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DOI: 10.1534/genetics.108.099002

GPC-1, a G Protein γ-Subunit, Regulates Olfactory Adaptation in *Caenorhabditis elegans*

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Manuscript received November 22, 2008
Accepted for publication January 24, 2009

ABSTRACT

Caenorhabditis elegans genome carries two Gγ genes, *gpc-1* and *gpc-2*, and two Gβ genes, *gpb-1* and *gpb-2*. Of these, *gpc-2* and *gpb-1* are expressed ubiquitously and are essential for viability. Through a genetic screen, we identified *gpc-1* as essential for olfactory adaptation. While wild-type animals show decreased chemotaxis to the odorant benzaldehyde after a short preexposure to the odorant, *gpc-1* mutants are still attracted to the odorant after the same preexposure. Cell-specific rescue experiments show that *gpc-1* acts in the AWC olfactory neurons. Coexpression of GPC-1 and GPB-1, but not GPB-2, caused enhanced adaptation, indicating that GPC-1 may act with GPB-1. On the other hand, knock down of *gpc-2* by cell-targeted RNAi caused reduced chemotaxis to the odorant in unadapted animals, indicating that GPC-2 mainly act for olfactory sensation and the two Gγ's have differential functions. Nonetheless, overexpression of *gpc-2* in AWC neurons rescued the adaptation defects of *gpc-1* mutants, suggesting partially overlapping functions of the two Gγ's. We further tested genetic interaction of *gpc-1* with several other genes involved in olfactory adaptation. Our analyses place *goa-1* Goα and *let-60* Ras in parallel to *gpc-1*. In contrast, a gain-of-function mutation in *egl-30* Gqα was epistatic to *gpc-1*, suggesting the possibility that *gpc-1* Gγ may act upstream of *egl-30* Gqα.

ANIMALS sense a variety of stimuli from the environment and change their response on the basis of prior experience to eventually maximize the chance of survival. One of the evolutionarily ancient types of plasticity of sensory responses is olfactory adaptation, in which the sensitivity of animals to volatile chemicals is decreased after prolonged exposure to the compound. Studies in vertebrate olfactory neurons, and on molecules that act therein, have revealed importance of several pathways in olfactory adaptation; calcium/calmodulin regulation of cyclic nucleotide-gated channel, calcium/calmodulin-dependent protein kinase II (CaMKII)-mediated regulation of adenylyl cyclase, and possibly the CO/cGMP pathway (Zufall and Leinders-Zufall 2000).

The soil nematode *Caenorhabditis elegans* also recognizes numbers of volatile chemicals or odorants (Bargmann *et al.* 1993). These compounds may be important cues for food, mating partners, or predators. Olfactory adaptation in this organism is usually assessed on the basis of its behavior, as a decrease of chemotaxis to odorants after continuous exposure to the same odorant for 0.5–1 hr (Colbert and Bargmann 1995). Chemoattractive behavior is mediated mainly by two pairs of amphid sensory neurons (olfactory neurons), AWA and AWC (Bargmann *et al.* 1993). In these neurons, odorants are thought to be received by olfactory receptors that are seven-transmembrane G protein-coupled receptors as is the case in mammals (Troemel *et al.* 1995). G protein α-subunits ODR-3, along with GPA-3, are required for odor sensing in both AWA and AWC (Lans *et al.* 2004). In addition, guanylyl cyclase ODR-1 (L'Etoile and Bargmann 2000) and cyclic nucleotide-activated channel TAX-2/TAX-4 (Coburn and Bargmann 1996) are essential in AWC neurons, while chemosensation in AWA neurons depends on TRPV channels (Colbert *et al.* 1997). Several mutants defective in olfactory adaptation have been reported, including OSM-9 TRPV channel, which is required for adaptation in AWC neurons (Colbert *et al.* 1997), EGL-4 cGMP-dependent protein kinase (L'Etoile *et al.* 2002), GOA-1 Goα (Matsuki *et al.* 2006), and TBX-2/TBX-3 transcription factors (Miyahara *et al.* 2004). Overexpression of guanylyl cyclase ODR-1 (L'Etoile and Bargmann 2000) and activating mutation in EGL-30 Gqα also cause adaptation defect (Matsuki *et al.* 2006). The Ras-MAPK pathway, which is required for efficient sensing of odorants (Hirotsu *et al.* 2000), was also shown to be important for olfactory adaptation in a modified adaptation assay in which olfactory adaptation is observed as early as after 5 min of preexposure to odorants (Hirotsu and Iino 2005). On the basis of these

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observations, various molecular mechanisms have been suggested for olfactory adaptation, but further studies are required for full understanding of the interrelationships of the molecules involved. For example, although multiple types of G protein α-subunits are involved in odor sensing and adaptation, roles of βγ subunits are currently unexplored.

C. elegans also shows other types of plasticity in its response to sensory stimuli. Of particular interest is salt chemotaxis plasticity, another well-described behavioral plasticity paradigm in *C. elegans*, which shows similar characteristics to olfactory adaptation. This behavioral modification is regulated by the availability of food (Saeki *et al.* 2001), which is also the case for olfactory adaptation (Colbert and Bargmann 1997; Nuttley *et al.* 2002). Furthermore, several common genes are known to regulate both olfactory adaptation and salt chemotaxis plasticity. For example, mutants of *goa-1* $G_o\alpha$ (Matsuki *et al.* 2006), *egl-30* $G_q\alpha$ (Matsuki *et al.* 2006; Tomioka *et al.* 2006), *arr-1* β-arrestin (Palmitessa *et al.* 2005; Hukema *et al.* 2006), *egl-4* cGMP-dependent protein kinase (L'Etoile *et al.* 2002; Hukema *et al.* 2006), and *osm-9* TRPV channel (Colbert *et al.* 1997; Jansen *et al.* 2002) are all defective in salt chemotaxis plasticity as well as olfactory adaptation.

C. elegans possesses only two G protein γ-subunit (Gγ) genes, *gpc-1* and *gpc-2*. Previous observations using reporter constructs revealed that *gpc-1* is expressed only in sensory neurons, whereas *gpc-2* was reported to be expressed in all neurons and muscles (Jansen *et al.* 2002). In agreement with the sensory neuron-specific expression of *gpc-1*, the mutant animals of *gpc-1* display defects in salt chemotaxis plasticity and plasticity of the response to Cu^{2+} (Jansen *et al.* 2002; Hilliard *et al.* 2005).

In a genetic screening for olfactory adaptation mutants, we found that *gpc-1* mutants also show olfactory adaptation defects. The mutant animals of *gpc-1* show strong olfactory adaptation defect especially when the preexposure time is short. In olfactory adaptation, *gpc-1* acts only in AWC sensory neurons, which sense the odorant benzaldehyde used in the experiments. Furthermore, our data suggest that the existence of functional Gβγ dimers of GPB-1 and GPC-1 are important for olfactory adaptation and the Gβγ dimers of GPB-1 and GPC-2 mainly act for olfactory sensation.

MATERIALS AND METHODS

Strains and culture conditions: *C. elegans* were cultured at 20° under standard conditions (Brenner 1974), except that the *Escherichia coli* strain NA22 was used as food. Bristol N2 was used as the wild type. The following mutants were used: *egl-30(js126)* I, *goa-1(n1134)* I, *che-1(p674)* I, *let-60(n1046)* IV, *arr-1(ok401)* X, *gpc-1(pk298)* X, and *gpc-1(pe372)* X.

Genetic screens for olfactory adaptation mutants: Mutagenesis was performed as described (Brenner 1974). Roughly 18,000 F_1 animals were divided into 48 independent groups and cultured separately. F_2 animals were tested for olfactory adaptation defects; animals that were attracted to 1:400 dilution of benzaldehyde after preexposure to 100 nl/ml of benzaldehyde for 5 min were isolated. These mutant candidates were cultured and the progeny were further screened with the same manipulation for an additional 5 generations to concentrate mutants. Finally, single worms were picked from each group and the progeny were tested for adaptation defects.

Mapping and cloning of *pe372*: Mutants with visible markers, MT464 [*unc-5(e53)* IV; *dpy-11(e224)* V; *lon-2(e678)* X] and MT465 [*dpy-5(e61)* I; *bli-2(e768)* II; *unc-32(e189)* III], were employed to allocate *pe372* to linkage group X. The position responsible for adaptation defects was finely mapped using single nucleotide polymorphisms (SNPs) between N2 and the Hawaiian strain CB4856 (Wicks *et al.* 2001).

Chemotaxis and adaptation assays: Chemotaxis assays to benzaldehyde were performed as described (Matsuki *et al.* 2006). Adaptation assays were performed as previously described (Hirotsu and Iino 2005) with modification. Briefly, cultured worms were washed three times with basal buffer (5 mM potassium phosphate, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 0.5 g/liter gelatin). Animals were then incubated with 200 μl of basal buffer with (preexposed) or without (mock preexposed) 100 nl/ml of benzaldehyde. Unless otherwise noted, preexposure was 5 min. After preexposure, the animals were washed once with basal buffer and placed at the center of the assay plates. On the plates of adaptation assays, 1 μl each of diluted benzaldehyde was spotted on two points separated by 2.5 cm at one end of the plates. One M NaN_3 (0.5 μl each) was spotted on two points with the same spacing of 2.5 cm at both ends of the plates (supplemental Figure 1A). The dilution of benzaldehyde in ethanol was 1:400 dilution for adaptation assays. Fifteen minutes after placing the animals at the center of the assay plates, the animals were counted by dividing the plates at the center into two regions, A and B, while the animals that remained within 0.5 cm from the center were not counted.

The chemotaxis index for adaptation assays is $(A - B)/(A + B)$ where A was the number of animals on the odorant-spotted side of the plate and B was the number of the animals on the opposite side.

RESULTS

A genetic screen for mutants with abnormality in olfactory adaptation: To improve the understanding of the mechanisms of olfactory adaptation, we performed a genetic screen to obtain adaptation-defective mutants. Rather than using the conventional adaptation assays, we employed a modified assay format we have previously reported (Hirotsu and Iino 2005). In this assay, olfactory adaptation is observed after a short odorant preexposure. Using this paradigm, animals that do not show reduction of chemotaxis to benzaldehyde after 5 min of preexposure to the odorant were collected. We screened 36,000 EMS mutagenized haploid genomes and isolated 26 independent mutants.

Several mutants showed severe defects in adaptation after both 5 min and 60 min of preexposure to benzaldehyde, while others exhibited no or minor defects after 60 min of preexposure. JN372 was one of the mutants showing the most severe defects after 5 min of preexposure with minimal defects after 60 min (Figure 1A, *pe372*). Adaptation defects can be caused by reduced sensitivity

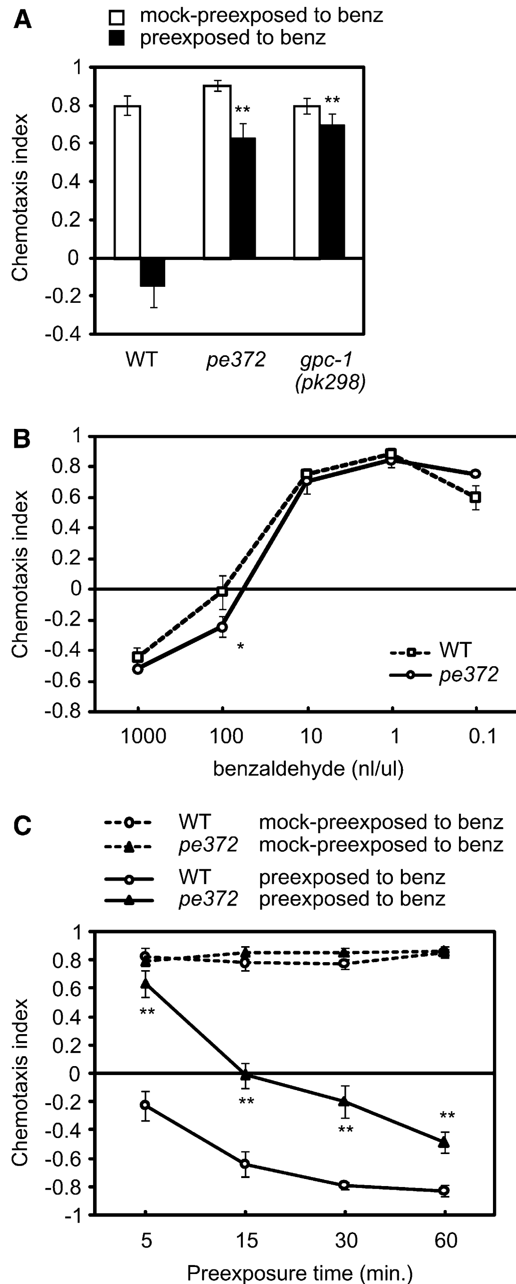

Figure 1.—Properties of olfactory adaptation and chemotaxis of wild-type animals and *gpc-1* mutant animals. (A) Olfactory adaptation of wild-type, *gpc-1(pe372)*, and *gpc-1(pk298)* animals. Worms were treated with 5 min of preexposure with basal buffer (open bars) or benzaldehyde (100 nl/ml; solid bars) and assayed for chemotaxis. Both *pe372* and *pk298* mutants are defective in olfactory adaptation. (B) Chemotaxis of untreated wild-type and *pe372* animals to different concentrations of benzaldehyde. *pe372* animals respond normally to a wide range of concentrations. (C) Olfactory adaptation assays with various durations of preexposure. Basal buffer (dotted lines), and benzaldehyde (100 nl/ml; solid lines). *pe372* animals adapt to benzaldehyde weakly compared to wild-type animals. $^{**}P < 0.001$; $^{*}P < 0.01$.

to odorants. However, chemotaxis assays using different concentrations of benzaldehyde revealed that JN372 animals have no defect in the sensitivity to the chemical (Figure 1B). In time-course experiments, wild-type animals quickly adapt after 5 min of odorant preexposure and the chemotaxis index further decreases by longer preexposure of up to 60 min, at which time the animals show strong avoidance of benzaldehyde, similar to the previous report (Nuttley *et al.* 2001). JN372 animals showed almost no adaptation after 5 min of preexposure, while the adaptation defect was small after 60 min of preexposure (Figure 1C). Therefore JN372 appears to be a mutant that adapts slowly and/or weakly to the odorant.

A G protein γ-subunit, GPC-1, is required for olfactory adaptation: We mapped the mutation responsible for the adaptation defect of JN372, *pe372*, using the linkage with the visible marker genes and SNPs between the Bristol N2 strain and the Hawaiian CB4856 strain (see materials and methods for details). *pe372* was linked to *lon-2* on linkage group X. Fine mapping using SNPs revealed that *pe372* resides between two SNPs, *uCE6-1303* and *uCE6-1307*.

There were only three predicted genes in the mapped region (Figure 2A). Three lines of experimental evidence identified *pe372* as an allele of *gpc-1*. First, the mutant of *gpc-1(pk298)* showed olfactory adaptation defect comparable to *pe372* (Figure 1A). Second, introduction of a cDNA for *gpc-1* driven by the 5.2-kb authentic promoter, *Pgpc-1::gpc-1*, rescued the olfactory adaptation defect of *pe372* mutant animals (Figure 3A). Finally, we found 1758 bp of deletion in *gpc-1* in the *pe372* mutant genome (Figure 2B). Because the deletion covers the half of the gene including the start codon, *pe372* is considered to be a null mutation of the *gpc-1* gene. Thus, we concluded that the deletion in *gpc-1* caused the adaptation defects in *pe372* animals.

gpc-1 encodes one of the two G protein γ-subunits (Gγ) in *C. elegans* (Jansen *et al.* 1999). It has been reported that *gpc-1* is involved in salt chemotaxis plasticity (Jansen *et al.* 2002), while no olfactory adaptation defect was observed in *gpc-1* mutants (Jansen *et al.* 2002; Law *et al.* 2004). Thus, this is the first report that *C. elegans gpc-1* is involved in olfactory adaptation. We surmise that our assay system for olfactory adaptation was somewhat more sensitive than that of conventional adaptation assays and therefore we could detect the adaptation defect of the *gpc-1* mutants (see discussion).

GPC-1 acts in AWC sensory neurons for olfactory adaptation: Previous studies suggested that olfactory adaptation depends on the functions of both sensory neurons and interneurons. To determine the cells in which *gpc-1* acts for adaptation, we expressed the *gpc-1* cDNA using cell-specific promoters in *gpc-1(pe372)* mutants and the transformants were tested for olfactory adaptation behavior (Figure 3A). The olfactory adaptation defect of *gpc-1(pe372)* mutants was rescued when *gpc-1* was expressed by the promoters of *gcy-10*, *odr-3*, *gpa-13*, and *ceh-36*. The expression patterns of these promoters overlap only in AWC neurons. On the other hand, expression of *gpc-1* in cells other than AWC neurons by

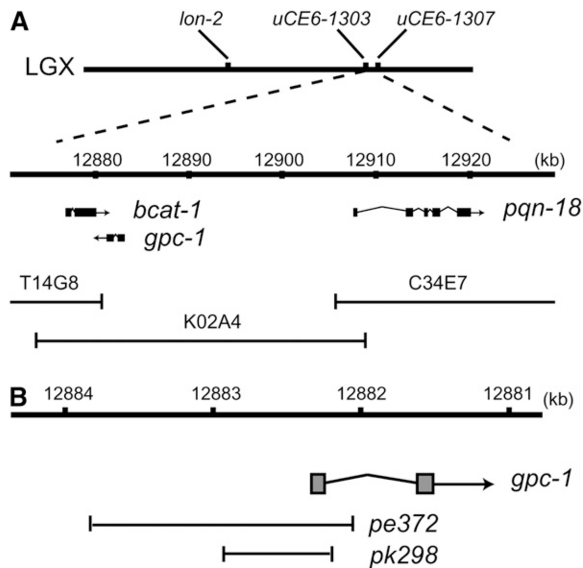

Figure 2.—Positional cloning of *pe372.* (A) Genetic and physical maps of chromosome X. *pe372* was mapped to an ∼50-kb interval between two SNPs, *uCE6-1303* and *uCE6-1307.* Only three genes, *bcat-1*, *gpc-1*, and *pqn-18*, were predicted within the mapped region. Three cosmids in this region, T14G8, K02A4, and C34E7 are also displayed at the bottom. (B) The gene model of *gpc-1* and the lesions of *gpc-1* mutants. *pe372* is a 1758-bp deletion that lacks the initiation codon and upstream sequence of the predicted ORF. The flanking sequences of the deletion are "tgatgaaaagaaaacataaa" and "gtgg caagcactaaactgta." The deletion of 720 bp in *gpc-1(pk298)* is also displayed.

the promoters of *gpa-4*, *sro-1*, *ttx-3*, *str-1*, and *gpa-11* did not rescue the defect.

To test more strictly whether the expression of *gpc-1* only in AWC neurons is sufficient for olfactory adaptation, we repeated the cell-specific rescue experiment by the *ceh-36* promoter, but in the *che-1(p674); gpc-1(pe372)* background (Figure 3B). In addition to AWC, the *ceh-36* promoter is expressed also in the salt-sensing ASE neurons in the wild type, but it is expressed only in AWC neurons in the *che-1* mutant in which ASE neurons fail to acquire its identity (Uchida *et al.* 2003; Koga and Ohshima 2004). *che-1* animals showed normal olfactory adaptation phenotype and *che-1; gpc-1* animals showed an olfactory adaptation defect. The expression of *gpc-1* cDNA by the *che-36* promoter rescued the olfactory adaptation defect of *che-1; gpc-1* animals. These results indicate that the function of *gpc-1* only in AWC neurons is sufficient for olfactory adaptation.

It has been previously reported that *gpc-1* is expressed specifically in sensory neurons ADL, ASH, ASJ, AFD, ASI, AWB, and PHB (Jansen *et al.* 2002). Because the olfactory neurons AWC were not included in this set, we reexamined the expression pattern of a *Pgpc-1::venus* transcriptional reporter fusion in wild-type background. At the first larval (L1) stage, reporter expression was mainly observed in the cells previously reported. At the adult stage, expression in AWC, ASE, and RIB was also observed (Figure 3, D–F). The reporter expression in AWC was confirmed by coexpression with *Podr-3::mRFP*, which drives the expression of mRFP in AWC and several other amphid neurons (Figure 3, E and F). AWC neurons are known as chemosensory neurons that receive attractive odor including benzaldehyde (Bargmann *et al.* 1993), which we used in the experiments. These data thus indicates that *gpc-1* acts only in the odor-sensing neurons for olfactory adaptation.

The seven-transmembrane olfactory receptor *str-2* is expressed in either the right or left member of the AWC neuron pair in a random manner, and the *str-2*-expressing neuron is defined as AWC^{ON} and the other as AWC^{OFF} (Troemel *et al.* 1999). The two AWC neurons are functionally distinct from each other. For example, AWC^{ON} detects the odorant butanone while AWC^{OFF} detects the odorant 2, 3-pentanedione. In contrast, benzaldehyde is detected by both AWC neurons (Wes and Bargmann 2001). To determine whether the expression of *gpc-1* in either type of AWC neuron is sufficient for olfactory adaptation, we expressed *gpc-1* in AWC^{ON}, AWC^{OFF}, or both of the AWC neurons (Figure 3C). The defect of *gpc-1* mutants was rescued only when *gpc-1* is expressed in both of the AWC neurons. These results indicate that GPC-1 is required in both of the AWC neurons for proper adaptation. When *gpc-1* is expressed in only one AWC neuron, the other neuron probably cannot adapt to the odorant, and the olfactory adaptation defect is revealed. This interpretation is consistent with the fact that animals with either of the AWC neurons ablated still show normal chemotaxis to benzaldehyde (Wes and Bargmann 2001).

GPC-1 acts with GPB-1 in olfactory adaptation: Gγ ordinarily acts with Gβ in a complex. In *C. elegans*, two Gβ-encoding genes, *gpb-1* and *gpb-2*, are predicted on its genome (Jansen *et al.* 1999). On the basis of the primary sequence, GPB-2 is categorized to the $G\beta_5$ subtype, which can function with either Gγ or regulator of G protein signaling (RGS). In contrast, GPB-1 belongs to the $G\beta_{1–4}$ subtype, which requires coupling with Gγ for their functions.

To determine which Gβ acts with GPC-1, we co-overexpressed *gpc-1* with *gpb-1* or *gpb-2* in AWC and observed the effect on chemotaxis. When *gpc-1* was co-overexpressed with *gpb-1* using the *odr-3* promoter, chemotaxis index was decreased significantly compared to the control, both in animals mock preexposed and those preexposed to the odorant (Figure 4A). This result indicates that *gpc-1*, along with *gpb-1*, has the ability to reduce the chemotaxis toward the odorant. Assuming that the effect of overexpression is opposite to that of the loss of *gpc-1*, which causes an adaptation defect, we call it enhanced adaptation. Reduced chemotaxis index of mock-preexposed animals could be caused by enhanced adaptation of the animals to the odorant encountered during the chemotaxis assay or the odor of bacterial food during cultivation. Such enhanced

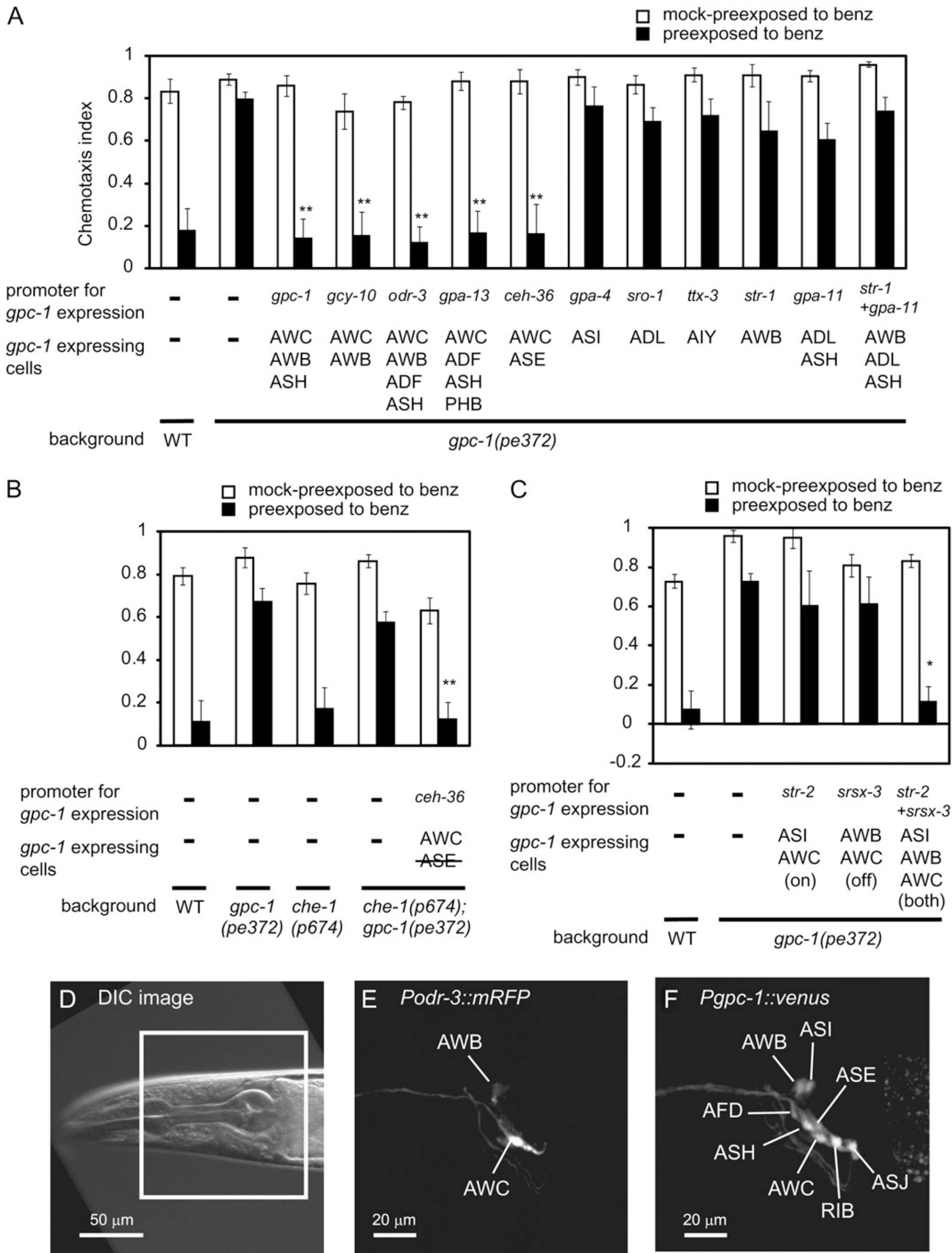

Figure 3.—*gpc-1* acts in AWC sensory neurons. (A–C) *gpc-1* is required in AWC sensory neurons for olfactory adaptation. *gpc-1* cDNA was fused to various promoters that drive cell-specific expression, and resulting constructs were introduced into *gpc-1(pe372)* (A and C) or *che-1(p674); gpc-1(pe372)* animals (B), as indicated. $^{**}P < 0.001$; $^{*}P < 0.01$. (D–F) Fluorescent images of the head region of an adult animal (depicted in the DIC image in D) transformed with *Podr-3::mRFP* and *Pgpc-1::venus*. *Podr-3::mRFP* is expressed in AWC and AWB (E). *Pgpc-1::venus* is expressed in AWC, ASE, and RIB in addition to the previously reported neurons, ADL, ASH, ASI, and AWB (F).

adaptation was not observed by co-overexpression of *gpb-2* and *gpc-1*. When *gpc-1*, *gpb-1*, or *gpb-2* were individually expressed, there was no effect on olfactory adaptation ($P > 0.01$ for each line; Figure 4A). These results are consistent with the idea that Gβγ consisting of GPB-1 and GPC-1, but not GPB-2 and GPC-1, regulates olfactory adaptation and acts negatively on odorant chemotaxis. They also suggest that endogenous expression levels of Gβ and Gγ are precisely matched because overexpression of neither component has an effect.

The *odr-3* promoter used in the overexpression experiment drives expression in AWC, AWB, AWA, ASH, and ADF (Roayaie *et al.* 1998). To determine which of these neurons are responsible for the enhanced adaptation observed by Gβγ overexpression, we used combinations of promoters for expression of *gpb-1* and *gpc-1*. For example, when *odr-3* promoter and *ceh-36* promoter were used for expression of *gpb-1* and *gpc-1*, respectively, co-overexpression can be achieved only in AWC. With this strategy, we found that the co-overexpression of *gpb-1* and *gpc-1* in AWC was sufficient for the enhanced adaptation, while co-overexpression in AWB or ASH had no effect (supplemental Figure 2A). Involvement of AWC for enhanced adaptation is consistent with the results of rescue experiments in which *gpc-1* is required in AWC for olfactory adaptation (Figure 3, A–C).

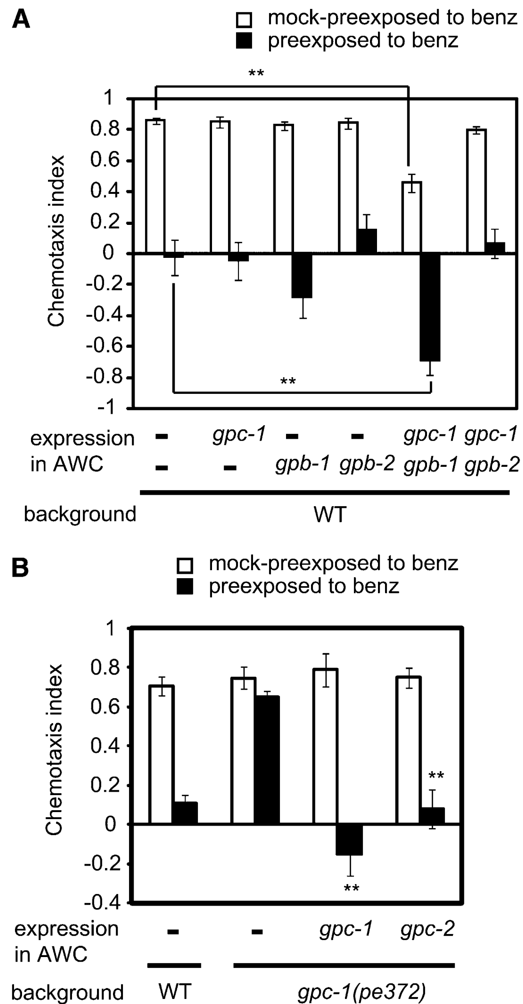

FIGURE 4.—GPB-1, but not GPB-2, may function with both GPC-1 and GPC-2 in olfactory adaptation. (A) The expression of *gpc-1*, *gpb-1*, or *gpb-2* in AWC sensory neurons using the *odr-3* promoter does not affect the olfactory adaptation of wild-type animals significantly ($P > 0.01$). Enhanced adaptation was observed only when *gpb-1* and *gpc-1* were co-overexpressed in AWC sensory neurons. (B) The expression of *gpc-2* in AWC sensory neurons fully rescues the olfactory adaptation defect of *gpc-1(pe372)* animals. $^{**}P < 0.001$.

The role of *gpb-1* was also assessed by observation of loss-of-function phenotypes. Unfortunately, *gpb-1* mutant animals are embryonic lethal (ZWAAL *et al.* 1996; GOTTA and AHRINGER 2001). Therefore we performed cell-specific RNAi by introducing transgenes that express both sense and antisense RNA of *gpb-1*, or other genes, by the *odr-3* promoter (ESPOSITO *et al.* 2007). The knockdown of *gpc-1* caused olfactory adaptation defect as expected, though the defect was weaker than the *gpc-1* mutants (Figure 5). Knockdown of *gpb-1* caused a strong adaptation defect, supporting the above model that GPB-1 and GPC-1 act together for olfactory adaptation. Knockdown of *gpb-1* also caused a reduction of chemotaxis in mock-preexposed animals, which will be discussed later.

GPC-2 mainly acts for the sensation in AWC neurons and have partially overlapping function with GPC-1 in olfactory adaptation: Of the two Gγ genes, knockdown of *gpc-2*, but not *gpc-1*, induces embryonic lethality caused by improper orientation of mitotic spindle during early embryogenesis (GOTTA and AHRINGER 2001). Because GPB-1 is also known to be involved in orientation of early cell division axes (ZWAAL *et al.* 1996), GPC-2 is considered to act with GPB-1 in this process. Although expression of *gpc-2* continues postembryonically in neurons, its neuronal function is not uncovered. Because *gpc-2* is reported to be expressed in all neurons and muscle cells (JANSEN *et al.* 2002), *gpc-2* is expected to be also expressed in AWC sensory neurons. To confirm this, we examined the coexpression of the transcriptional reporters, *Pgpc-2::venus* and *Podr-3::mRFP*. At the first stage (L1) larvae, the expression of *gpc-2* in AWC was actually observed (supplemental Figure 3).

To test if *gpc-2* has any function in olfactory adaptation, we performed cell-specific RNAi by expression of sense and antisense RNA of *gpc-2* by the *odr-3* promoter as described above. In contrast to knockdown of *gpc-1*, which caused olfactory adaptation defect, the knockdown of *gpc-2* did not cause olfactory adaptation defect but instead showed a small decrease of chemotaxis in mock-preexposed animals (Figure 5). The double knockdown of *gpc-1* and *gpc-2* caused more severe chemotaxis defect. The same was true in the knockdown of *gpc-2* in the *gpc-1(pe372)* mutant background. These results indicate that *gpc-2* acts mainly in olfactory sensation in AWC neurons and the function of *gpc-1* is also contributing weakly in olfactory sensation.

To test further the functional relationship between *gpc-1* and *gpc-2*, we overexpressed *gpc-2* in *gpc-1(pe372)* mutant animals using the *odr-3* promoter. Interestingly, expression of *gpc-2* in AWC rescued the adaptation defect of *gpc-1* mutants (Figure 4B). This result indicates that *gpc-1* and *gpc-2* have somewhat overlapping functions in olfactory adaptation. We also tested the effect of overexpression of *gpc-2* in wild-type background (supplemental Figure 2B). In contrast to the overexpression of *gpc-1* (Figure 4A), the overexpression of *gpc-2* with neither *gpb-1* nor *gpb-2* showed an enhanced adaptation phenotype. In summary, the above results suggest that GPC-1 mainly acts for olfactory adaptation while GPC-2 mainly acts for olfactory sensation, but they have partially overlapping functions.

As described above, the knockdown of *gpb-1* caused a decrease in chemotaxis in mock-preexposed animals, as well as a defect in olfactory adaptation. Therefore GPB-1 seems to act for both olfactory adaptation and sensation, namely, the Gβγ dimer GPC-1/GPB-1 and GPC-2/GPB-1 appear to act mainly for olfactory adaptation and olfactory sensation, respectively. We note that knockdown of *gpb-1* causes much more severe defect in olfactory adaptation than double knockdown of *gpc-1* and *gpc-2*. This might be due to the difference in RNAi

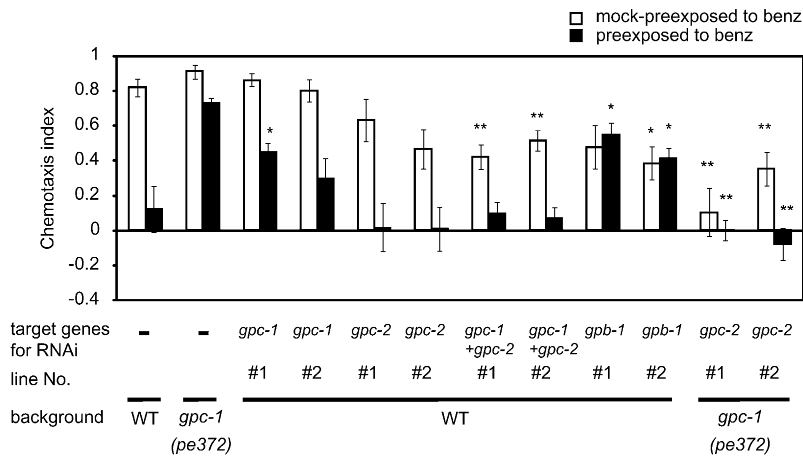

FIGURE 5.—Cell-specific knockdown revealed the functional features of *gpc-1*, *gpc-2*, and *gpb-1*. Sense and antisense RNA of *gpc-1*, *gpc-2*, or *gpb-1* were expressed by the *odr-3* promoter. For *gpc-2*, knockdown was performed also in the *gpc-1(pe372)* background. The results of two lines for each RNAi construct are shown. $^{**}P < 0.001$; $^{*}P < 0.01$.

efficiency between *gpb-1* and *gpc-1/gpc-2* (*e.g.*, compare *gpc-1* RNAi with *gpc-1* mutants), or GPB-1 may have an unknown role in adaptation that requires neither GPC-1 nor GPC-2. Developmental defects in AWC or other neurons in some of the knockdown strains cannot be ruled out because GPC-2 and GPB-1 are known to be involved in mitotic spindle orientation (see DISCUSSION).

GPC-1 is mainly localized to cilia: To observe the localization of GPC-1 in AWC sensory neurons, a construct for a fluorescent protein-fused GPC-1, *venus::gpc-1*, was generated and expressed in animals under the control of the *odr-3* promoter. When introduced into *gpc-1* mutants, the fusion gene fully rescued the adaptation defects of the animals, indicating that the Venus::GPC-1 fusion protein is functional (supplemental Figure 4E). Venus::GPC-1 was mostly localized to the cilia and a small fraction localized to the cell bodies and axons (supplemental Figure 4B). Interestingly, they were not localized to the proximal region of the axons where synapses do not exist. Thus, the localization at axons might reflect the functions of GPC-1 near the synapses. The localization of GPC-2 was also observed using *venus::gpc-2*. Venus::GPC-2 showed subcellular localization virtually identical to Venus::GPC-1 (supplemental Figure 4D).

Though most of the overexpressed Gγ probably lack Gβ to couple with, they clearly localized to the cilia. Furthermore, overexpression of *gpb-1* did not affect the localization of Venus::GPC-1 (supplemental Figure 4C). Therefore, Gγ seems to be transported to the cilia in sensory neurons regardless of binding to Gβ.

Genetic interactions of *gpc-1* with previously characterized pathways that regulate olfactory adaptation: In mammals, G protein-coupled receptors (GPCRs) are known to be downregulated by G protein-coupled receptor kinase (GRK) and β-arrestin (FERGUSON 2001). In this process, GRK, which is recruited to the cell membrane by Gβγ dimer, phosphorylates GPCR. The phosphorylation of GPCR is followed by the recruitment of β-arrestins and internalization of GPCRs. A recent study has shown that olfactory receptors can also be inactivated by this mechanism (MASHUKOVA *et al.* 2006).

ARR-1, the *C. elegans* homolog of β-arrestin, is reported to regulate olfactory adaptation (PALMITESSA *et al.* 2005). According to this report, *arr-1* null mutants exhibit normal chemotaxis but have defects in olfactory adaptation, whereas overexpression of *arr-1* induces enhanced adaptation. However, the mutants of *grk-2*, which encode the *C. elegans* neuronal GRK, show defects of chemotaxis to various chemicals rather than adaptation defect (FUKUTO *et al.* 2004). Thus *grk-2* is unlikely to be involved in the regulation by *arr-1* and it is currently unknown how *arr-1* regulates adaptation.

Given the possible link between Gβγ, β-arrestin, and olfactory adaptation, we tested the *arr-1* mutants in our adaptation assay. Unexpectedly, however, the *arr-1* mutants showed no adaptation defect in our assay system. This discrepancy is probably due to the difference in the assay formats of chemotaxis; our assay format is more sensitive to the avoidance response and weak chemotaxis to the odorant (see DISCUSSION). As expected, enhanced adaptation by overexpression of *gpb-1* and *gpc-1* was observed in the *arr-1(ok401 lf)* background similar to the wild-type background (Figure 6B).

We further tested genetic interactions between *gpc-1* and other genes associated with olfactory adaptation, *let-60* (Ras), *goa-1* ($G_o\alpha$), and *egl-30* ($G_q\alpha$). *let-60(n1046 gf)*, *goa-1(n1134 rf)*, and *egl-30(js126 gf)* mutant animals show olfactory adaptation defects (HIROTSU and IINO 2005; MATSUKI *et al.* 2006); *gf* refers to gain-of-function alleles and *rf* refers to reduction-of-function alleles). We first generated double mutants of *gpc-1(pe372 null)* and each of the three mutants: *let-60(gf); gpc-1(null)*, *goa-1(rf); gpc-1(null)* and *egl-30(gf); gpc-1(null)*. The combination of *gpc-1* and *egl-30* behaved differently from the other two. Namely, *egl-30(gf); gpc-1(null)* showed adaptation defect identical to that of the *egl-30(gf)* single mutants. The double mutants *let-60(gf); gpc-1(null)* and *goa-1(rf); gpc-1(null)* showed severe defects after 60 min of odor preexposure, in contrast to the milder effects observed in either single mutant (Figure 6A).

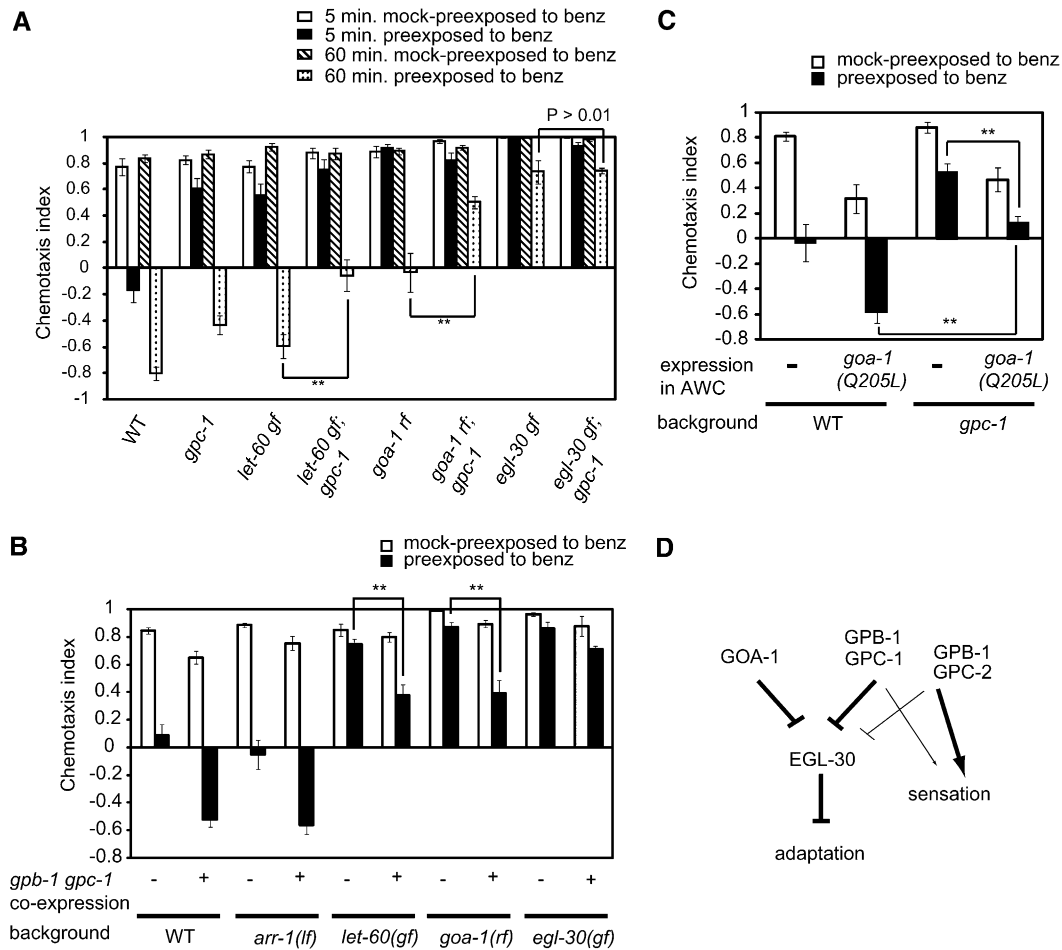

FIGURE 6.—*egl-30*, but not *arr-1*, *let-60*, or *goa-1*, is genetically epistatic to *gpc-1*. (A) The double mutants of *let-60(gf)* and *gpc-1*, and of *goa-1(n1134 rf)* and *gpc-1(pe372)*, show additive defects in olfactory adaptation after 60 min of preexposure. In contrast, no additive defect was observed in the double mutant of *egl-30(js126 gf)* and *gpc-1(pe372)*. (B) *gpb-1* and *gpc-1* were co-overexpressed in AWC sensory neurons in wild-type, *arr-1(ok401lf)*, *let-60(n1046gf)*, *goa-1(n1134rf)*, and *egl-30(js126gf)* animals using the promoter of *odr-3*. *arr-1(lf)* animals are not defective in our paradigm and *arr-1(lf)* mutation does not impair the effect of coexpression of *gpb-1* and *gpc-1*. The olfactory adaptation defects of *let-60(gf)* and *goa-1(rf)* are partially suppressed by co-overexpression of *gpb-1* and *gpc-1*, while the olfactory adaptation phenotype of *egl-30(gf)* is less significantly affected ($P > 0.01$) by the co-overexpression. (C) Expression of a constitutively active form of *goa-1*, *goa-1(Q205L)*, in AWC neurons of wild-type animals results in an enhanced adaptation phenotype. The expression of *goa-1(Q205L)* in *gpc-1(pe372)* background caused an intermediate reduction of chemotaxis. (D) The summary of genetic interaction. GPC-1 and the dimerization partners, GPB-1 act in parallel to the Go (GOA-1) pathway and may act upstream of Gq (EGL-30). GPC-2 also contributes weakly to olfactory adaptation but mainly regulate olfactory sensation. $^{**}P < 0.001$; $^{*}P < 0.01$.

To further examine the relationship between *gpc-1* and the other genes, we observed the effect of Gβγ overexpression in AWC sensory neurons in the background of *let-60(gf)*, *goa-1(rf)*, or *egl-30(gf)*. Effect of co-overexpression of *gpb-1* and *gpc-1* on adaptation was still observed in the *let-60(gf)* and *goa-1(rf)* background, but not in the *egl-30(gf)* background (Figure 6B).

These results suggest that *egl-30* acts downstream of *gpc-1* because when *egl-30* is mutated to the activated form *egl-30(js126gf)*, the adaptation phenotype is no more affected by either loss or overexpression of *gpc-1*. In contrast, *goa-1* and *let-60* appear to act genetically in parallel to *goa-1*, because the double mutants showed more severe defects than each single mutant, and overexpression of *gpc-1* and *gpb-1* still had effect in the *goa-1(rf)* or *let-60(gf)* background.

It has been reported that the expression of constitutively active form of *goa-1*, *goa-1(Q205L)*, in the AWC neurons of wild-type animals causes enhanced adaptation similar to overexpression of *gpc-1* and *gpb-1* (MATSUKI *et al.* 2006; Figure 6C). Expression of *goa-1(Q205L)* in the *gpc-1(pe372)* background caused an intermediate phenotype between the *gpc-1(pe372)* mutant and wild-type animals expressing *goa-1(Q205L)* (Figure 6C). These results further suggest that *goa-1* acts in parallel to *gpc-1* because if *gpc-1* acts downstream of *goa-1(Q205L)*, the phenotype would not be altered by *goa-1(Q205L)* overexpression in *gpc-1(null)*.

In summary, the above tests of genetic interactions suggest that *egl-30* acts downstream of *gpc-1* while *goa-1* and *let-60* acts in parallel to *gpc-1*. Reservations about the latter conclusion would be that in contrast to *gpc-1*,

neither *goa-1* nor *let-60* alleles used were null, leaving the possibility open that either of these genes actually acts downstream of *gpc-1*. We consider this possibility unlikely, though, because the pattern of genetic interaction of *gpc-1* with *goa-1(n1134rf)* or *let-60(n1046gf)* was very different from that with *egl-30(js126gf)*.

DISCUSSION

The role of *gpc-1* in the early phase of olfactory adaptation: In this article, we showed that *gpc-1* is required for adaptation to the AWC-sensed odorant, benzaldehyde. The defects were prominent after 5 min of preexposure, at which time wild-type animals mostly lost chemotaxis and tended to show weak aversive response to the odorant, while *gpc-1* mutants were strongly attracted. However, after 60 min of preexposure, the *gpc-1* mutants adapted to the odorant to the extent close to the wild type. We previously reported that *gpc-1* mutants show defects in salt chemotaxis plasticity when the animals were preexposed to NaCl for 10 min but not for 60 min (Tomioka *et al.* 2006). *gpc-1* mutants are also defective in adaptation to Cu^{2+} when the animals were exposed to the stimulus for 1 min (Hilliard *et al.* 2005). These observations indicate that *gpc-1* is involved in adaptation to multiple kinds of stimuli, and the defects of *gpc-1* mutants may be observed only after a short preexposure to the stimuli.

There are two possible explanations for the early time point-specific effect of *gpc-1* in olfactory adaptation. First, there may be two or more different mechanisms of adaptation, and *gpc-1* may act specifically in one of these adaptation mechanisms. In this view, the mechanism involving GPC-1 may act only at early time points. Second, the *gpc-1* mutants may not be directly involved in olfactory adaptation, but rather play a modulatory role. Although both interpretations explain why *gpc-1* mutants adapt only after a long preexposure to the odorant, we prefer the first possibility because overexpression of *gpc-1* and *gpb-1* reduced chemotaxis in mock-treated animals, causing a behavior similar to adapted animals.

In this study, we employed the "early adaptation assay" (Hirotsu and Iino 2005). This assay is different in several aspects from the conventional adaptation assays. First, we used the bacterial strain NA22, rather than OP50, to grow worms before the assay. Second, worms are preexposed to the odorant in solution. Finally, and probably most importantly, the plate format used in chemotaxis assays (supplemental Figure 1A) is different from that typically used in the conventional adaptation assays (supplemental Figure 1B). In our format, spots of odorants, worms, and control spots are aligned in linear geometry and in close proximity. Therefore, if a fraction of worms avoid the odorant, the assay is very sensitive to this behavior, because essentially all the worms avoiding the odorant move into area B (supplemental Figure 1A). In addition, the chemotaxis index we used is more sensitive to weak positive chemotaxis. Because of these differences, worms show greatly reduced chemotaxis indexes after as short as 5 min of odorant preexposure, hence the nomenclature early adaptation. We have previously shown that mutants in the Ras-MAPK pathway show severe defects in early adaptation, while the same mutants show small or no defects in the conventional adaptation assays. We also showed that early adaptation depends on the function of interneurons AIY. On the other hand, although the *arr-1* β-arrestin mutant was reported to have deficits in the conventional olfactory adaptation assay, we did not see any defect in the early adaptation assay (Figure 6B). Therefore, it is likely that there are multiple molecular and behavioral mechanisms for olfactory adaptation that probably act in different time courses, and they are manifested differently in the two types of adaptation assays. This view accounts for the previous failure to detect olfactory adaptation defect in the *gpc-1* mutant in the conventional adaptation assay (Jansen *et al.* 2002).

We have shown that *gpc-1* acts in the AWC olfactory neurons for olfactory adaptation. In salt chemotaxis plasticity, *gpc-1* was reported to act in multiple sensory neurons, ASI, ASH, and probably also ADL (Hukema *et al.* 2006). Of these, ASI mediates attractive behaviors to salt (Bargmann and Horvitz 1991). On the other hand, ASH and ADL are known to mediate aversive behaviors, and salt chemotaxis plasticity is likely to be generated by an interplay of these neurons (Hukema *et al.* 2006). Thus the role of *gpc-1* at the cellular level is still unclear in salt chemotaxis plasticity. *gpc-1* was also shown to be required for adaptation to Cu^{2+}-avoidance behavior (Hilliard *et al.* 2005). In this case, *gpc-1* is likely to act in the Cu^{2+}-sensing neuron ASH, because decrease of calcium response of ASH neurons to repeated Cu^{2+} stimuli is reduced in the *gpc-1* mutant. The role of *gpc-1* in AWC neurons might be similar to its role in ASH neurons for Cu^{2+} response.

In the sensory regulation described above, *gpc-1* is assumed to act in response to sensory stimuli. To see when *gpc-1* acts for olfactory adaptation, we performed stage-specific *gpc-1* rescue experiments using the *hsp-16.2* heat-shock promoter, and also tested stage-specific coexpression of *gpb-1* and *gpc-1* in wild-type background. However, the olfactory adaptation defect of *gpc-1* mutants was not rescued by the heat shock neither in the adult stage nor the embryonic stage. Similarly, enhanced adaptation was not observed by expression of *gpb-1* and *gpc-1* in either developmental stage (data not shown). Therefore, at present we cannot judge whether *gpc-1* acts in mature neurons for olfactory adaptation or whether it affects olfactory adaptation through its functions in development. In our heat-shock experiments, the expression by the heat-shock promoter may have not been appropriate for the function of GPC-1, for example in terms of the expression level. Alterna-

tively, the expression of *gpc-1* may be continuously required for a long period, for example from embryo to adulthood for generation and maintenance of competence for olfactory adaptation. We looked at the morphology of AWC neurons by cell-specific expression of Venus in the *gpc-1* mutants, but no abnormality was observed (data not shown).

The complex of GPB-1 and GPC-1 is important for olfactory adaptation: We demonstrated several features about the composition of the Gβγ dimers involved in olfactory responses. First, GPB-1, but not GPB-2, is required for the proper function of GPC-1 (Figure 4A). Second, GPC-2 can substitute for GPC-1 in olfactory adaptation, at least when overexpressed (Figure 4B). Third, GPC-2 is required for olfactory sensation rather than adaptation under normal conditions and GPC-1 is also weakly required for olfactory sensation (Figure 5). Finally, GPB-1 is required for both olfactory adaptation and sensation (Figure 5).

These results suggest that two Gγs GPC-1 and GPC-2, and a Gβ of the $G\beta_{1-4}$ subtype, GPB-1, act in AWC sensory neurons for olfactory responses probably by forming Gβγ heterodimers. The other Gβ of *C. elegans*, GPB-2, belongs to the $G\beta_5$ subtype. $G\beta_5$ acts not only with Gγ but also with RGS proteins. In egg laying and locomotion, GPB-2 is known to act with RGS, EGL-10, and EAT-16 (Koelle and Horvitz 1996; Hajdu-Cronin *et al.* 1999). We have previously reported that *gpb-2* weakly contributes to olfactory adaptation probably through EGL-10 RGS signaling (Matsuki *et al.* 2006). However, we did not observe any functional association of *gpb-2* with *gpc-1* in the co-overexpression experiments in this study (Figure 4A). This result suggests that GPB-2 regulates olfactory adaptation without coupling with GPC-1, but probably by directly coupling with the RGS.

In the cell-specific knockdown, we detected differential functions of GPC-1 and GPC-2: GPC-1 biased to adaptation and GPC-2 biased to sensation. Therefore the functions of GPC-1 and GPC-2 are basically distinct but partially overlapping. Comparison of the knockdown phenotype of *gpb-1* with that of *gpc-1* and *gpc-2* suggests that GPB-1 acts with both GPC-1 and GPC-2. Therefore it is presumed that GPB-1/GPC-1 and GPB-1/GPC-2 serve differential but overlapping functions. This functional differentiation could be explained by assuming that each of the two Gβγ complexes interact with multiple target proteins with different affinities, and this assumption is consistent with the known mode of interaction where the Gβγ dimers interact with their effectors on the Gβ face with an influence by the Gγ subunit (Cabrera-Vera *et al.* 2003).

In G protein signaling, either Gα or Gβγ subunits can act as an effector subunit. For Gβγ, various targets are known, such as K^+ and Ca^{2+} channels, adenylyl cyclase, phospholipase Cβ (PLC-β), PI 3-kinase, G protein-coupled receptor kinase (GRK), and other protein kinases (Clapham and Neer 1997; Cabrera-Vera *et al.* 2003). Of these, GRK acts with β-arrestin for down-regulation of many GPCRs (Ferguson 2001). The mutant animals of *arr-1*, which is the β-arrestin homolog in *C. elegans*, are reported to have defects in olfactory adaptation. Furthermore, they also showed defects in salt chemotaxis plasticity comparable to that of *gpc-1* mutants (Hukema *et al.* 2006). Therefore, GRK-β-arrestin pathway was one of the strongest candidates for a GPC-1 target in olfactory adaptation. However, in our assay, the *arr-1* mutant did not show any discernible defect, in clear contrast with *gpc-1*. Therefore, GPC-1 is unlikely to act on the GRK/β-arrestin regulatory system.

By testing other mutants that show abnormality in olfactory adaptation, we showed that *gpc-1* genetically acts in parallel to *let-60* and *goa-1*, and upstream of *egl-30*. In olfactory adaptation, we previously reported that *goa-1* genetically acts upstream of *egl-30* (Matsuki *et al.* 2006). Thus, both *goa-1* and *gpc-1* may regulate *egl-30* in parallel (Figure 6D). Other than the role as effector subunits of G proteins, Gβγ dimers possibly regulate the activity of Gα directly. A Gβγ dimer binds the GDP-bound form of Gα most tightly and thus suppresses spontaneous Gα activation. In contrast, binding of a Gβγ dimer to the GTP-bound form of Gα is also reported to compete with GTPase activating proteins (GAPs) (Tang *et al.* 2006), which accelerate hydrolysis of GTP, hence inactivation of Gα. Therefore, both activation and inactivation are possible for the effect of Gβγ on Gα. Our data suggest that GPC-1 negatively regulates EGL-30 Gqα. The activity of EGL-30 may be directly affected by the Gβγ dimers, or through an intervening signaling pathway. The possibilities suggested by our genetical analyses needs to be biochemically tested in future studies.

We thank Yuji Kohara for providing cDNA clones and Caenorhabditis Genetic Center, which is funded by National Institutes of Health National Center for Research Resources, for all the strains used in this study. We also thank members of our laboratory for useful advice and discussion.

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Communicating editor: K. Kemphues