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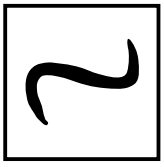

Primary Research Paper

Proteins interacting with *Caenorhabditis elegans* Gα subunits

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Abstract

To identify novel components in heterotrimeric G-protein signalling, we performed an extensive screen for proteins interacting with *Caenorhabditis elegans* Gα subunits. The genome of *C. elegans* contains homologues of each of the four mammalian classes of Gα subunits (Gs, Gi/o, Gq and G12), and 17 other Gα subunits. We tested 19 of the Gα subunits and four constitutively activated Gα subunits in a large-scale yeast two-hybrid experiment. This resulted in the identification of 24 clones, representing 11 different proteins that interact with four different Gα subunits. This set includes *C. elegans* orthologues of known interactors of Gα subunits, such as AGS3 (LGN/PINS), CalNuc and Rap1Gap, but also novel proteins, including two members of the nuclear receptor super family and a homologue of human haspin (germ cell-specific kinase). All interactions were found to be unique for a specific Gα subunit but variable for the activation status of the Gα subunit. We used expression pattern and RNA interference analysis of the G-protein interactors in an attempt to substantiate the biological relevance of the observed interactions. Furthermore, by means of a membrane recruitment assay, we found evidence that GPA-7 and the nuclear receptor NHR-22 can interact in the animal.

Keywords: heterotrimeric G-protein; signal transduction; protein interaction; nuclear receptor

Introduction

Heterotrimeric G-proteins, consisting of α, β and γ-subunits, are signal transduction molecules that couple ligand-bound seven transmembrane (7-TM) receptors to a wide variety of intracellular second messenger systems (Kaziro *et al*., 1991; Simon *et al*., 1991). However, there is a growing body of evidence that 7-TM receptors can also transmit extracellular signals through mechanisms that function independently of G-protein coupling (Brzostowski and Kimmel, 2001). In addition, non-receptor modulators and receptor-independent activators of heterotrimeric G-proteins have been identified (Cismowski *et al*., 1999; Takesono *et al*., 1999).

All higher eukaryotic organisms possess G-protein-coupled signal transduction, which has been implicated in many important biological functions, ranging from photoreception to neurotransmission and exocytosis, as well as processes such as embryogenesis, angiogenesis, tissue regeneration, and normal and aberrant cell growth. Analysis of the complete *C. elegans* genome (The *C. elegans* Sequencing Consortium, 1998) resulted in the prediction of 21 Gα, two Gβ, and two Gγ genes (E. Cuppen, unpublished results; Jansen *et al*., 1999). Besides one member (*gsa-1, goa-1, egl-30* and *gpa-12*) of each of the mammalian Gα classes (Gs, Go/i, Gq and G12), there are 17 *C. elegans* specific genes (*gpa-1–11*, *13–17* and *odr-3*) that most closely resemble the mammalian Go/i

class but cannot be clearly classified into any of the existing families (Jansen *et al*., 1999).

The Gs homologue *gsa-1* is required to survive the first larval stage (Korswagen *et al*., 1997), and *goa-1* (Go/i) and *egl-30* (Gq) reciprocally function in neuromuscular processes that regulate behaviours such as locomotion, egg laying, defecation and pharyngeal pumping (Hajdu-Cronin *et al*., 1999; Mendel *et al*., 1995; Miller *et al*., 1999). Furthermore, *goa-1* and *gpa-16* are functionally redundant in asymmetric cell division (Gotta and Ahringer, 2001). At least 14 of the *C. elegans*-specific Gα subunits are thought to function in chemosensory signalling. They are expressed almost exclusively in the specialized structures that sense the environment, such as the amphid neurons, and loss-of-function and overexpression studies revealed chemotactic defects (Jansen *et al*., 1999; Roayaie *et al*., 1998). At this point, it is largely unknown how these G-proteins transduce their signal and how the observed specificity in signalling is established.

In this study, we tried to identify new regulatory mechanisms in heterotrimeric G-protein signalling by performing yeast two-hybrid interaction screens. Such screens may result in the identification of both upstream and downstream components in the signal transduction cascade, as well as modulators. We report here on the identification of 11 different interacting proteins, and a systematic approach by which we attempted to establish biological relevance for the observed interactions, using promoter–GFP reporter constructs and loss-of-function studies using RNAi and genetic mutants. Finally, we provide *in vivo* evidence to support the interaction between the *C. elegans*-specific Gα subunit GPA-7 and the nuclear receptor NHR-22 using a membrane-recruitment assay.

Materials and methods

cDNA cloning

The predicted coding sequence of all *C. elegans* Gα subunits was amplified from total RNA (strain N2) by RT–PCR. First strand synthesis was initiated using a mixture of random hexamers and oligo-dT oligonucleotides and cDNAs were amplified by PCR, using specific oligonucleotides that include the start and stop codons and contain recognition sites for restriction enzymes for in-frame cloning in the appropriate two-hybrid bait or prey vector (pGBK-T7 or pGAD-T7, Clontech). Detailed cloning information can be obtained from the authors upon request. The resulting cDNA constructs were checked by sequencing for potential differences with the spliced gene-product predicted by ACeDB and for the absence of mutations. Mutations resulting in constitutive active GTPase-deficient Gα subunits were introduced by site-directed mutagenesis using PCR and oligonucleotides harbouring the desired mutation. The amino acid changes that were induced were: GPA-5-QL, Gln203Leu; GPA-6-QL, Gln215Leu; GPA-7-QL, Gln203Leu; GPA-12-QL, Gln205Leu; GOA-1-QL, Gln205Leu; GSA-1-QL, Gln208Leu.

All cDNA sequences were submitted to GenBank: GPA-1, AY008124; GPA-2, AY008125; GPA-3, AY008126; GPA-4, AY008127; GPA-5, AY008128; GPA-6, AY008129; GPA-7, AY008130; GPA-8, AY008131; GPA-10, AY008132; GPA-11, AY008133; GPA-12, AY008134; GPA-13, AY008135; GPA-14, AY008136; GPA-15, AY008137; GPA-16, AY008138; EGL-30, AY008139; GOA-1, AY008140; GSA-1, AY008141; ODR-3, AY008142.

Two-hybrid library screening and yeast mating assay

Yeast two-hybrid interaction library screenings were done according to the manufacturer's protocol (Clontech). The *C. elegans* prey cDNA library that was screened (mixture of a random primed and a oligo-dT primed mixed stage *C. elegans* N2 cDNA library in pACT) was a kind gift of R. Barstead, OMRF Oklahoma City, OK. For mating assays, bait and prey plasmids were transformed to yeast strain AH109 (*MAT***a**) and Y187 (*MAT*α,), respectively, and grown overnight in selective medium lacking tryptophan or leucine, respectively. 10 µl of each culture was mixed in 80 µl YPDA in 96-well microtitre plates in the appropriate matrix and grown overnight at 30 °C with shaking. 5 µl drops were seeded on plates lacking both tryptophan and leucine (LT^-) as a control for mating efficiency and on selection plates lacking tryptophan, leucine, histidine and adenine ($LTHA^-$) for detection of interactions.

Prey plasmids were rescued from yeast and sequenced. cDNA sequences of the specific interactors were submitted to GenBank: Y45G5AM.1, AF408754; C05D2.6, AF408755; F32A6.4, AF408756; F44A6.1, AF408757; K06A1.4, AF408758; F44B9.6, AF408759; T22A3.3, AF408760; F53A10.2, AF408761; F59H5.1, AF408762; C01H6.9, AF408763.

Promoter–GFP reporter constructs

To study the *in vivo* expression pattern for candidate interacting genes, we generated PCR fragments harbouring 2–3 kb promoter sequence and the first 1–3 exons of the gene of interest, which are fused in-frame to the GFP coding sequence, as described previously (Hobert *et al*., 1999). PCRs were performed using Accutaq LA DNA polymerase mix (Sigma, St. Louis, MO), which has proofreading activity. Purified PCR fragments were directly injected at 50 μg/ml in *dpy-20(e1282)* animals together with the plasmid pMH86 (Han and Sternberg, 1991) at 150 μg/ml. For each promoter–GFP reporter construct, we created at least three transgenic lines. Transgenic animals carrying an extra-chromosomal array were analysed under a Zeiss Axioskop 2 fluorescence microscope for GFP expression. Cells were identified in reference to Sulston and Horvitz (1977) and White *et al*. (1986).

Nematode strains, culturing and manipulation

General methods used for culturing, manipulation, and genetics of *C. elegans* were as described (Lewis and Fleming, 1995). DNA transformation assays in *C. elegans* by microinjection were as described (Mello and Fire, 1995). Deletion mutants were identified as described (Jansen *et al*., 1997). The following strains were used in this study: Bristol N2, CB1282 [*dpy-20(e1282)*IV], NL561 [*goa-1(pk62)*I], MT363 [*goa-1(n363)*I], DA0823 [*egl-30(ad805)*I], NL795 [*gpa-7(pk610)*IV], NL1586 [*pkIs523(gpa-7-XS)*], NL573 [*pkIs299(hs-goa-1XS)*], CB444 [*unc-52(e444)*II], MT1799 [*lin-36(n766)unc-32(e189)*III], MT555 [*lin-8(n111)-dpy-10(e128)*II], MT111 [*lin-8(n111)*II] and NL4256 [*rrf-3(pk1426)*II]. The CalNuc deletion mutant, *nucb-1(pk1654)*, was identified using oligonucleotides nuc1 (ACACTTCGCGCTCTTTGTTT), nuc2 (CGTGTTCGATGCAAATATGG), nuc3 (ATGAAAGGCGTACCGATGTC) and nuc4 (ATCGAACATCGGAAAAATCG). A genomic region of about 3.4 kb, including all coding exons for CalNuc (F44A6.1), is deleted in this mutant. In the *nhr-22(n2034)* mutant strain (kindly provided by P. Sengupta and the NemaPharm Group of Axys Pharmaceuticals; Liu *et al*., 1999) a genomic region of 1.2 kb is deleted (exons 3–5), resulting in an out-of-frame mutation, deleting the ligand binding domain but leaving the DNA-binding domain intact. Male mating efficiency and behaviour of *nhr-22* mutants was assayed as described (Hodgkin, 1983).

RNA interference

The cDNA inserts of the interacting candidates were subcloned from the prey vector into the *Xho*I site of the L4440 vector (Timmons and Fire, 1998) and transformed into the HT115(DE3) bacterial strain using standard methods. NGM-agar plates containing 1 mM IPTG were inoculated with 50 μl of an overnight bacterial culture and grown overnight. Ten L3-L4 wild-type or *rrf-3* mutant animals (a strain hypersensitive to dsRNA; Simmer *et al*., 2002) were placed on the plate, incubated overnight and transferred to a fresh plate with nutrient that produces dsRNA of the specific cDNA insert. The F_1 progeny was monitored closely for any aberrant behavioural or developmental phenotype.

Membrane recruitment assay

We made expression constructs driven by the *myo-3* promoter for NHR-22 (pRP2231), NHR-22 fused to GFP (pRP2228), NHR-22 fused to the transmembrane domain of Pat-3 (pRP2232), GPA-7 (pRP2233), GPA-7 fused to GFP (pRP2229), GPA-7 fused to the transmembrane domain of Pat-3 (pRP2234) and GPA-7QL fused to GFP (pRP2230). All transmembrane and GFP tags were fused to the N-terminal end of the proteins. Constructs were injected at 50 μg/ml with 100 μg/ml pMH86 (Han and Sternberg, 1991) into *dpy-20(e1282)* or with 100 μg/ml pRF4 (Kramer *et al*., 1990) into N2 Bristol animals. At least two transgenic lines were generated for each co-localization experiment. We produced transgenic lines carrying pRP2232 (25 μg/ml) with *gpa-15::gfp* (50 μg/ml) (Jansen *et al*., 1999) and pMH86 (100 μg/ml) as marker plasmids. Males from these transgenic

lines were crossed with transgenic lines carrying pRP2229 or pRP2230 to give rise to transgenic animals that express both *gpa-15::gfp* and *myo-3::gfp::gpa-7WT* (pRP2229) or *myo-3::gfp::gpa-7QL* (pRP2230). GFP expression was observed under a Zeiss Axiskop 2 equipped with a fluorescent light source and Nomarski optics. Laser scanning confocal microscopy was used to visualize GFP expression at the plasma membrane.

Results

cDNA cloning of all *C. elegans* Gα subunits

C. elegans Gα subunit cDNAs were cloned based upon gene predictions and EST data present in ACeDB (release 12/98). When RT–PCRs failed, predictions were re-examined using three-frame translations of genomic sequence in ACeDB, compared with a multiple protein sequence alignment of the known *C. elegans*, mouse and human Gα subunits. We cloned the cDNAs for all Gα subunits, except for GPA-9, for which we were not able to amplify the 5′ half of the gene, and GPA-17, which was discovered only recently. Sequence analysis shows that about 12% of the intron–exon boundaries (19 out of 154), including the manually corrected start and stop codons, were not correctly predicted by GENEFINDER and annotated in ACeDB (Version WS 12/98). Furthermore, no evidence for alternatively spliced isoforms was found.

Yeast two-hybrid interaction screen

Each of the cloned *C. elegans* Gα subunits and constitutively active GTPase-deficient mutants of GPA-5, GPA-7, GPA-12 and GSA-1 were used as bait in a yeast two-hybrid interaction screen (Table 1). 815 colonies grew on HTLA- plates, of which 547 turned blue on X-gal. 537 prey plasmids were successfully rescued and used to confirm the interaction specificity in a mating assay with the original and control baits. 100 interactions could not be reproduced and 137 were unspecific. The inserts of the 300 remaining clones were sequenced and compared with database sequences. Based upon the molecular identity, 103 clones were excluded from further analysis, as they encode proteins that reside in cellular compartments such as the mitochondrion, or are extracellular, these being locations in which Gα proteins are not expected to be functional. Furthermore, a set of common false-positives, the heat shock proteins, was discarded (a full list can be obtained from the authors upon request). The remaining 197 candidates were tested in a large yeast mating matrix for their interaction characteristics with all 19 cloned Gα subunits. In this experiment, additional control baits (empty vector, p53, *C. elegans* Gβ subunits) were included. At this point, we found that the original GPA-1 bait contained a frameshift mutation in the middle of the cDNA insert, resulting from a RT–PCR cloning error. We repeated the screen for GPA-1 with a corrected bait and used the frameshifted GPA-1 as a control for unspecific interactions. All other bait inserts were once more sequenced to exclude the presence of mutations and, in addition, all bait inserts were cloned into a bacterial expression vector. No other mutations were found and all bacterially expressed proteins were of the expected size (data not shown).

The majority of the clones (173) was found to interact with one or more of the control baits and thus could be considered unspecific interactors (a full list of the identity of these clones can be obtained from the authors upon request). The remaining 24 candidates did not interact with any of the control baits. As expected, all of these candidates did interact with the bait with which they were originally retrieved from the library. Furthermore, we found that none of the candidates interacted with any of the other *C. elegans* Gα subunits (data not shown), illustrating a high degree of specificity for the identified interactions.

Characteristics of the interacting candidates

The 24 remaining clones were found to encode 11 different proteins that interact with four different baits (Table 2). To confirm the observed interaction in an independent assay, we switched the bait and prey vector inserts and included GTPase-deficient mutants in a mating assay (Figure 1). Artificial interaction interfaces can be created at the junctions of the DNA binding domains and the bait, and/or the transcription activation domain and the prey. Changing the configuration can eliminate such false-positives, whereas strong interactions are expected to work in both configurations. Four prey inserts activated transcription by themselves, making them uninformative as bait.

Table 1. Summary of the two-hybrid interaction library screening for all *C. elegans* Gα subunits

		Screened x10^6	Colonies picked	X-gal		Mating		Sequencing		Matrix mating	
				Positive	Negative	Aspecific	Specific	Excluded	OK	Aspecific	Specific*
1	GPA-1†	0.6	96	85	15	1	69	42	27	27	0
2	GPA-1	4.2	65	59	9	20	30	2	28	28	0
3	GPA-2	1	35	10	7	3	0				
4	GPA-3	1.1	55	33	3	2	28	21	7	7	0
5	GPA-4	1.3	30	11	9	0	2	0	2	2	0
6	GPA-5	0.5	2	0							
7	GPA-6	1.3	53	42	10	9	23	2	21	15	6 (1)
8	GPA-7	2.1	3	3	1	1	1	0	1	1	0
9	GPA-8	1.8	3	1	1	0	0				
	GPA-9‡										
10	GPA-10	0.9	7	3	1	2	0				
11	GPA-11	0.6	1	0							
12	GPA-12	0.3	3	0							
13	GPA-13	0.5	68	64	4	25	25	5	20	17	3 (2)
14	GPA-14	0.6	3	2	0	2	0				
15	GPA-15	3.2	1	1	1	0	0				
16	GPA-16	2.9	12	5	2	3	0	0	0		
	GPA-17§										
17	ODR-3	3.1	108	73	5	29	39	11	28	28	0
18	EGL-30	4.5	86	39	11	1	27	12	15	15	0
19	GSA-1	1.8	15	1	0	0	1	0	1	1	0
20	GOA-1	0.3	143	108	20	35	53	8	45	31	14 (7)
21	GPA-5-QL	0.5	4	0							
22	GPA-7-QL	2	15	5	1	3	1	0	1	0	1 (1)
23	GPA-12-QL	1	6	1	0	0	1	0	1	1	0
24	GSA-1-QL	0.9	1	1	0	1	0				
	Total	37	815	547	100	137	300	103	197	173	24 (11)

* Numbers between brackets indicate the number of unique proteins.

† After the two-hybrid screens a frameshift mutation was found in the middle of this bait construct.

‡ We were not able to clone the 5'end of GPA-9 cDNA.

§ This subunit was identified in a recently finished gap in the genome sequence during preparation of this manuscript.

For the other candidates, the interaction was confirmed in the reversed configuration assay, with the exception of T22A3.3 (a protein of unknown function). Interestingly, however, T22A3.3 did interact with K06A1.4 (NHR-22) and was also found to interact with LAG-1 [Su(H)] and MPK-1 (MAP-kinase) in other interaction screens (Walhout *et al*., 2000), suggesting either broad or unspecific interaction activity. We observed a strong homotypic interaction for Y50F7A.1 (Rap1GAP) and a weak homotypic interaction for F44B9.6 (LIN-36). Interaction analysis with GTPase-deficient mutants revealed that the interactions of the nuclear receptors K06A1.4 and Y45G5AM.1 with GPA-7 and GPA-13, respectively, are independent of the activation state of the Gα subunit. T22A3.3 was found to interact exclusively with the wild-type form of GPA-6 and only two (AGS3.1 and Rap1GAP) out of the seven candidates for GOA-1 interact with the GTPase-deficient mutant of GOA-1.

Expression analysis of the interacting candidates

Gene-expression patterns for the interacting candidates were obtained by fusing promoter regions to GFP-encoding sequences. Complete and partial overlap in expression pattern for the candidate genes and their respective bait can be observed for a subset of the candidates (Figure 2 and summarized in Table 3). For example, both NHR-22 and GPA-7 are expressed in different types of muscle cells and neurons, but also in the male specific neurons in the tail required for mating (Figure 2I). GOA-1 is ubiquitously expressed in neuronal tissue and in muscles (Mendel *et al*., 1995; Segalat *et al*., 1995), and

Table 2. Properties of proteins interacting with Gα subunits

Protein ID	Description	Bait	*n**	Fragment†	Chr	QL‡	rev§	RNAi¶	Mutant	Remarks
T22A3.3	Protein of unknown function	GPA-6	6(4)	121–418	I	–	–	No obvious phenotype	Not available	Interacts also with LAG-1, MPK-1[III], and K06A1.4; has no clear homologues in other organisms
K06A1.4	Nuclear receptor (NHR-22)	GPA-7-QL††	1	271–579	II	+	nd‡‡	No obvious phenotype	*nhr-22:* deletion of ligand binding domain: no obvious phenotype. Also not in *gpa-7* or GPA-7-XS background	DNA-binding domain is not present in the interacting fragment
Y45G5AM.1	Nuclear receptor	GPA-13	2(2)	15–400	V	+	nd‡‡	No obvious phenotype	nd	
F15D3.1	Dystrophin (DYS-1)	GPA-13	1	1948–2241	I	+	nd	No obvious phenotype	*dys-1*; hyperactivity and hypercontraction of animal[§§]	
C05D2.6	Protein of unknown function**)	GOA-1	1	15–650	III	–	nd‡‡	Sterile progeny or reduced broodsize in *rrf-3* mutant background.	Not available	Has no clear homologues in other organisms
C01H6.9	Contains protein kinase domain	GOA-1	1	307–920	I	–	nd	No obvious phenotype; also not in combination with a closely related protein F22H10.5	Not available	Homology to haploid germ cell-specific nuclear protein kinase (haspin)
F44B9.6	LIN-36	GOA-1	6(5)	682–962	III	–	+	No obvious phenotype; also not in *goa-1* or GOA-1 XS background	MuV in combination with LIN8/38/15[¶¶] : *goa-1;lin-8* or *goa-1;lin-36* mutants do not have a synMuv phenotype	Weak homotypic interaction

F59H5.1	Protein of unknown function	GOA-1	1	501–742	II	–	+	No obvious phenotype; also not in *goa-1* or GOA-1 XS background	Not available	Has no clear homologues in other organisms
F32A6.4[||]	AGS3.1, LGN, Pins (partner of inscuteable)	GOA-1	2(2)	432–579	X	+	+	No obvious phenotype; also not in *goa-1* or GOA-1 XS background	Not available	Interacting fragment contains three GoLoco motifs out of four
F53A10.2[||]	Rap1GAP	GOA-1	2(2)	2–694	II	+	+	No obvious phenotype; also not in *goa-1* or GOA-1 XS background	Not available	Interacting fragment contains 1 GoLoco motif; homotypic interaction
F44A6.1[||]	Nucleobindin/CalNuc EF-hand Ca-binding protein	GOA-1	1	1–453	X	–	nd[‡‡]	No obvious phenotype, also not in *goa-1* or GOA-1 XS background	*nucb-1*: complete coding region deleted: no phenotype (also not in *goa-1* or *egl-30* background)	No homologues in *C. elegans*, one orthologue in *Drosophila*, two orthologues in mouse and human

* Number of clones identified in the screen. Between brackets the number of independent clones is indicated.
† Smallest region of the protein sufficient for interaction.
‡ Interaction with GTPase-deficient form of the original bait.
§ Interaction detectable when bait and prey protein are switched.
¶ Results are in line with data published by Fraser *et al.* (2000), Gonczy *et al.* (2000) and Miyabayashi *et al.* (1999).
|| Interologs: interaction between homologous proteins was previously described in another species
** previously annotated as the homologue of human LIS-1 protein; now the gene is split in two and the interacting fragment has no homology to any known protein.
†† Does also interact with wild-type GPA-7.
‡‡ nd, not determined, because these baits autoactivate the reporter genes.
§§ Bessou, *et al.* (1998);
¶¶ Thomas and Horvitz (1999);
|||| Walhout *et al.* (2000).

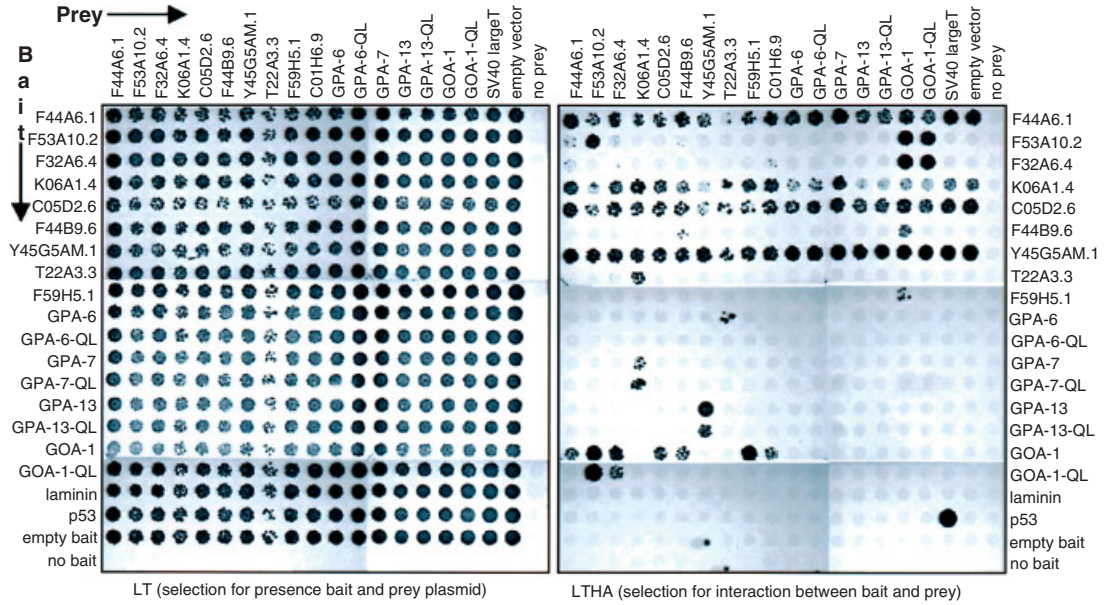

Figure 1. Yeast mating assay. The interacting candidates identified in the screen and the corresponding Gα-subunits were cloned in both bait and prey vectors and tested in a mating matrix. Diploid strains were plated on Leu^-/Trp^- medium (left panel) to control for the presence of both bait and prey plasmids, and on $Leu^-/Trp^-/His^-/Ade^-$ medium to select for two-hybrid interactions. Baits F44A6.1, K06A1.4, C05D2.6 and Y45G5AM.1 are not informative, because they autoactivate transcription of the reporter genes

several of the interacting candidates are predominantly expressed in muscle cells such as AGS3.1 (Figure 2C–E) and CalNuc, or neuronal cells like LIN-36, F59H5.1 and C01H6.9, whereas others are expressed in both neurons and muscle cells such as C05D2.6.

As for human haspin, expression of the *C. elegans* haspin homologue C01H6.9 is mainly observed in the nucleus (Figure 2G). Some candidates seem to have no or little overlap with their respective bait. T22A3.3 is expressed in several neuronal cells, including a specific chemosensory neuron, AWB (Figure 2H), but its binding partner GPA-6 is expressed in the chemosensory neurons AWA, ASI and PHB (Jansen *et al.*, 1999).

We cannot exclude the possibility that the observed expression patterns are not complete, due to the lack of important regulatory promoter elements or to low expression levels. Furthermore, we may have missed co-expression in earlier developmental stages, as we determined the specific cells in L4 and adult stages. However, our data does show a correlation between the expression of the Gα subunits and their specific interacting partners.

Functional analysis of the Gα interactors

We performed RNAi experiments (Timmons and Fire, 1998) to functionally inactivate the genes encoding the proteins interacting with Gα. Wild-type (N2) and a mutant strain (*rrf-3*) that is hypersensitive to dsRNA (Simmer *et al.*, 2002) were fed bacteria expressing dsRNA derived from the cDNA inserts of the prey clones. F_1 progeny was analysed for aberrant behavioural or developmental phenotypes (e.g. lethality, brood size, development, locomotion and egg-laying activity, morphology, etc.). Only RNAi of C05D2.6 in an *rrf-3* mutant background resulted in a phenotype, e.g. sterile progeny (44%) and a reduced brood size (13 ± 4, $n = 27$). In a wild-type background, no obvious phenotype for C05D2.6 RNAi is observed (brood size is 223 ± 21, $n = 23$). The C05D2.6 (RNAi) phenotype correlates with the reduced broodsize observed in *goa-1* genetic mutants (Mendel *et al.*, 1995) and animals fed with dsRNA directed against GOA-1 (our own observations; Fraser *et al.*, 2000). Interestingly, both GOA-1 and C05D2.6 are co-expressed in the distal tip cell of the gonad (Figure 2A; Mendel *et al.*, 1995), and ablation of this cell is

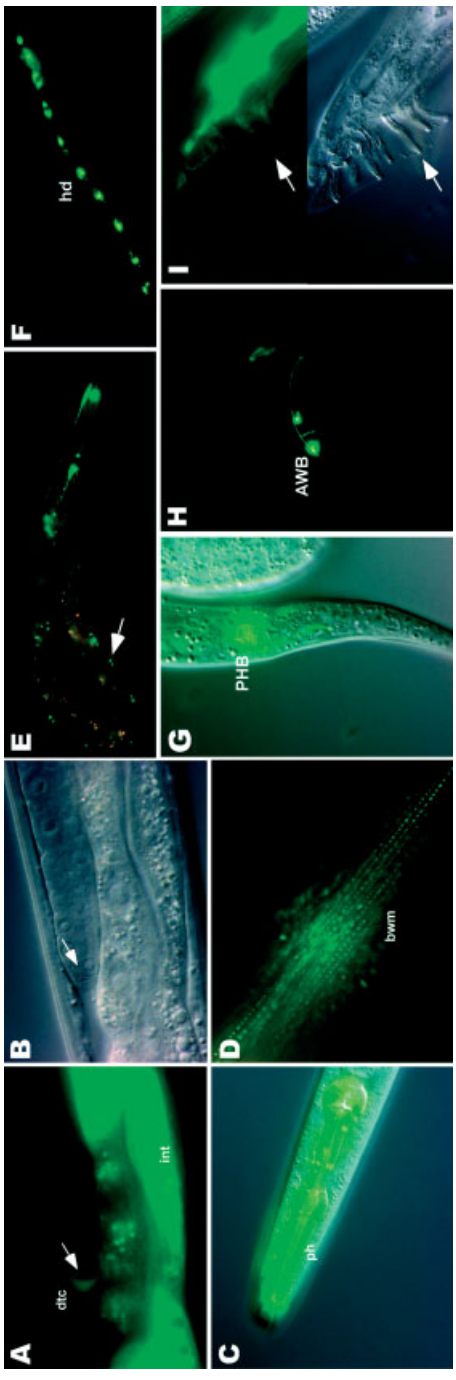

Figure 2. Expression pattern of C05D2.6 (A–B), AGS-3.1 (LGN/Pins; C–E), RapIGAP (F), C01H6.9 (haspin; G), T22A3.3 (H) and NHR-22 (K06A4.1; I), as determined by promoter–GFP reporter constructs. An overview of all expression patterns is shown in Table 3. (A) C05D2.6 is expressed in the distal tip cell (dtc, arrow) of the gonad and in the intestine (int). (C) Expression of AGS-3.1 is seen in the pharynx (ph), (D) in body wall muscles (bwm) and (E) in a punctuated pattern in the ventral nerve cord (arrow). (F) Expression of RapIGAP in hypodermal cells (hd). (G) C01H6.9 expression is shown in one of the phasmid neurons, PHB. (H) Expression of T22A3.3 is seen in a set of neurons in the head, including AWB. (I) K06A4.1 expression is seen in male-specific neurons (arrow)

Table 3. Expression profile of the interacting candidates and their corresponding Gα subunits

	Description	Bait	Expression
T22A3.3	Unknown	GPA-6	Neurons in the head (AWB, ADF, ASG (very faint), various interlabial sensory neurons), socket cell, sheath cell in tip of nose, pharyngeal muscle (anterior strong, posterior weak), intestine
K06A1.4*	NHR-22	GPA-7-QL	Neurons in the head (including sensory neurons and various interlabial neurons), interneuron (PVT), many neurons in male tail, pharyngeal and vulval muscle, coelomyciten, intestine
Y45G5AM.1	Nuclear receptor	GPA-13	Intestine, weak in hypodermal cells, predominantly nuclear staining
F15D3.1†	DYS-1	GPA-13	Muscles
C01H6.9	Kinase	GOA-1	Sensory neurons (PHB) and interneurons (PVT) in tail, several uncharacterized neuronal cells in the head with projection to the nose and into the nerve ring, intestinal cells, weak expression in hypodermal seam cells, mainly nuclear staining
C05D2.6	Unknown	GOA-1	Several neurons in head and tail, pharyngeal muscle, intestine, distal tip cell of the gonad, weak expression in the ventral nerve cord
F32A6.4	AGS3.1	GOA-1	All muscles (body wall, pharyngeal, sphincter, vulval), intestine, weak expression in a set of neurons in the head and neurons in the ventral nerve cord, typical subcellular localization in strong dots
F44A6.1	CalNuc	GOA-1	Pharyngeal muscle, body wall muscle, vulval muscle, weak expression in neurons in head and tail, weak staining in intestinal cells (mainly posterior)
F44B9.6‡	LIN-36	GOA-1	Vulval (precursor) cells, many neurons in head, tail and ventral nerve cord; weak expression in hypodermal and intestinal cells; nuclear localization
F59H5.1	Unknown	GOA-1	Neuronal head ganglia, intestinal cells, hypodermal cells, coelomyciten, highly expressed in embyros
F53A10.2	rap1GAP	GOA-1	Hypodermal seam cells, various neurons, intestinal cells
GPA-6§			Subset of sensory neurons (AWA, ASI (faint), PHB)
GPA-7§			Many neurons, muscle cells, many neurons in male tail
GPA-13§			Subset of sensory neurons (ADF, ASH, AWC, PHA, PHB)
GOA-1¶			Ubiquitously in neurons and muscles, distal tip cell of the gonad

* A similar expression pattern was described in Miyabayashi *et al*., (1999).
† Bessou *et al*. (1998);
‡ Thomas and Horvitz (1999);
§ Jansen, *et al*. (1999);
¶ Mendel *et al*. (1995).

shown to result in a decreased number of germ cells (Kimble and White, 1981).

Furthermore, RNAi against the candidate proteins that interact with GOA-1 in a mutant background did not modulate the *goa-1* loss- and gain-of-function phenotypes (Table 2). As knockdowns by RNAi may not be complete loss-of-function, we analysed three genetic mutants of interacting candidates: *nhr-22* (K06A4.1), *lin-36* (F44B9.6; Thomas and Horvitz, 1999) and *nucb-1* (F44A6.1) but did not observe any phenotype for these mutants. As *lin-36* has a synthetic multivulva phenotype when combined with mutations in class A synMuv genes (e.g. *lin-8*), we generated double mutants between *goa-1* and *lin-36* or *lin-8*. However, no synMuv phenotype was observed. Although there is only a single homologue for *nucb-1* in *C. elegans*, whereas there are two in mammals, mutants behave completely like wild-type animals and no modulation of *goa-1* loss- or gain-of-function mutant phenotypes could be observed (Table 2), suggesting a non-essential role for this protein in *C. elegans*. Similarly, for a genetic mutant of *nhr-22,* no behavioural, developmental or male-mating defects were observed, nor modulation of *gpa-7* loss- or gain-of-function mutant phenotypes (Table 2). As NHR-22 is a member of a very large, well-conserved gene family in *C. elegans* of more than 250 members (Enmark and Gustafsson, 2000; Miyabayashi *et al*., 1999; Sluder and Maina, 2001), functional redundancy, similar to that observed for the *C. elegans* Gα protein family (Jansen *et al*., 1999), cannot be excluded.

GPA-7 and NHR-22 interact *in vivo*

Because *nhr-22* mutant animals do not show any obvious phenotypes, we examined whether there is *in vivo* evidence to support an interaction using a membrane recruitment assay. We co-expressed GFP coupled to wild-type or activated GPA-7 and NHR-22 coupled to a membrane localization signal in muscle cells. In the absence of NHR-22 (not shown) or the presence of wild-type NHR-22 (Figure 3A), wild-type GPA-7 is localized in both the nucleus and cytoplasm. However, in the presence of membrane-targeted NHR-22, wild-type GPA-7 is recruited to the plasma membrane (Figure 3B). Similar data was found for activated GPA-7 (not shown), consistent with the two-hybrid data (Figure 1). Furthermore, the reversed experiment with GFP-tagged NHR-22 (which is, like GPA-7, localized in the cytoplasm and the nucleus) co-expressed with membrane-targeted GPA-7, results in the same membrane recruitment (not shown). Taken together, these results show that GPA-7 and NHR-22 can interact both *in vitro* and *in vivo*. Such an interaction between a G-protein and a nuclear receptor has not (to our knowledge) been reported before.

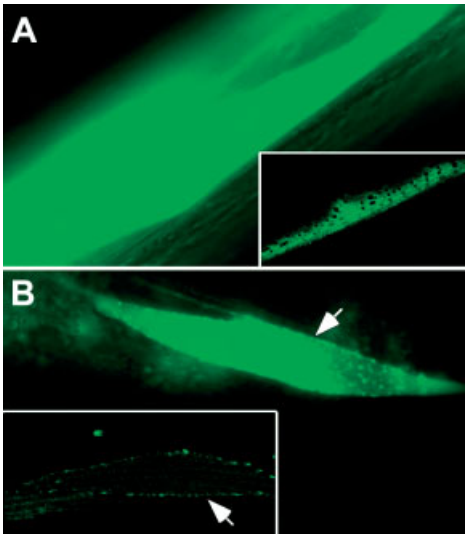

Figure 3. *In vivo* interaction between GPA-7 and NHR-22. GFP-tagged GPA-7 is localized in the cytosol and nucleus of muscle cells in the presence of untagged NHR-22 (A) and is recruited to the membrane in the presence of NHR-22 tagged with a membrane localization signal (B), as indicated by multiple foci (arrow). The panels inside the pictures show a confocal image of the muscle cell

Discussion

This study aimed to identify specific components in G protein signalling pathways by systematically using all *C. elegans* Gα subunits in yeast two-hybrid interaction screens. Although initially a large set of potential interactors was obtained, application of a series of controls resulted in a specific, but surprisingly small, set of only 11 different *C. elegans* proteins that interact with four Gα subunits. Among these are orthologues of proteins known to interact with Gα subunits and novel proteins with homologues in mammalian species.

The low number of interactors identified raises the questions: why did we not identify more interacting proteins?; and why did we not find interactions for the majority of the Gα subunits? First, the Gα or partner protein may not be folded properly, due to its fusion to the DNA-binding or transcription activation domain, prohibiting an interaction. Second, the proximity of the plasma membrane or the presence of additional co-factors may be necessary for proper protein folding or interaction. Indeed, we failed to reconstitute a trimeric complex of an α-, β- and γ-subunit in a yeast three-hybrid system (E. Cuppen, unpublished results; data not shown), although we were able to demonstrate two-hybrid interactions between β-, γ- and γ-like-subunits (van der Linden *et al*., 2001). Thirdly, Gα subunits may have to be in their active, GTP-bound, state to interact with effectors. However, we only identified a single protein (K06A1.4) in the four screens with the GTPase-deficient mutants and none of the expected downstream effectors, such as adenylyl cyclases (Gilman, 1987) or phospholipases (Simon *et al*., 1991), were retrieved. The latter may be due to the way Gα proteins activate these downstream effectors. When these are short-lived 'kiss-and-goodbye'-type interactions, it may be impossible to detect them in an *in vivo* environment, limiting this screen to the identification of more structural interactions. Finally, it is possible that the interacting candidates are not present in the library that was screened, due to under-representation as a result of low expression levels, as is the case for some of the Gα subunits, which are expressed in only a few specific neuronal cells (Jansen *et al*., 1999). Illustrative of this is that most of the GPA sequences (except for GPA-4, GPA-12, GOA-1, GSA-1, EGL-30) are not present in EST and/or cDNA libraries (source: Wormbase).

For the reasons mentioned, the two-hybrid interaction system chosen will not result in the identification of all up- and downstream interactors for Gα proteins. However, we identified both known interactors (Rap1GAP, CalNuc and AGS3.1), thus validating our screen, and novel interacting proteins including the nuclear receptors NHR-22 and Y45G5AM.1 and the homologue of human haspin, C01H6.9, illustrating the value of the approach.

What is the biological relevance of the observed interactions? First, using GFP–reporter constructs, we showed that there is a correlation between the expression in specific cells or cell types of the interacting proteins and their partner. Of the 11 preys, three had a complete overlap (C05D2.6, AGS3.1, and CalNuc) and four had a partial overlap (NHR-22, C01H6.9, LIN-36, and rap1GAP), but for four no overlap was found (T22A3.3, Y45G5AM.1, DYS-1 and F59H5.1). Differences in expression may indicate that the interaction should be considered false-positive, but may also reflect tissue- or cell-specific functions in the case of partial overlap, or be a result of the technical limitations of the approach (GFP sensitivity and silencing of transgenes in early embryonal stages).

Second, we performed RNAi experiments to inactivate gene function. However, except for C05D2.6 in an *rrf-3* mutant background, we did not observe any obvious phenotype using this approach. This may be caused by the predominant neuronal localization of both Gα subunits and interacting partners, where RNAi by feeding is known to be less effective (Timmons *et al*., 2001). Moreover, chromosome-wide analyses have shown that RNAi mimics the genetic knock-out phenotype in only about 50% of cases (Fraser *et al*., 2000; Gonczy *et al*., 2000), making results obtained by this method not fully conclusive. Nevertheless, the lack of phenotype in both RNAi experiments and when using genetic mutants, as shown for three of the interactors, may be associated with a non-essential or redundant role for these proteins. Particularly for NHR-22, redundancy may be an issue, as the nuclear receptor family, together with the G protein-coupled receptors, belongs to one of the largest in *C. elegans*. Interestingly, as for the Gα subunits, many of the nuclear receptors are expressed in (a limited set of) neuronal cells. Nevertheless, we do provide support for an *in vivo* interaction between NHR-22 and GPA-7 using a membrane recruitment assay. As this assay does not formally prove that the endogenous proteins interact *in vivo*, other assays such as co-precipitation or co-localization assays for endogenous proteins will be needed. However, these approaches would require highly specific antibodies for the individual members of these large gene families of nuclear receptors and Gα-proteins, which might be hard to obtain.

While large-scale two-hybrid screens have been shown to be helpful for uncovering networks of interactions in biological processes (Drees *et al*., 2001; Walhout *et al*., 2000), our systematic study of the individual members of the complete Gα-protein family has resulted in only limited results. Although the combination of complete genome information, large-scale two-hybrid screens, and the powerful genetic and molecular techniques that are available for *C. elegans* potentially form an ideal combination for doing (large-scale) functional genomics studies, such an approach may not be suitable for every gene family. In addition, large-scale two-hybrid approaches are now known to be prone to considerable amounts of both false-positives and false-negatives (Mrowka *et al*., 2001; von Mering *et al*., 2002). Therefore, one may have to consider alternative high-throughput technologies, such as mass spectrometry-based approaches like TAP (tandem-affinity purification; Gavin *et al*., 2002) and HMS-PCI (high-throughput mass-spectrometry protein complex identification; Ho *et al*., 2002) to identify relevant interacting proteins and novel candidates in signal transduction cascades.

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