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Probable occurrence of toxin-susceptible G proteins in the nematode *Caenorhabditis elegans*

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Pertussis toxin, islet-activating protein (IAP), and cholera toxin ADP-ribosylated 40 kDa and 45 kDa proteins in membrane preparations from *Caenorhabditis elegans*. Proteins with the same molecular weights were recognized in the same membranes by an antibody that had been raised against a peptide common to α-subunits of mammalian $\alpha\beta\gamma$-heterotrimeric G proteins. The antibody produced immunoprecipitation with the 40 kDa protein ^{32}P-labeled by IAP. A 35 kDa protein immunochemically indistinguishable from the β-component of mammalian G proteins was also found in *C. elegans* membranes. The membranes displayed adenylate cyclase activity which was highly sensitive to forskolin and GTP analogues, whose action was antagonized by GDPβS. Receptor-coupled regulation of adenylate cyclase thus appears to be mediated by mammalian-type G proteins in *C. elegans* as well.

GTP-binding protein; Pertussis toxin; Islet-activating protein; Cholera toxin; Adenylate cyclase; *Caenorhabditis elegans*

1. INTRODUCTION

GTP-binding proteins (G proteins) form a family of proteins that serve as mediators of hormonal and sensory transduction processes in eucaryotes [1]. G proteins acting as membrane signal transducers consist of a guanine nucleotide-binding α subunit, a β subunit, and a γ subunit. These G proteins can be ADP-ribosylated by bacterial toxins such as pertussis toxin (islet-activating protein; IAP) [2] and/or cholera toxin (CTX) [3]. Recently, G proteins in *Drosophila melanogaster* [4–9], *Dictyostelium discoideum* [10,11], and yeast [12,13] have been reported.

The nematode *Caenorhabditis elegans* (*C. elegans*) could be a very useful organism for the study of the molecular events underlying differentiation and development. Genetic analysis of *C. elegans* is well developed and a variety of mutants have been obtained [14,15]. Many genes encoding proteins that could play roles in signal transduction pathways, such as protein kinase A [16] or C [17] homologues, *let*-23 [18], *lin*-12 [19], and *let*-60 [20], have been cloned.

Genes encoding the G protein α [21] or β [22] subunit homologue of *C. elegans* have also been cloned. However, the α subunit homologue gene thus cloned does not code for any cysteine residue that should be a candidate for an amino acid ADP-ribosylated by IAP. In this report, we will describe the occurrence of a 40 kDa G protein and a 45 kDa G protein that serve as the substrate for ADP-ribosylation catalyzed by IAP and CTX, respectively. These G proteins could be coupled to membrane adenylate cyclase in *C. elegans* membranes.

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2. MATERIALS AND METHODS

2.1. *Preparation of the crude membrane fraction from* C. elegans

Wild-type *C. elegans* as cultured and harvested as described in [15]. Worms were suspended in TEDANa0 (20 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 50 kIU/ml of aprotinin, 20 mM NaCl), crushed in liquid nitrogen and homogenized using a Polytron homogenizer (max power, 10 min × 2). The lysate was centrifuged at 500 × g for 5 min and the supernatant was further centrifuged at 10,000 × g for 30 min, and the final pellet was stocked in liquid nitrogen (the *C. elegans* crude membrane fraction).

2.2. *1% Lubrol-PX extract of the* C. elegans *crude membrane fraction*

An equal volume of TEDANa containing 2% Lubrol-PX was added to the crude membrane fraction. The mixture was shaken vigorously for 60 min on ice and was centrifuged at 100,000 × g for 60 min. The supernatant was stored at −80°C.

2.3. *IAP- or CTX-catalyzed ADP-ribosylation*

IAP- [23] or CTX- [24] catalyzed ADP-ribosylation was conducted as described before. Briefly, samples were incubated for 60 min at 30°C with preactivated IAP (10 mg/ml) or CTX (50 mg/ml) in the presence of [α-^{32}P]NAD. The reaction was stopped by adding Laemmli's sample buffer [25]. The mixture was applied to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, stained with Coomassie brilliant blue, and subjected to autoradiography.

2.4. *Measurement of adenylate cyclase activity*

The reaction mixture for the adenylate cyclase assay contained 10 mM HEPES-NaOH (pH 7.5), 0.5 mM EGTA, 0.1% BSA, 2.5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxantine (IBMX), 0.5 mM ATP, 5 mM phosphocreatine, and 50 U/ml of creatine kinase. A crude membrane preparation (25 μg protein) was incubated in the reaction

mixture further supplemented with stimulants for 10 min at 22°C. The reaction was stopped by adding 1 N HCl to make a final concentration of 0.1 N and boiling for 1 min. cAMP was measured by the use of radioimmunoassay as described before [26].

2.5. *UI-1 antibody*

Affinity-purified rabbit antibody UI-1 directed against the common region of the α subunits of G proteins was obtained using a synthetic peptide (CGAGESGKTIVKQMK)-KLH conjugate as an antigen according to Goldsmith et al. [27]. The peptide was synthesized by the Pharmaceutical Development Laboratory, KIRIN Brewery Co., Ltd. (Gunma, Japan).

2.6. *Immunoblotting*

Samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to a nitrocellulose filter. Using UI-1, anti-rabbit IgG, rabbit peroxidase-antiperoxidase complex as 1st, 2nd, and 3rd antibody, respectively, positive bands were detected by diaminobenzidine/H_2O_2.

2.7. *Immunoprecipitation*

IAP-catalyzed by ADP-ribosylation was stopped by diluting on ice with concentrated stocks of 'RIPA' buffer to make a final concentration of 50 mM Tris-HCl (pH 7.4), 75 mM NaCl, 1% deoxycholate, 1% Nonidet P-40, 50 kIU/ml of aprotinin, 1 mM DTT, and 0.5% SDS. Antibody UI-1, normal rabbit serum, or antibody UI-1 preincubated with the antigen (the UI-1 peptide) was then added to the mixture, which was kept at 4°C overnight before a 2-h incubation on ice with the further addition with protein A-Sepharose CL-4B. The precipitates were boiled in Laemmli's sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

3. RESULTS AND DISCUSSION

Two proteins with molecular weights of 40 kDa and 45 kDa were ADP-ribosylated by IAP and CTX, respectively, when the crude membrane fraction from *C. elegans* was incubated with these toxins in the presence of [α-^{32}P]NAD (lane 3 in Fig. 1A and B). No radioactivity was incorporated into the protein bands unless the incubation medium was supplemented with toxins (lane 4). Proteins with the same molecular weights occurring in the cholate extract of bovine brain membranes were also ADP-ribosylated by these toxins under the same conditions (lane 1 in Fig. 1A and B), suggesting that the nematode 40 kDa and 45 kDa proteins are $G_i\alpha$- (or $G_o\alpha$-) and $G_s\alpha$-like proteins, respectively. An additional CTX- substrate protein (52 kDa) in brain membranes seems to be another α-subunit of G_s, a product of alternative splicing of $G_s\alpha$ gene exons.

An antibody, termed antibody UI-1, was raised against a highly conservative amino acid sequence that forms a region responsible for GTPase activity and is common to mammalian G protein α-subunits [28]. The UI-1 antibody, which is capable of interacting with $G_s\alpha$'s (45 kDa and 52 kDa), $G_i\alpha$'s (39–41 kDa) in the bovine brain membrane preparation (lane 1 in Fig. 2A), recognized 40 kDa and 45 kDa proteins when an immunoblot analysis was applied to the crude membrane fraction from *C. elegans* (lane 2). Neither protein was detected in the soluble fraction of the nematode (lane 3). The 35 kDa protein was recognized by an antibody for

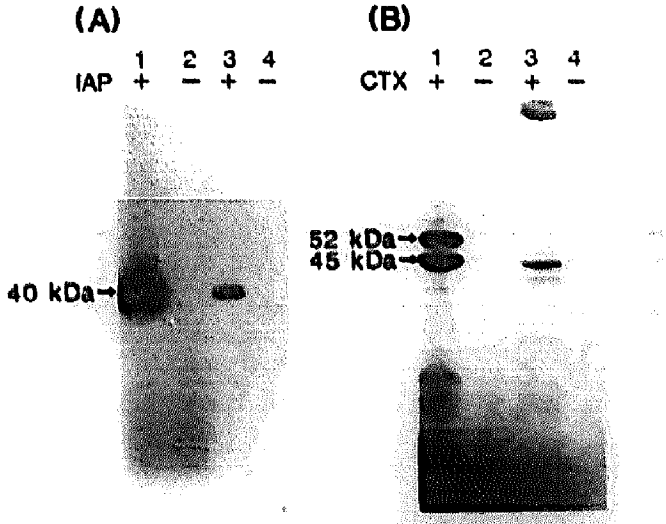

Fig. 1. Electrophoretogram of membrane proteins ADP-ribosylated by IAP or CTX. Membrane preparations were incubated in the presence of [α-^{32}P]NAD with (lanes 1 and 3) or without (lanes 2 and 4) IAP (Panel A) or CTX (Panel B) at 30°C for 60 min before SDS-polyacrylamide gel electrophoresis and autoradiography. Lanes 1 and 2, 1% cholate extract of bovine brain membrane fraction; lanes 3 and 4, *C. elegans* crude membrane fraction, in each panel.

the βγ-component of mammalian G proteins in membrane preparations from *C. elegans* as well as from bovine brain (Fig. 2B). Probably, nematode 40 kDa and 45 kDa proteins and 35 kDa protein, like those in brain membranes, are the GTP-hydrolyzing α-subunits and the β-subunit, respectively, of heterotrimeric G proteins. In accordance with this notion, the elution profile for proteins susceptible to IAP from an anion-exchange column or a gel-filtration column was essentially the same as the profile for the fractions exhibiting GTPγS-binding activity (data not shown). The activity peak

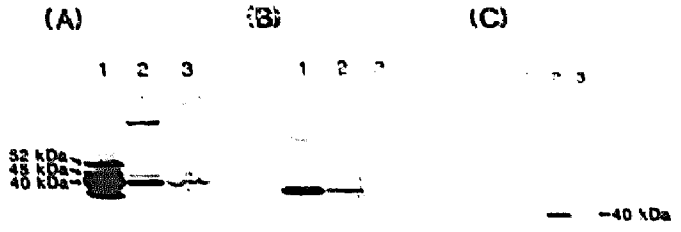

Fig. 2. *C. elegans* proteins recognized by antibodies directed against mammalian G proteins. Panels A and B: immunoblot; bovine brain membrane fraction (lane 1), *C. elegans* crude membrane fraction (lane 2) and *C. elegans* supernatant fraction (lane 3) were subjected to immunoblot analysis using UI-1 antibody (A) or anti-βγ antibody (B). Panel C: Immunoprecipitation of protein ADP-ribosylated by IAP; 1% Lubrol-PX extract of the *C. elegans* crude membrane fraction were incubated with [α-^{32}P]NAD and IAP, and submitted to immunoprecipitation with the use of normal rabbit serum (lane 1), 4 mg/ml UI-1 antibody (lane 2) or UI-1 antibody preincubated with the UI-1 peptide (lane 3).

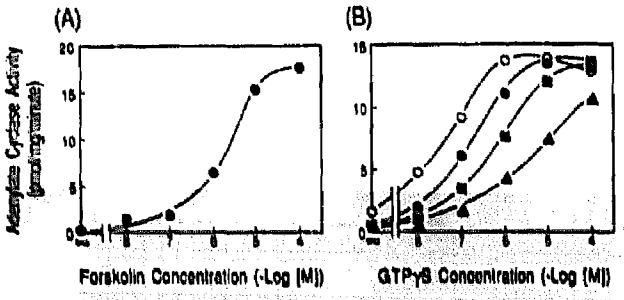

Fig. 3. Adenylate cyclase activity in *C. elegans* crude membrane fraction in the presence of forskolin (Panel A) or GTPγS (Panel B). 100 μM (▲), 10 μM (■), 1 μM (●) or no (○) GDPβS was also included in the assay mixture in Panel B.

contained 40 kDa and 35 kDa proteins immunologically indistinguishable from mammalian Gα and Gβ, respectively (not shown).

In order to ascertain that the IAP-substrate 40 kDa protein is really the α-subunit of G_i- or G_o-like protein, we tried to immunoprecipitate the 40 kDa protein once ADP-ribosylated by the toxin with the use of the UI-1 antibody. The membrane preparation from *C. elegans* was incubated with IAP *plus* [α-^{32}P]NAD before immunoprecipitation. The ^{32}P-labeled 40 kDa protein was precipitated with the UI-1 antiserum (lane 2 in Fig. 2C). No precipitation occurred if the antiserum was replaced by non-immune serum (lane 1) or if the UI-1 antiserum had been deprived of the antibody by prior to treatment with the antigen peptide (lane 3). Thus, the 40 kDa IAP-substrate protein in nematode membranes must be the α-subunit of G_i- or G_o-like protein.

The nematode membrane preparation possessing the 40 and 45 kDa toxin-substrate proteins displayed an adenylate cyclase activity highly sensitive to the concentration-dependent activation by forskolin (Fig. 3A) that is known to be a direct activator of G-protein-coupled membrane cyclase in a variety of mammalian cells [29]. The addition of NaF, together with $AlCl_3$, to membranes also increased the cyclase activity (not shown). The adenylate cyclase activity increased progressively upon the addition of increasing concentrations of GTPγS (Fig. 3B) or GppNHp (not shown) instead of forskolin. The GTPγS-induced stimulation of adenylate cyclase was antagonized by GDPβS in a competitive and concentration-dependent manner (Fig. 3B). Thus, adenylate cyclase must be coupled to G protein in *C. elegans* membranes, although no evidence has been provided so far for an involvement of the CTX-substrate 45 kDa protein in GTP-induced activation of the cyclase activity.

In summary, the membrane fraction, but not the soluble fraction, from *C. elegans* are provided with at least two proteins indistinguishable from mammalian G proteins in their susceptibility to pertussis and cholera toxins as well as in their capability of interaction with the antibody directed against the peptide region responsible for GTPase activity of G proteins. The γ-component of the G proteins is likely to occur in nematode membranes, though no direct evidence is available, because the real substrate of IAP-catalyzed ADP-ribosylation should be G proteins with an αβγ-heterotrimeric structure [23]. Conceivably, the nematode G proteins would act as signal transducers communicating between adenylate cyclase and membrane receptors for neurotransmitters that have proven to be present in the nematode neuronal tissues. We are now investigating the possible modification of the receptor-evoked cellular responses by prior ADP-ribosylation of these proteins by IAP or CTX.

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