BL#39007), UAS-KDEL:RFP (BL#7119), UAS-sec-RFP (Letchworth et al., 2009) and chico2 (BL#10738). The UAS-dGPHR, UAS-dGPHR::HA, UAS-huGPHR, UAS-huGPHR::HA and UAS-hams-GPHR flies were obtained by P element mediated insertion of pCASY constructs containing full length cDNA coding for dGPHR, dGPHR::HA, huGPHR, huGPHR::HA and hams-GPHR, respectively (molecular details available upon request).

Mouth hook contractions

Larvae were rinse 30 seconds in PBS and transferred to agar plate and quantified for the frequency of mouth-hook contraction by individual larva at 22°C. Typically, the assay time was 20 min. The contraction frequencies of individual larva remained consistent throughout the assay.

Results

CG8090 encodes the invertebrate ortholog of GPHR

Following an EMS mutagenesis, we isolated an allele carrying a point mutation in a gene referred as CG8090 in Flybase (http://flybase.org). Protein prediction analysis indicates that CG8090 transcript encodes the invertebrate ortholog of GPHR (dGPHR), a ubiquitously expressed protein containing nine transmembrane domains whose AA sequence is conserved among vertebrates, insects and plants (Maeda et al., 2008) and Fig. 1A. Genomic DNA sequencing of the EMS dGPHR allele (called dGPHRk34) revealed the presence of a missense mutation in the dGPHR coding region that substitute an highly conserved proline into a lysine in the third transmembrane domain of the protein (Fig. 1B.C). A second non-complementing allele of dGPHR (dGPHR2LoD674) corresponding to a PiggyBac transposon insertion into the fifth intron of dGPHR was also used in this study. We found that both dGPHRk34 and dGPHR2LoD674 mutations affect dGPHR mRNA stability as demonstrated by qRT-PCR quantification (Fig. 1D).

dGPHR mutant escapers develop into dwarf flies

Phenotypic analysis of dGPHRk34 and dGPHR2LoD674 homozygous mutants and transheterozygous allelic combinations points to an essential role of dGPHR in regulating developmental growth and body size of Drosophila (Fig. 2). When raised in standard medium conditions, most dGPHR mutants die as late larvae. However, some individuals go through the pupal stage and give rise to...
parameters in adult wing blades. As shown in Fig. 2E, both cell size and cell number were reduced in \(dGPHR\) mutant when compared to controls. The similar penetrance and strength of the phenotype observed between \(dGPHR\) homozygous mutants and \(dGPHR^{k34}/Df(2R)ED2426\) or \(dGPHR^{LL03674}/Df(2R)ED2426\) allelic combinations (data not shown) demonstrate that \(dGPHR^{k34}\) and \(dGPHR^{LL03674}\) are complete loss-of-function alleles as far as growth control is concerned.

\(dGPHR\) is not a component of the Insulin pathway

\(dGPHR\) mutant larvae present a reduced growth rate and a developmental delay, which are hallmarks of mutants affecting the Insulin signaling (IS) pathway (Hietakangas and Cohen, 2009). To investigate whether \(dGPHR\) is implicated in IS, we analyzed the expression of two known transcriptional targets of the IS pathway in \(dGPHR\) mutants, namely 4E-BP (Karim and Thummel, 1991), encoding a translational repressor and \(InR\), encoding the Insulin receptor (Puig and Tjian, 2005). Both 4E-BP and \(InR\) transcription is upregulated in response to repressed Insulin signaling. We found that transcription of 4E-BP and \(InR\) mRNAs is unaffected in first instar larvae mutant for \(dGPHR\) (Fig. 3A). This suggests that \(dGPHR\) does not directly act in the IS pathway. However, as expected when larval growth is reduced, \(dGPHR\) mutants displayed reduced Insulin and Ecdysone levels (measured by the transcriptional rate of \(E74B\), a direct target of the Ecdysone receptor), signaling activities in later stages of larval development (supplementary material Fig. S1). To further confirm that \(dGPHR\) does not act via the Insulin pathway, we followed the progeny of \(dGPHR\) mutant mitotic clones in wing imaginal disc. Indeed, previous reports indicated that cells defective in IS pathway component present defective growth rate and hence give rise to smaller clones than wild-type cells (Hietakangas and Cohen, 2009). As shown in Fig. 3B, \(dGPHR\) mutant cells grew and divided as well as neighboring cells giving rise to clones of similar size than the one derived from wild-type cells. Since imaginal cells can compensate their reduced division rate by increasing cell size, we compared the size of \(dGPHR\) mutant and wild-type cells by phalloidin staining and found no major difference (Fig. 3C). It is known that larval growth is largely based on an increase in cell size, which is accomplished by endoreplication, a modified cell cycle, consisting of successive rounds of DNA synthesis without intervening mitoses. We thus investigated the requirement of \(dGPHR\) in endoreplication by analyzing the nuclei size of \(dGPHR\) mutant cells induced during embryogenesis and observing in late third instar larvae (120 hr AED). Although \(dGPHR\) mutant larvae (120 hr AED) display salivary gland cells with reduced nuclei size when compared to controls (supplementary material Fig. S2), no obvious differences in nuclei size were noticed between \(dGPHR\) mutant cells and the wild-type cells, in salivary glands with MARCM clones mutant for \(dGPHR\) (Fig. 3D). Altogether, our data indicate that \(dGPHR\) does not directly act via the Insulin signaling pathway or in the endoreplication process, which are both critical regulators of larval growth. Moreover, by using a food intake assay, such as feeding with colored yeast (data not shown), analyzing metabolic markers (supplementary material Fig. S3A) or quantifying mouth hook contractions (supplementary material Fig. S3B), we could exclude the hypothesis that the smaller sizes of \(dGPHR\) larvae reflect the inability of \(dGPHR\) larvae to feed properly.
dGPHR protein localizes to the endoplasmic reticulum and to the Golgi

The vertebrate GPHR protein has been shown to be localized and to function in the Golgi apparatus (Maeda et al., 2008). In order to test whether its invertebrate ortholog has a similar sub-cellular localization, we overexpressed an HA-tagged version of the dGPHR via the Gal4-UAS system. When expressed in a stripe of cells along the antero-posterior boundary of the wing imaginal disc using the patched-Gal4 driver (Fig. 4A), dGPHR-HA was detectable in two distinct subcellular patterns, one surrounding the nucleus and the other inside the cytoplasm and more punctiform (Fig. 4B,C). Double labeling with specific organelle markers demonstrated that the ring-like staining largely co-localized with the ER marker RFP::KDEL and the punctiform

Fig. 2. Phenotypic characterization of the dGPHR mutants. (A–C) dGPHR mutations affect the size of larvae, pupae and adults. For panel B, the weights of 30 adults male and female were measured. (D) Larval surface over time, pupariation and adult emergence period of control and dGPHR− grown in rich diet medium. (E) Adult wing size, wing cell size and numbers in a given area of wild-type and dGPHR mutant animals. Values indicated by * are statistically significant (p<0.05).
one to a fraction of the Golgi apparatus (labeled with Galt::RFP) (Okajima et al., 2005). Similar expression patterns were observed when the human GPHR was expressed in wing disc cells (Fig. 4D,E).

The vertebrate GPHR proteins are the functional orthologs of dGPHR

The differential subcellular localization of GPHR in human and Drosophila cells prompted us to test the functional relationship between the vertebrate and invertebrate GPHRs. For that, we asked whether the vertebrate GPHR protein could functionally compensate for the lack of dGPHR function in flies. To do so, the human or the hamster GPHR proteins were expressed in dGPHR mutant flies using a ubiquitous Gal4 driver (daughterless-Gal4) and the weight of the emerging adult flies was used as a readout of body size. As shown in Fig. 5A, expression of the dGPHR protein or its two vertebrate orthologs was sufficient to fully rescue dGPHR mutant growth defects and developmental lethality (data not shown). This indicated that the vertebrate GPHRs can compensate for a lack of dGPHR and that these proteins are therefore functional orthologs. Although dGPHR is predicted to be ubiquitously expressed (Flyatlas, http://flyatlas.org), we decided to investigate in which tissue dGPHR function was required to ensure optimal growth during Drosophila development. We took advantage of the ability of UAS-dGPHR transgene to rescue the growth defect of dGPHR

![Graph](image1)

![Image](image2)

![Image](image3)

**Fig. 3. dGPHR mutations do not impact the Insulin pathway signaling.** (A) mRNA quantification by qRT-PCR of two transcriptional targets of the IS pathway. dGPHR mutations do not affect InR and 4E-BP transcription. (B–D) Confocal microscope section of a third instar wing imaginal disc containing mitotic clones of dGPHR<sup>k34</sup> mutant cells (absence of green staining), and wild-type (bright green staining) sister clones. (B) dGPHR mutant clones and wild-type sister clones have similar size. (C) Size of wild-type and dGPHR mutant cells labeled with phalloidin is similar. (D) Nuclei size of salivary gland cells is not affected by a mutation in the dGPHR. Scale bars: 50 μm (B,D), 20 μm (C).
dGPHR inactivation affects both ER and Golgi organization and impairs protein secretion

We then tested whether dGPHR inactivation could have effects on ER and Golgi organization. Using Galt::RFP and RFP::KDEL as Golgi and ER markers, respectively, we analyzed salivary glands of third instar larvae at 96 hr AED (a time window that precedes the growth defect phenotype of dGPHR mutants, Fig. 2D) and demonstrate that dGPHR inactivation was associated with severe ER and Golgi disorganization phenotypes (Fig. 6A,B). Whereas RFP::KDEL forms a uniform network in wild-type salivary gland cells, it accumulates basally and asymmetrically in the center of the cell in proximity to the nucleus in mutant tissue (Fig. 6A). The global organization of the Golgi apparatus was also affected by dGPHR inactivation. The regular dot-like pattern detected in wild-type cells carrying a Galt::RFP transgene was disrupted in mutant cells although not uniformly. Whereas in some regions of the mutant cells (labeled ‘a’ in Fig. 6B), Golgi structure appears similar to control, in others (labeled ‘b’), Galt::RFP staining appears more fragmented and fainter than in controls. To insure that these effects were not an indirect consequence of the dGPHR mutation on growth retardation (i.e. on Insulin signaling reduction), we tested whether similar phenotypes can be observed in chico" mutant salivary glands (at 96 hr (data not shown) and 120 hr AED). As shown in supplementary material Fig. S4, chico" salivary gland cells present a homogeneous distribution of both RFP::KDEL and Galt::RFP, similar to wild-type glands. To further characterize the defaults observed in organelles associated with the secretory pathway, wild-type and mutant cells were labeled with the ER exit site marker sec16. As already observed with Golgi and ER markers, the distribution of sec16 positive structures was quite heterogeneous in mutant cells. Whereas in some compartments of mutant cells, tER sites were uniformly scattered and spaced (labeled ‘a’ in Fig. 7B) as in wild-type controls, in others (labeled ‘b’) the sec16 positive structures were more fragmented (Fig. 7B). The cell compartments in which sec16 staining was fragmented correspond to domains in which RFP::KDEL was abnormally accumulated (Fig. 7B). This disorganization of ER was confirmed using electron microscopy. Whereas TEM pictures of wild-type salivary gland cells present large vesicles of secretion surrounded with well-organized ER ribbon, the cytoplasmic organization of dGPHR mutant cells was strikingly different (supplementary material Fig. S5). The size of secretory granules was 5 times smaller in mutant than in wild-type cells. In addition, the very well-organized ER ribbon with associated ribosomes seen in wild-type cells was no longer detectable in dGPHR mutant cells. The abnormal ER organization associated with dGPHR mutation, led us to test the functionality of secretory pathway. For this purpose, we make use of a sec::GFP reporter construct that allows us to visualize the secretory process in vivo. In contrast to control cells in which the sec::GFP protein is present throughout the cytoplasm in a uniform pattern, its expression pattern was very similar to that of the ER markers in dGPHR mutant cells (supplementary material Fig. S5). Altogether, these data indicate that in the absence of functional dGPHR, both the Golgi apparatus and the ER are disorganized indicating that the dGPHR protein is required to shape the ER-Golgi apparatus and that its inactivation affect the secretory pathway functionality.

Discussion

We report here the first analysis of a Golgi pH Regulator mutant in Drosophila melanogaster. In contrast to what is expected for a loss-of-function mutation in a gene thought to be involved in fundamental cellular processes, complete removal of maternal (data not shown) and zygotic dGPHR function is not fully lethal and can generate viable flies. This is rather unexpected knowing...
Fig. 5. The dGPHR is the functional ortholog of the mammalian GPHR. Rescue experiment of the dGPHR mutant phenotype through ubiquitous (A) or tissue specific (B) overexpression of the Drosophila and mammalian GPHR cDNAs. The hamster and human GPHR cDNAs are as efficient as the Drosophila ortholog to rescue the growth defects observed in dGPHR mutant flies (A). This indicates that the vertebrate and invertebrate orthologs are functionally equivalent. (B) Whereas overexpression of the dGPHR cDNA in muscle is not sufficient to rescue dGPHR mutant growth defects, gut or neuronal overexpressions are. Values indicated by * are statistically significant (p < 0.05).

Fig. 6. dGPHR inactivation induces ER and Golgi disorganization. Wild-type and dGPHR mutant salivary gland cells carrying an endoplasmic reticulum marker (RFP::KDEL, A), a Golgi marker (Galt::RFP, B). (A) The RFP::KDEL marker is uniform in control cells and concentrated both basally and nearby the nuclei in mutant cells. (B) The Galt::RFP expression pattern is form a regular network in control cells. In mutant cells, Galt::RFP staining is heterogeneous within the cells. In some regions, such as region ‘a’, Galt::RFP expression is similar to wild type, whereas in region ‘b’ it is more fragmented and weaker. This indicates that dGPHR inactivation is affecting organization of both ER and Golgi. Scale bars: 25 μm.
that functional redundancy is relative rare in flies and clearly less pronounced than in vertebrates. Although the complete mouse knockout phenotype has not been yet reported, a recent report indicate that mice lacking GPHR in keratinocytes exhibited hypo-pigmented skin, hair loss and scaliness, indicating that mammal GPHR is essential for epidermis homeostasis (Tarutani et al., 2012). One possible explanation for the relative milder phenotype in Drosophila could be that pH acidification is under the regulation of multiples molecules or pathways. Another possibility would be that dGPHR is not functioning as Golgi lumen acidificator. This later hypothesis is, however, unlikely as we have shown that the vertebrate GPHR can compensate for the absence of the Drosophila ortholog, suggesting that the two proteins are performing similar functions. It should, however, be mentioned that our results indicate that the mammalian and flies GPHR do not have the same sub-cellular localization. Whereas the vertebrate GPHR is a Golgi only associated protein, its Drosophila ortholog seems to be associated with both the Golgi and the ER. We cannot, however, exclude that accumulation in the ER in secondary to the overexpression of these proteins. Although this do not definitively ruled out this hypothesis, it should be noted that dGPHR::HA staining nicely colocalize with ER marker whereas it only partially overlaps with Golgi staining. The different sub-cellular localization between Drosophila and mammalian GPHR could also relate to different organization of the pathway in invertebrates and vertebrates. Indeed, if the molecular principles underlying the secretory pathway are largely conserved between mammals and Drosophila, some differences exist (Kondylis and Rabouille, 2009). Whereas the Drosophila secretory pathway consist of tER sites closely associated to individual Golgi stacks, its mammalian homolog structure is made of Golgi stacks linked to form a single copy organelle forming a ribbon (Rabouille et al., 1999; Ripoche et al., 1994). Further studies will be required to understand whether these differences could be explained by morphological or functional differences between flies and vertebrate secretory pathway. Although dGPHR mutant embryos can develop into adult, those are much smaller that wild-type flies indicating that Golgi function is regulating growth. In metazoans, the Insulin/Insulin-like growth factor signaling pathway controls the growth rate of tissues according to nutrient availability. Recent studies have described that communication between different larval tissues is essential to influence overall body growth and development although in many cases, the nature of the secreted factors that mediate this inter-organ signaling is still unclear. For example, Drosophila growth is mediated by Insulin-like peptides that are produced by specific neurons in the brain and released into the hemolymph to couple nutrient uptake with systemic growth (Colombani et al., 2003; Gémardin et al., 2009). Upstream of the event is the secretion by the fat body of unknown signalling molecules that circulate in the hemolymph to reach the brain in order to control Insulin secretion (Gémardin et al., 2009). In addition, animal growth relies on nutrient processing, which involves the secretion of digestive enzymes by gut cells. Hence, one putative explanation for the dwarf phenotype associated with the dGPHR mutation could correspond to the impairment of some cells that normally produced and release proteins that favour growth, to do so. For instance, one could imagine that the Insulin producing cells and/or the fat body, and/or the gut cells have reduced capacity in secreting such molecules when the function of dGPHR is impaired. The ability of UAS-dGPHR transgene to rescue the growth defect of dGPHR mutants when expressed either in neuronal cells or gut cells are in agreement with this hypothesis and suggest that restoring secretion in one tissue can compensate for defects in other tissues. Nevertheless, further work will be required to address these issues.

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Competing Interests
The authors have no competing interests to declare.

References

Fig. 7. tER sites are affected in dPGHR mutants. Wild-type (A) and dPGHR mutant (B) salivary gland cells stained with anti-sec16 antibodies that detect tER sites. In mutant cells, the region in which the ER is disrupted (b) presents an abnormal pattern of sec16 expression. The tER seems more numerous but of smaller size in region ‘b’ than in region ‘a’ or than in control cells. Scale bar: 25 μm.

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