Characterization of heterotrimeric G-proteins in adult *Acanthocheilonema viteae*

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Heterotrimeric G-proteins have been found in eukaryotic cells, from yeast to humans, but have received little attention, to date, with respect to parasitic organisms. We now present the first report of the characterization of heterotrimeric G-proteins expressed in a filarial nematode, *Acanthocheilonema viteae*. Using a combination of (i) affinity labelling with [α-^{32}P]GTP; (ii) ADP-ribosylation with cholera toxin and pertussis toxin; (iii) Western blotting with a panel of anti-G-protein antibodies; and (iv) reverse transcriptase-PCR with degenerate G-protein oligonucleotide primers followed by hybridization analysis using oligonucleotides specific for individual G-protein subunits, we demonstrate that adult *A. viteae* expresses homologues of the β1- and/or β2-like subunits and α-subunits of the G_s, G_i, G_q and G_{12} subfamilies found in mammals. The role which these G-proteins may play in the biology of the organism is discussed.

INTRODUCTION

Guanine nucleotide binding proteins (G-proteins) have been shown to be involved in the regulation of a diverse range of biological processes, such as intracellular trafficking, cell movement, growth, proliferation and differentiation (reviewed in [1–3]). This G-protein superfamily predominantly comprises two major subfamilies: the small, monomeric, ras-like G-proteins, and the heterotrimeric G-proteins. Heterotrimeric G-proteins regulate the transduction of transmembrane signals from cell-surface receptors to a variety of intracellular effectors, such as adenylate cyclase and phospholipase C [1–4]. These G-proteins consist of three distinct classes of subunits, α (39–46 kDa), β (37 kDa) and γ (8 kDa), and, in general, effector specificity is conferred by the α-subunit, which contains the GTP-binding site and an intrinsic GTPase activity [2–4]. However, it is now widely accepted that $\beta\gamma$ complexes can also directly regulate effectors such as phospholipase A_2, phospholipase C-β, adenylate cyclase and ion channels in mammalian systems and, in addition, cellular responses such as mating factor receptor pathways in yeast [5–10].

Several distinct subfamilies of heterotrimeric G-proteins have been isolated and cloned; at least 16 different α-subunit genes have been identified in mammals, and these have been divided into four major subfamilies: G_s, G_i, G_q and G_{12} [2–4,11]. Moreover, cDNA clones encoding at least four related, but distinct, β-subunits and six γ-subunits have been identified [12–16]. The G_s family contains G_s and G_{olf}; these α-subunits stimulate adenylate cyclase, and can be irreversibly activated by ADP-ribosylation by cholera toxin [2–4]. The G_i family contains G_i, G_o and G_z subunits, which can inhibit adenylate cyclase and modulate potassium and calcium channels [2–4,17,18]. In addition, $\beta\gamma$-subunits resulting from activation of pertussis toxin-sensitive G_i-like G-proteins ($G_i\alpha$ and $G_o\alpha$) have been shown to regulate the β-isoforms of phospholipase C [2–4,9,10]. Interestingly, the α-subunits of the G_q subfamily (G_q, G_{11}, G_{14}, G_{15} and G_{16}) have also been shown to activate the β-isoforms of phospholipase C [2–4,19,20]. Although the G_{12} subfamily, which contains G_{12} and G_{13}, is as yet less well characterized, these G-proteins have been shown to be coupled to thrombin and thromboxane A_2 receptors [21] and have been implicated in the regulation of Na^+–H^+ exchange (G_{13}) and eicosanoid production (G_{12}) [22,23], but not phospholipase C [24]. Moreover, G_{12} and G_{13} have recently been shown to be involved in Rho-dependent stress fibre formation and focal adhesion assembly [25], suggesting a role for these G-proteins in the functional regulation of the actin cytoskeleton.

Heterotrimeric G-proteins have been found in eukaryotic cells, from yeast to humans, but have received little attention to date with respect to parasitic organisms. One group of parasitic organisms which is found throughout the Tropics and which has yet to receive any attention with respect to the employment of G-proteins is the filarial nematodes. Some species of this group of organisms, e.g. *Wuchereria bancrofti*, *Brugia malayi* and *Onchocerca volvulus*, are of major medical importance as they cause a range of pathological lesions, the most important of which are elephantiasis, chronic debilitating skin diseases and blindness. Methods of control for filarial nematodes (particularly *O. volvulus*) are currently inadequate, and could in the long term benefit from an understanding of how the worms interact with signals from their environment, the parasitized host. In relation to this, we have characterized the heterotrimeric G-proteins expressed in the rodent filarial nematode, *Acanthocheilonema viteae*, as a first approach to addressing the role(s) of these important regulatory molecules in signal transduction pathways in filarial parasites. We have used a combination of different techniques designed to specifically target G-proteins: affinity labelling with [α-^{32}P]GTP, ADP-ribosylation with cholera and pertussis toxins, Western blotting with a panel of anti-G-protein antibodies, and reverse transcriptase-PCR (RT-PCR) with degenerate G-protein oligonucleotide primers followed by hybridization analysis using oligonucleotides specific for in-

Abbreviation used: RT-PCR, reverse transcriptase-PCR.

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dividual G-protein subunits. We now report that *A. viteae* expresses β1- and/or β2-like subunits and α-subunits of the G_s, G_i, G_q and G_{12} subfamilies.

EXPERIMENTAL

Reagents

ATP, cholera toxin, collagenase, Coomassie Brilliant Blue, dithiothreitol, GTP, β-NAD^+, $NaBH_3CN$, $NaBH_4$, $NaIO_4$, PMSF, sodium orthovanadate, Tween-20 and thymidine were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). [α-^{32}P]GTP, [α-^{32}P]NAD^+ and the anti-'pan-G-protein' antibody NEI-800 were from NEN/DuPont (Stevenage, U.K.). Pertussis toxin was obtained from Porton Products Ltd. (Porton House, Berks., U.K.). Donkey serum was from the Scottish Antibody Production Unit (Law Hospital, Carluke, Scotland, U.K.). Donkey anti-rabbit Ig conjugated to horseradish peroxidase was purchased from Amersham International (Little Chalfont, Bucks., U.K.). Reagents for RNA extraction were from Stratagene (Cambridge, U.K.). Reverse transcriptase was obtained from Life Technologies (Inchinnan, Scotland, U.K.). The random hexadeoxynucleotide primer, rRNasin, *Taq* polymerase and 10 × *Taq* polymerase buffer were from Promega (Southampton, U.K.). Oligonucleotides were produced by Cruachem (Glasgow, Scotland, U.K.). The deoxynucleotides were purchased from Pharmacia Biotech (St. Albans, Herts., U.K.).

Parasite

Jirds (*Meriones libycus*) were infected by subcutaneous injection of third-stage *A. viteae* larvae recovered from infected ticks (*Ornithodorus tartakovskyi*) according to the method of Worms and colleagues [26]. Adult parasites were recovered from patent jirds after direct visual examination of the skin and underlying body surfaces of the animals.

Parasite extracts

Adult *A. viteae* worms were cut into 3–5 mm pieces with sharp scissors and then incubated in collagenase (1 mg/ml in PBS, pH 7.4, for 30 min.) to digest the cuticle. The resultant pieces of parasite were washed three times in PBS by microcentrifugation. The parasite pieces were then ground up in a glass tissue homogenizer (Anachem, Luton, Beds., U.K.) in 1 ml of homogenization buffer [10 mM phosphate buffer, pH 7.4, containing NaCl (140 mM), EDTA (1 mM) and the protease inhibitor PMSF (1 mM)]. The homogenate was centrifuged at a low speed (40 ***g*** for 5 min) to remove tissue debris and the resulting pellet was solubilized in 0.1 M Tris/HCl buffer, pH 8.0, containing 5 mM EDTA, 3 % (w/v) SDS and 1 mM PMSF. The supernatant was centrifuged at 130000 ***g*** for 30 min and the membrane pellet was solubilized in homogenization buffer. The solubilized low-speed pellet (PBS-insoluble extract), the high-speed pellet (membrane extract) and the supernatant from the high-speed spin (cytosolic extract) were retained for analysis.

GTP labelling

Parasite membrane and cytosolic extracts and a rat brain membrane preparation [27] were labelled with [α-^{32}P]GTP using a method adapted from [28]. Briefly, 50 μl samples, adjusted to contain (final concentrations) 40 mM Hepes (pH 7.5) and 2 mM $MgCl_2$, were incubated with 1 μM [α-^{32}P]GTP (sp. radioactivity 100 Ci/mmol) for 5 min at 37 °C. The ribose on the bound nucleotide was then oxidized to form a reactive dialdehyde by addition of $NaIO_4$ (1 mM, final concentration) and the samples were incubated for 1 min at 37 °C. This reaction was followed by the addition of $NaBH_3CN$ (20 mM, final concentration) for 1 min at 37 °C, to stabilize Schiff bases formed between the oxidized nucleotide and nearby lysine residues. The reactions were then terminated by the addition of $NaBH_4$ (20 mM, final concentration) for 1 min at 37 °C to reduce the excess dialdehyde. The samples were placed on ice for 10 min. An equal volume of pervanadate loading buffer [50 mM Tris buffer, pH 8, containing 5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, 0.01 % (w/v) Bromophenol Blue and 1 μM sodium pervanadate] was added. The samples were subjected to SDS/PAGE on a 12.5 % gel, stained with Coomassie Brilliant Blue, destained, dried and autoradiographed.

ADP-ribosylation with cholera toxin and pertussis toxin

ADP-ribosylation was carried out according to the method of Harnett [29]. Cholera toxin (900 μg/ml) and pertussis toxin (90 μg/ml) were activated by incubation with 25 mM dithiothreitol for 30 min at 37 °C in toxin reaction buffer [0.3 M potassium phosphate buffer, pH 7.0, containing (final concentrations) 25 mM Tris/HCl, 10 mM thymidine, 1 mM ATP, 10 mM $MgCl_2$, 1 mM EDTA and 0.1 mM GTP]. The toxin preparations were then adjusted to 60 μg/ml (pertussis toxin) and 600 μg/ml (cholera toxin) with 75 mM Tris/HCl buffer (pH 7.5) containing 1 mg/ml BSA. Aliquots (10 μl) of parasite and rat brain membrane extracts (50 μg) were incubated with 10 μl of activated cholera toxin (6 μg) or pertussis toxin (0.6 μg) and 5 μl of [α-^{32}P]NAD^+ (sp. radioactivity 10–50 Ci/mmol, adjusted to 0.6 mCi/ml with 200 μM unlabelled NAD^+), made up to a total volume of 60 μl with toxin reaction buffer, for 45 min at 32 °C. The reaction was stopped by the addition of 1 ml of ice-cold 20 % trichloroacetic acid, followed by 20 min on ice and microcentrifugation at 15640 ***g*** for 20 min at 4 °C. The pellets were washed twice with 0.5 ml of ice-cold acetone and resuspended in pervanadate loading buffer. The samples were then subjected to SDS/PAGE on a 12.5 % polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue, destained, dried and autoradiographed.

SDS/PAGE and Western blotting

Parasite and rat brain membrane extracts were resolved by SDS/7.5 %-PAGE according to the method of Laemmli [30]. Loading buffer [50 mM Tris/HCl buffer, pH 8, containing 5 M urea, 0.17 M SDS, 0.4 M dithiothreitol, 0.01 % (w/v) Bromophenol Blue] was added in equal volume to the samples. Following SDS/PAGE, proteins were semi-dry blotted according to the manufacturer's instructions (Sartorius Ltd., Epsom, Surrey, U.K.) on to nitrocellulose membranes (Hybond-ECL; Amersham). Blots were blocked in 5 % (w/v) gelatin and 10 % (v/v) donkey serum in TBS (Tris-buffered saline), pH 7.6, containing 0.1 % (v/v) Tween-20 (TBS-T) for 1 h at 37 °C. Blots were then incubated overnight at 4 °C with primary antibodies (described in Table 1) diluted 1:1000 in 0.25 % (w/v) gelatin and 3 % (v/v) donkey serum in TBS-T. Donkey anti-rabbit Ig conjugated to horseradish peroxidase was used as secondary antibody [diluted 1:1000 in 3 % (v/v) donkey serum in TBS-T] for 2 h at room temperature. Blots were developed using an enhanced chemiluminescence system (ECL Detection Kit and Hyperfilm-ECL; Amersham). In order to check the specificity of binding, in some experiments antibody solutions were pre-incubated for 2 h with an excess (1 mg/ml) of the peptide to which they were raised.

RNA extraction, RT-PCR and slot blotting

Total RNA was isolated from adult *A. viteae* and mouse brain (positive control) using a guanidinium thiocyanate/phenol/chloroform single-step procedure [31]. Reverse transcription was performed on total RNA (approx. 1 μg) using 200 units of Moloney murine leukaemia virus reverse transcriptase, 0.5 μg of random hexadeoxynucleotide primers and 20 units of rRNasin (RNase inhibitor) in 20 μl of 1 × *Taq* polymerase buffer. This reaction mixture was incubated for 1 h at 37 °C. PCR was performed in a Hybaid thermal cycler. The following primers were employed: oMP19 (forward), CGGATCCAARTGGATHCAYTGYTT; oMP20 (reverse), GGAATTCRTCYTTYTTRTTNAGRAA; oMP21 (reverse), GGAATTCRTCYTTYTTRTTYAARAA. These are degenerate primers which recognize sequences common to all mammalian G-protein α-subunits [32]. The reverse transcription reaction was made up to 100 μl with 10 ng/l of each primer and 2 units of *Taq* polymerase in 1 × *Taq* polymerase buffer. A total of 35 cycles, each of which consisted of 1 min at 94 °C, 1.5 min at 41 °C and 2 min at 72 °C, were performed, with the final cycle having an extension time of 10 min at 72 °C. As a negative control, mouse brain RNA was amplified using primers specific for β-actin (5′ primer, GTGGGCCGCTCTAGGCACCAA; 3′ primer, CTCTTTGATGTCACGCACGATTTC [33]). A total of 25 cycles, each of which consisted of 1 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C, were performed, with the final cycle having an extension time of 10 min at 72 °C. A 60 μl sample of each PCR product was alkali-denatured with 60 μl NaOH (1.7 M) and incubated at room temperature for 10 min, then 120 μl of 0.2 × SSC (3 mM sodium citrate, 30 mM NaCl) was added.

The PCR products were analysed by electrophoresis on agarose gels stained with ethidium bromide, with visualization under UV light. The samples were then applied to a sheet of Hybond-N^+ nylon (Amersham) on a slot blot apparatus (Bio-Rad), and left for 30 min. A vacuum was applied, and each slot was given two washes with 150 μl of 0.4 M NaOH, followed by two washes with 150 μl of 2 × SSC. The membrane was allowed to air dry and then baked for 2 h at 80 °C. Hybridization was carried out using oligonucleotide primers designed to recognize internal sequences specific to individual G-protein subunits [34]. The primers used were: SG1 (G_s/G_{olf}), GCAGCAGCTACAACATGGT; CT35 (G_{i1}), CTTCAGCAAGAACCAG; OP1 (G_{i3}), CAATTTCATGCTTTCA; OP4 (G_{oB}), GAGCTTCAGGGAATTCG; GQ4 (G_q), ATTCGCTAAGCGCTACTAGA; CT106 (G_{11}), CTCGCTTAGTGCCACC; CT109 (G_{13}), TTCACTTGAAGAGACAAGGAAA; OP3 (G_{oA}), GAGCATGAGAGACTCG. The primers were labelled with fluorescein and detected using an anti-fluorescein antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (ECL 3′-oligolabelling and detection systems; Amersham), according to the manufacturer's instructions. Probes were used at a concentration of 10 ng/ml, except for CT109 and SG1, which were used at 30 ng/ml. Hybridization and stringency washes were carried out at 42 °C using 5 × SSC.

RESULTS

Identification of G-proteins in adult *A. viteae*

[α-^{32}P]GTP affinity labelling studies (Figure 1) demonstrated that several putative GTP-binding proteins were present in membrane extracts of adult *A. viteae*. For example, at least three proteins of molecular mass 38–44 kDa (a range consistent with that of α-subunits of the heterotrimeric G-proteins) could be detected (Figure 1, lane 8). The possibility that these bands represent α-subunits of heterotrimeric G-proteins was supported by the finding that rat brain membranes, which are known to contain such GTP-binding proteins, were also clearly labelled in this region (lane 2). The specificity of such [α-^{32}P]GTP binding was established by labelling *A. viteae* and rat brain membranes in the presence of deoxyribonucleotides as non-oxidizable competitors [28]. Thus labelling of GTP-binding proteins was strongly inhibited by 100 μM dGTP or dGDP (rat brain, lanes 3 and 4; *A. viteae*, lanes 9 and 10), but not by 100 μM dATP (lanes 5 and 11) or dCTP (lanes 6 and 12). Moreover, labelling in the absence of $NaIO_4$ cross-linking completely abrogated [α-^{32}P]GTP binding to rat brain (lane 1) and *A. viteae* (lane 7) membranes. Putative α-subunits were also detected in *A. viteae* cytosolic fractions, although detection was very faint (results not shown). This finding is consistent with heterotrimeric G-protein subunits generally being membrane-associated [1–4,19,35]. It is also likely that *A. viteae* expresses ras-like G-proteins, as a major band at 25 kDa was labelled; such a molecular mass corresponds to the expected size range of monomeric GTPases. A band of similar molecular mass was also observed in cytosolic extracts of *A. viteae* (results not shown), suggesting that low-molecular-mass GTP-binding proteins are found both free in the cytosol and associated with membranes in *A. viteae*. Finally, this technique also identified a putative GTP-binding protein of approx. 71 kDa, which could be similar to the novel high-molecular-mass GTP-binding proteins found to be associated with α_1-adrenergic receptors in rat liver membranes (74 kDa) [36] and expressed in the parasitic trematode *Schistosoma mansoni* (66 kDa) [37].

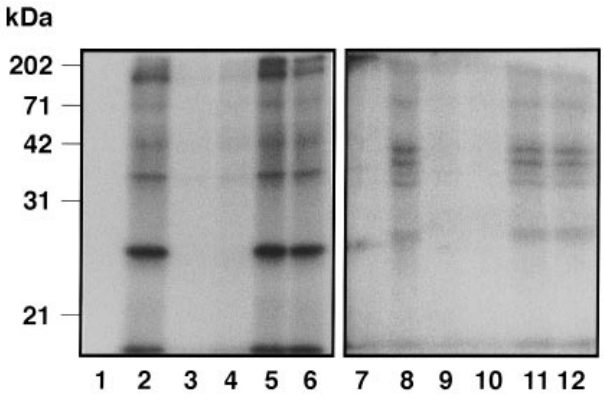

Figure 1 Affinity labelling of G-proteins with [α-^{32}P]GTP in membrane extracts of *A. viteae*

Membrane extracts of adult *A. viteae* and rat brain were prepared and incubated with [α-^{32}P]GTP followed by $NaIO_4$ cross-linking, resolved by SDS/PAGE and subjected to autoradiography as described in the Experimental section. Lanes 1–6, rat brain membranes; lanes 7–12, membrane extract of *A. viteae*. Lanes 1 and 7, [α-^{32}P]GTP binding in the absence of NaIO4 cross-linking; lanes 2 and 8, [α-^{32}P]GTP binding; lanes 3 and 9, [α-^{32}P]GTP binding in the presence of dGTP (100 μM); lanes 4 and 10, [α-^{32}P]GTP binding in the presence of dGDP (100 μM); lanes 5 and 11, [α-^{32}P]GTP binding in the presence of dATP (100 μM); lanes 6 and 12, [α-^{32}P]GTP binding in the presence of dCTP (100 μM).

ADP-ribosylation by cholera toxin and pertussis toxin

The affinity labelling studies outlined above suggested that at least three putative Gα-subunits were expressed in *A. viteae* membranes. In order to investigate whether any of these represented G_s- or G_i-like α-subunits, *A. viteae* membranes were treated with either cholera toxin or pertussis toxin in the presence of [^{32}P]NAD^+ to specifically target such members of the Gα

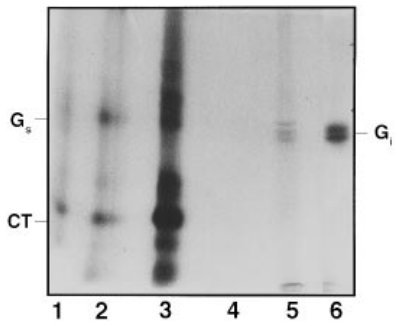

Figure 2 ADP-ribosylation of *A. viteae* membrane extracts by cholera toxin and pertussis toxin

Cytosolic and membrane extracts of *A. viteae* and rat brain membrane extracts were treated with cholera toxin (CT; lanes 1–3) or pertussis toxin (lanes 4–6) in the presence of [^{32}P]NAD$^+$, as described in the Experimental section. Lanes 1 and 4, *A. viteae* cytosolic extracts; lanes 2 and 5, *A. viteae* membrane extracts; lanes 3 and 6, rat brain membrane extracts.

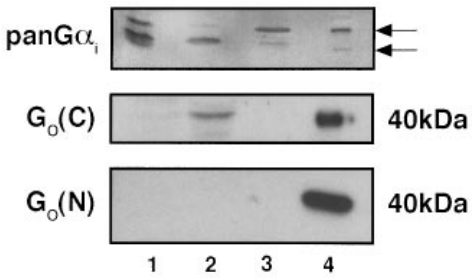

Figure 3 Expression of G_i-like α-subunits in *A. viteae* membrane fractions

Western blots are shown of *A. viteae* extracts treated with NEI-800, an antibody which predominantly recognizes proteins of the G_i subfamily (top); OC1, an antibody against the G_o C-terminal decapeptide (middle); and ON1, an anti-G_o (N-terminus) antibody (bottom). Lane 1, *A. viteae* PBS-insoluble extract; lane 2, membrane extract; lane 3, cytosolic extract; lane 4, rat brain membrane extract.

families by ADP-ribosylation. Cholera toxin specifically ADP-ribosylates members of the G_s subfamily [1–4,19,35]; in the presence of cholera toxin, a band of approx. 44 kDa was labelled in *A. viteae* membrane extracts (Figure 2, lane 2), corresponding to a band of equivalent size labelled in a rat brain membrane extract (Figure 2, lane 3), indicating that a G_s-like α-subunit is indeed expressed in membrane fractions of *A. viteae*. In contrast, no labelling was detected in *A. viteae* cytosolic extracts. The 23 kDa band which is present in all cholera toxin-treated samples corresponds to auto-ADP-ribosylated cholera toxin [38], and the other bands seen in heavily overexposed lanes presumably reflect the fact that, although α_s is the physiological substrate, cholera toxin is a promiscuous enzyme capable of ADP-ribosylating to a low stoichiometry any highly expressed protein possessing a suitably accessible arginine (reviewed in [39,40]).

Pertussis toxin specifically labels members of the G_i subfamily of G-proteins [1–4,19,35]. Three bands of 40–44 kDa were labelled in response to pertussis toxin treatment of *A. viteae* membrane extracts (Figure 2, lane 5). Taken together with the finding that at least two heavily labelled bands of 40–42 kDa were observed in rat brain membranes (Figure 2, lane 6), which are a good source of such G-proteins, these results suggest that *A. viteae* may express three G_i-like α-subunits. Again, no labelling of such G-proteins was detected in cytosolic extracts of *A. viteae* (Figure 2, lane 4).

Table 1 G-protein antibodies used, with corresponding peptide sequence and isotypes recognized

The pan-G antibody was supplied by DuPont (NEI-800). Peptides were conjugated to keyhole limpet haemocyanin. The production and specificities of the antibodies have been described previously [46,49,54–56].

Antibody	Peptide	Corresponding sequence	Subunit(s) recognized
BN3	MSELDQLRQE	β1-(1–10)	β1, β2
CS	RMHLRQYELL	α_s-(385–394)	α_s
IM1	NLKEDGISAAKDVK	α_o-(22–35)	α_o
ON1	GCTLSAEERAALERSK	α_o-(2–17)	α_o
OC1	ANNLRGCGLY	α_o C-terminal decapeptide	α_o
SG1	KENLKDCGLF	α_i-(341–350)	α_i, α_{i1}, α_{i2}
I1C	LDRIAQPNYI	α_{i1}-(159–168)	α_{i1}
I3C/I3B	KNNLKECGLY	α_{i3}-(345–354)	α_{i3}
IQB	EKVSAFENPYVDAIKS	α_q-(119–134)	α_q
CQ	QLNLKEYNLV	α_o-(351–360) α_{11}-(350–359)	α_q/α_{11}
13CB	HDNLKQLMLQ	α_{13} C-terminal decapeptide	α_{13}
Pan-G		GTP-binding sequences	α_i subunits

Western blotting with anti-(G_i family) antibodies

The pertussis toxin ADP-ribosylation studies suggested the expression of multiple G_i-like G-proteins in *A. viteae*. Western blotting studies, involving an antibody (NEI-800) raised against the GTP-binding site common to all G-protein α-subunits, were then used to provide corroborative evidence for expression of these G_i-like α-subunits, as such 'pan-G-protein' antibodies are known to preferentially recognize the G_i subfamily [41]. While this antibody detected two bands of 38–42 kDa in a rat brain membrane extract (Figure 3, top panel, lane 4, indicated by arrows), it also recognized differential expression of at least two similar bands in *A. viteae* cytosolic (lane 3), membrane (lane 2) and PBS-insoluble (lane 1) extracts, indeed providing further corroborative evidence for the expression of multiple G_i-like α-subunits by *A. viteae*. That this antibody recognized α_i-subunits in the cytosolic and PBS fractions may, at first sight, appear inconsistent with the lack of pertussis toxin substrates in such fractions of *A. viteae* (Figure 2). However, the lack of ADP-ribosylated α-subunits in these soluble fractions presumably simply reflects the fact that pertussis toxin can only modify α-subunits in $\alpha\beta\gamma$ complexes; thus, as cytosolic α-subunits cannot associate with $\beta\gamma$-subunits, which are restricted to membrane fractions, these cytosolic α-subunits are not substrates for ADP-ribosylation.

In order to investigate further the $G_i\alpha$ profile of *A. viteae*, a panel of antibodies raised against synthetic peptides specific to individual mammalian G-protein α-subunits (Table 1) was tested. Antibody OC1, raised against the C-terminal decapeptide of G_o (Figure 3, middle panel), recognized, as expected, a 40 kDa protein in the rat brain membrane extract (Figure 3, middle panel, lane 4). No proteins of a similar molecular mass were detected in the cytosolic or PBS-insoluble extracts of *A. viteae* (lanes 3 and 1 respectively). However, a protein of approx. 40 kDa was strongly detected in membrane extracts of *A. viteae*, suggesting that a G_o-like α-subunit may be expressed in this

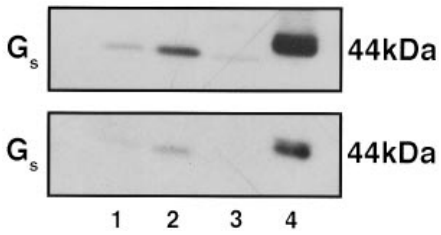

Figure 4 Expression of G_s-like α-subunits in *A. viteae* membrane fractions

Western blots were developed with the anti-G_s antiserum CS1 (upper panel) and following preincubation of CS1 with 1 mg/ml of the peptide to which the antibody was raised (lower panel). Lane 1, PBS-insoluble extract of *A. viteae*; lane 2, membrane extract; lane 3, cytosolic extract; lane 4, rat brain membrane extract.

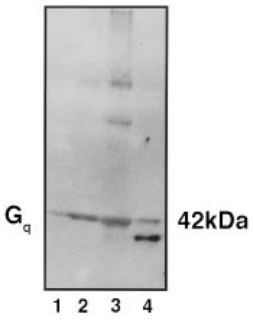

Figure 5 Expression of G_q-like α-subunits in *A. viteae* membrane fractions

The Western blot was probed with anti-G_q antiserum IQB. Lane 1, PBS-insoluble extract of *A. viteae*; lane 2, cytosolic extract: lane 3, membrane extract; lane 4, rat brain membrane extract.

parasite. In contrast, antibodies raised against the N-terminal region of G_o (ON1, IM1) did not detect such Gα expression in any of the *A. viteae* fractions (Figure 3, bottom panel, lanes 1–3), despite strongly recognizing a 40 kDa protein in the rat brain membrane extract (lane 4, and results not shown). This apparent discrepancy may simply suggest that G_o-like subunits in *A. viteae* may be more evolutionarily related to their mammalian homologues at their C-terminal rather than their N-terminal regions, a proposal consistent with the fact that the C-terminal decapeptide contains the pertussis toxin ADP-ribosylation site. *A. viteae* extracts were also probed with antibodies specific for G_{i1} (I1C), $G_{i1/i2}$ (SG1), G_{i3} (I3C) and G_z; no parasite proteins were recognized by these antibodies (results not shown).

Western blotting using an antibody against G_s

The cholera toxin ADP-ribosylation studies suggested the expression of a G_s-like G-protein in *A. viteae*. An antiserum which recognizes G_s (CS1) was used to confirm expression of such an α-subunit in *A. viteae*. This antibody not only recognized a 44 kDa protein in the rat brain membrane extract (Figure 4, upper panel, lane 4), but also recognized a protein of slightly decreased molecular mass in all of the *A. viteae* fractions, and most strongly in the membrane extract (lanes 1–3). The observed different molecular masses of the rat brain G_s and the putative parasite homologue may raise a note of caution with respect to the parasite protein truly being a G_s-like α-subunit. However, in parallel experiments, preincubation of the antibody with the peptide to which it was raised completely blocked (Figure 4, lower panel, lanes 1 and 3) or greatly abrogated (lanes 2 and 4)

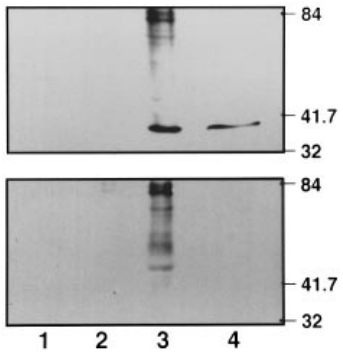

Figure 6 Expression of G-protein β-subunits in *A. viteae* fractions

Western blots were treated with the anti-β-subunit antiserum BN3 (upper panel) and with BN3 that had been preincubated with BN3 peptide (1 mg/ml) (lower panel). Lane 1, PBS-insoluble extract of *A. viteae*; lane 2, cytosolic extract; lane 3, membrane extract; lane 4, rat brain membrane extract. Numbers on the right indicate molecular mass in kDa.

antibody recognition of p44 in both *A. viteae* and rat brain extracts, providing strong evidence that the p44 recognized by anti-G_s in *A. viteae* is indeed a G_s-like α-subunit.

Western blotting using anti-G_q antibodies

The ADP-ribosylation studies only target G_s- or G_i-like subunits; since the [α-^{32}P]GTP binding studies suggested the existence of at least three putative Gα-subunits, it was decided to investigate whether *A. viteae* expresses any G_q or G_{12} family members by further Western blotting studies using antisera specific for such α-subunits (Table 1). An antibody specific for an internal sequence of G_q (IQB) recognized a band of 42 kDa not only in the rat brain membrane extract but also in all samples of *A. viteae*, with the membrane extract exhibiting the strongest signal of the parasite fractions (Figure 5). However, *A. viteae* extracts were not recognized by antiserum CQ, which was raised against a synthetic decapeptide corresponding to the C-terminal region common to both G_q and G_{11} (results not shown), again suggesting that particular regions of parasite G-proteins may be more evolutionarily related to their mammalian homologues than others. Finally, *A. viteae* extracts were not recognized by an antibody (13CB) which recognizes G_{13} (results not shown).

Western blotting using an anti-Gβ antibody

The combination of affinity labelling, ADP-ribosylation and Western blotting studies suggested that *A. viteae* expresses at least three α-subunits of the G_i, G_s and G_q families. If this is the case, then it was also to be expected that *A. viteae* would express one or more β-subunits. This was confirmed by experiments using an antibody (BN3) that recognizes β1 and β2 subunits (Figure 6). In these experiments, rat brain membrane extracts showed a single band of 37 kDa (Figure 6, upper panel, lane 4) and, while the *A. viteae* membrane extract also showed a major band at 37 kDa, a number of high-molecular-mass bands were also detected (lane 3). Parallel experiments, in which the blot was probed with antibody preincubated with the β-subunit peptide to which it had been raised (Figure 6, lower panel), confirmed that the p37 band detected in *A. viteae* membrane extracts did indeed represent a G-protein β-subunit; the bands of 37 kDa in both the rat brain and *A. viteae* membrane extracts were completely abrogated by this treatment, whereas the higher-molecular-mass bands in the *A. viteae* membrane extract remained detectable,

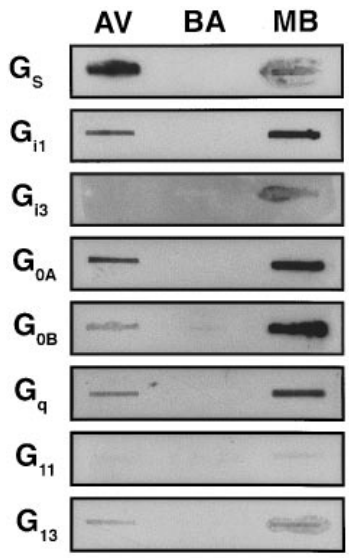

Figure 7 Investigation of Gα-subunit expression in *A. viteae* by RT-PCR and oligonucleotide hybridization

RT-PCR products amplified for G-protein α-subunit expression were slot-blotted and probed with oligonucleotide probes specific for individual α-subunits, as described in the Experimental section. AV, *A. viteae* RNA amplified using G-protein-specific primers; BA, mouse brain RNA amplified using β-actin-specific primers; MB, mouse brain RNA amplified using G-protein-specific primers. RT-PCR products were probed with the indicated oligonucleotides specific for individual G-proteins.

presumably due to non-specific binding of the primary or secondary antibodies.

RT-PCR and oligonucleotide hybridization

The above studies suggested that *A. viteae* expresses $G\alpha_o$, $G\alpha_s$, $G\alpha_q$ and β1 and/or β2 G-protein subunits. To further confirm these results and to investigate whether other subunits not detected by the anti-peptide antibodies are expressed in *A. viteae*, a different approach was adopted. RT-PCR using oligonucleotide primers of sequences common to all G-proteins was performed on parasite RNA. Aliquots of the PCR products were then resolved by agarose gel electrophoresis, and bands corresponding to the expected size of α-subunits (203 bp) were observed. A band of the correct size was also observed when PCR was undertaken with mouse brain RNA and β-actin primers. No PCR products were observed in the absence of reverse transcriptase (results not shown). The remainder of the PCR products were slot-blotted on to nylon filters and probed for α-subunit expression by hybridization with oligonucleotides specific for individual G-protein α-subunits. While all of the individual G-protein probes tested hybridized (G_{11} weakly), as expected, to mouse brain PCR products amplified with degenerate G-protein primers, none of them hybridized to the product amplified using β-actin primers (Figure 7). Hybridization to the *A. viteae* PCR products was also seen with the probes specific for G_q, G_s, G_{i1}, G_{13}, G_{oA} and G_{oB}. In contrast, the G_{i3} probe did not hybridize with the *A. viteae* PCR products. Hybridization was also seen with the G_{11} probe, but this (as with the mouse brain PCR products) was rather weak/non-reproducible (Figure 7).

DISCUSSION

Heterotrimeric G-proteins are highly conserved throughout evolution, and have been identified in organisms as diverse as mammals, birds, amphibia, invertebrates, yeast, slime moulds and green plants (reviewed in [1–4,19]). Such G-proteins have also been found to be expressed in the free-living nematode *Candida elegans* [42,43] and in the trematode parasite *S. mansoni* [37,44]. We now report for the first time, after analysis by four independent techniques, that a filarial nematode, *A. viteae*, expresses a number of heterotrimeric G-proteins; indeed, RT-PCR analysis indicates that α-subunits of each major class of heterotrimeric G-proteins are expressed by this parasite. Moreover, Western blotting indicates that β1 and/or β2 subunits are also expressed.

Affinity labelling studies using [α-^{32}P]GTP indicated the presence of several putative G-proteins in *A. viteae* membrane fractions. While at least three of these were resolved in the molecular mass range (38–44 kDa) consistent with heterotrimeric G-protein α-subunits (Figure 1), there was also evidence for the expression of low-molecular-mass ras-like GTPases and a novel 71 kDa GTP-binding protein. Studies using cholera toxin and pertussis toxin, to target G_s and G_i α-subunits respectively by ADP-ribosylation, indicated the existence of a G_s- and three G_i-like G-proteins in *A. viteae* (Figure 2). In addition, the use of antiserum BN3, which is specific for the β1- and β2-subunits of G-proteins (Figure 6), suggests that *A. viteae* membrane extracts contain homologues of the mammalian G-protein β-subunits. Indirect evidence to support this finding also comes from studies investigating pertussis toxin-mediated ADP-ribosylation of *A. viteae* membrane fractions, as the G_i-like substrates of this type of covalent modification are required to be in their intact αβγ form for the reaction to occur [29]. Moreover, it is perhaps not surprising that β-like subunits are expressed by *A. viteae*, as these subunits are the most highly conserved components of heterotrimeric G-proteins throughout evolution [1–4,19,35], and have already been identified in the parasitic trematode *S. mansoni* [37] and in the free-living nematode *C. elegans* [45].

The possibility that *A. viteae* expresses a G_s-like G-protein was confirmed by (i) specific detection of a 44 kDa parasite protein by an antiserum, CS1 (Figure 4), raised against C-terminal peptides of mammalian G_s [46], and (ii) hybridization of a G_s-specific oligonucleotide probe to *A. viteae* PCR products amplified by degenerate primers common to all G-proteins (Figure 7). Similarly, Western blotting studies with a 'pan-G-protein' antibody, which predominantly recognizes α-subunits of the G_i subfamily (Figure 3), identified G_i-like parasite proteins of similar molecular masses to those identified by pertussis toxin targeting (Figure 2). However, antibodies specific for the different isotypes of mammalian G_i did not recognize *A. viteae* proteins (results not shown). Moreover, while an antibody to the C-terminal region of G_o (OC1) recognized a parasite homologue of approx. 40 kDa, this protein was not recognized by antibodies (ON1, IM1) to the N-terminal domain of mammalian G_o (Figure 3). This apparent discrepancy may simply reflect the fact that G_o-like subunits in *A. viteae* may be more evolutionarily related to their mammalian homologues at their C-terminal rather than their N-terminal regions, a proposal consistent with the fact that the C-terminal decapeptide contains the pertussis toxin ADP-ribosylation site. Further support for this proposal is provided by the finding that, while the sequence of the G_o α-subunit of *C. elegans* shows no amino acid substitutions in the region corresponding to the OC1 decapeptide, there are three amino acid changes in the sequence eqivalent to the ON1 decapeptide [42]. Taken together, these results may suggest that the pertussis

toxin-susceptible parasite proteins are not very similar to the mammalian G_i or G_o proteins except in the regions of the pertussis toxin ADP-ribosylation site and the highly conserved GTP-binding site (as shown by the recognition of at least two parasite proteins of 38–42 kDa by the antibody NEI-800).

Although most of the anti-G_i antibodies tested were raised against peptides that contain the C-terminal cysteine which is ADP-ribosylated by pertussis toxin, these antibodies are G_i-specific and hence their specificity is likely be directed to other isotype-specific amino acids in this region which may have been subject to mutation during evolution. This proposal that parasite G_i-like α-subunits differ from mammalian homologues in their C-terminal region was supported by analysis of *A. viteae* PCR products by oligonucleotide probes specific for internal sequences of mammalian G_i-like α-subunits. These results showed that, while the probe for G_{i3} did not hybridize to the parasite PCR products, hybridization with probes specific for G_{i1}, G_{oA} and G_{oB} indicated that the pertussis toxin-sensitive G-protein homologues found in *A. viteae* probably comprise both G_o- and G_i-like G-proteins. Interestingly, while G_{i3} is considered to be ubiquitously expressed in mammalian tissues, G_{i1} and G_o expression appears to be restricted to the brain, neural and endocrine tissue, perhaps suggesting that parasite homologues expressed in *A. viteae* may be involved in the regulation of cellular responses in primitive specialized tissues. The somewhat conflicting results obtained with the isotype-specific antibodies and oligonucleotide probes may simply reflect (i) that the parasite homologues are more similar to mammalian G-proteins in the region where the oligonucleotide probes bind than in the areas of antibody recognition; (ii) that the oligonucleotide probes recognize regions equivalent to a shorter amino acid sequence than that recognized by the antibodies, or (iii) the relative sensitivities of RT-PCR and Western blotting assays.

The G_q and G_{12} subfamilies of α-subunits are insensitive to both pertussis and cholera toxins, and so the expression of putative homologues in *A. viteae* was investigated by Western blotting (Figure 5) and RT-PCR (Figure 7) analysis. Blotting with an antiserum (IQB) specific for G_q suggested that *A. viteae* expresses a G_q-like protein (Figure 5). However, this G_q-like protein is not identical to mammalian G_q in the C-terminal region which is common to G_q and G_{11}, as an antibody (CQ) specific for this sequence did not recognize any parasite proteins (results not shown). Furthermore, this result also suggests that *A. viteae* is perhaps unlikely to express a G_{11}-like protein. These findings were corroborated and extended by RT-PCR (Figure 7), which confirmed that, while *A. viteae* expresses a G_q-like homologue, the probe for G_{11} did not reproducibly hybridize to *A. viteae* PCR products, indicating that it is, indeed, unlikely that this worm expresses G_{11} (Figure 7). Interestingly, a recent study by Knol and colleagues [47] describes the cloning of a G_q-like α-subunit from the pond snail *Lymnaea stagnalis* which cannot be definitively identified as either a $G\alpha_q$- or $G\alpha_{11}$-like protein on the basis of amino acid sequence comparison. Indeed, as the *Lymnaea* $G\alpha_q$ shares 80–82% overall amino acid sequence identity with vertebrate $G\alpha_q$ and $G\alpha_{11}$ proteins, it was suggested that the cloned molecule may represent a molluscan homologue of a common ancestor of these two mammalian G-proteins. The *Lymnaea* $G\alpha_q$ protein was found to be expressed in the central nervous system of the mollusc and, since members of the $G\alpha_q$ subclass of G-proteins have also been shown to be expressed in neuronal and chemosensory cells of other invertebrates [47,48], a similar location seems warranted for the G_q homologue of *A. viteae*.

An antibody raised against the C-terminal decapeptide of G_{13} failed to recognize any parasite proteins (results not shown); however, an oligonucleotide probe specific for G_{13} hybridized to *A. viteae* PCR products amplified for G-protein α-subunit expression (Figure 7). Interestingly, and perhaps reflecting the apparently conflicting results obtained by Western blotting and RT-PCR studies in the nematode system, $G_{13\alpha}$ can only be detected in mammalian brain tissues at the mRNA level, as the protein is not expressed in sufficient quantities to be detected by Western blotting [49]. Alternatively, it is possible that our results may be due to the G_{13} homologue found in *A. viteae* having diverged considerably at its C-terminal region. Certainly there is a precedent for homologues of this class of G-protein being expressed in invertebrates, as the *Drosophila* concertina gene has been shown to encode a Gα-like protein with identity greatest to the $G\alpha_{12/13}$ subclass of G-proteins [50].

In summary, we have demonstrated that a filarial nematode, *A. viteae*, expresses homologues of several heterotrimeric G-protein subunits, including the α-subunits of G_s, G_{i1}, G_{oA}, G_{oB}, G_q and G_{13}, and β1 and/or β2. In addition, there is also evidence for the expression of at least two low-molecular-mass ras-like G-proteins and a novel, as yet undefined, 71 kDa GTP-binding protein. The biological processes that these proteins regulate remain to be defined, but it is likely that at least some of them transduce signals derived from the parasitized host, as host–parasite interactions at the worm surface are considered to play a role in parasite development, growth and maintenance. Certainly this has been observed in *S. mansoni*, where 5-hydroxytryptamine receptors are coupled to adenylate cyclase activity via G_s [51] and are involved in the regulation of glycolysis in this parasite [52]. Our finding that G_s and G_{i1} are expressed in *A. viteae* makes it seem likely that the filarial nematode, like *S. mansoni*, expresses a homologue of the mammalian adenylate cyclase system. The receptor(s) to which this is linked, however, remain to be established. The free-living nematode *C. elegans* has also been shown to express a G-protein-coupled adenylate cyclase activity [43] as well as other homologues of mammalian signalling molecules, such as a receptor tyrosine kinase, let-23, and a ras-like protein, let-60. This suggests that, in general, signalling pathways appear to be well conserved throughout multicellular organisms [53] and that the G-protein subunits identified in *A. viteae* may be coupled to homologues not only of adenylate cyclase (G_s/G_i) but perhaps also of phospholipase C (G_i/G_q) and ion channels/transporters ($G_{13}/G_o/G_i$). Differences in sequence/structure between G-proteins of parasitic worms and humans ([37,42,51]; the present study), however, may ultimately identify these regulatory molecules as novel targets for chemotherapeutic intervention.

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