Guanine nucleotide-binding protein in sea urchin eggs serving as the specific substrate of islet-activating protein, pertussis toxin

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A GTP-binding protein serving as the specific substrate of islet-activating protein (IAP), pertussis toxin, was partially purified from Lubrol extract of sea urchin egg membranes. The partially purified protein possessed two polypeptides of 39 and 37 kDa; the 39 kDa polypeptide was specifically ADP-ribosylated by IAP and the 37 kDa protein cross-reacted with the antibody prepared against purified $\beta\gamma$-subunits of $\alpha\beta\gamma$-heterotrimeric IAP substrates from rat brain. Incubation of this sea urchin IAP substrate with a non-hydrolyzable GTP analogue resulted in a reduction of the apparent molecular mass on a column of gel filtration as had been the case with purified rat brain IAP substrates, suggesting that the sea urchin IAP substrate was also a heterooligomer dissociable into two polypeptides in the presence of GTP analogues. Thus, the 39 and 37 kDa polypeptides of the sea urchin IAP substrate correspond to the α- and β-subunits, respectively, of mammalian IAP substrates which are involved in the coupling between membrane receptor and effector systems.

Islet-activating protein *GTP-binding protein* *(Sea urchin egg)*

1. INTRODUCTION

It is currently known that a family of structurally and functionally homologous, membrane-associated GTP-binding proteins is present in a variety of vertebrate cells. Identified and characterized members of this family include the stimulatory (G_s) and inhibitory (G_i) GTP-binding proteins of the hormone-sensitive adenylate cyclase system [1], a new GTP-binding protein (G_o) of unknown function discovered in brain tissues [2–4] and the transducin (T) of vertebrate retina that communicates between light activation of rhodopsin and stimulation of cyclic GMP-dependent phosphodiesterase [5]. These proteins have been purified from a variety of vertebrate species as oligomeric proteins with an $\alpha\beta\gamma$-subunit structure. Each of these α-subunits contains a single guanine nucleotide-binding site with high affinity and a site for NAD-dependent ADP-ribosylation catalyzed by cholera toxin or pertussis toxin (islet-activating protein or IAP). Several lines of evidence have been provided [6] for the additional role of some GTP-binding proteins in Ca^{2+}-related signal transduction. In fact, IAP substrate protein(s) including G_i and G_o was found to couple Ca^{2+}-mobilizing receptors to phospholipase C activation in rat mast cells [7], guinea pig neutrophils [8,9] and differentiated human leukemic (HL-60) cells [10], although there are some reports showing that the non-IAP substrate GTP-binding protein(s) is functioning in some of the other cell types [11,12].

An involvement of a GTP-binding protein(s) in the signal transduction system has also been suggested in invertebrates [13,14]. In fertilization of

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sea urchin eggs, it has been reported that there is an increase in polyphosphoinositide breakdown immediately before cortical vesicles of the eggs begin to fuse with the plasma membrane (i.e. exocytosis) [15]. The exocytosis results in the elevation of the fertilization envelope, which prevents the entry of additional sperm. Interestingly, microinjection of a non-hydrolyzable GTP analogue caused exocytosis [16], suggesting that a GTP-binding protein plays a role in the pathway leading to exocytosis in sea urchins. Sea urchins are evolutionally far from mammals. Thus, it is of interest to ascertain whether similar GTP-binding proteins are preserved in these two systems. Here we explored homologies between the invertebrate and vertebrate GTP-binding proteins, both of which serve as the specific substrate of IAP-catalyzed ADP-ribosylation.

2. MATERIALS AND METHODS

2.1. *Materials*

Rat brain IAP substrate proteins (G_i and G_o) were purified as in [4]. Guanosine 5′-*O*-(thio)triphosphate, GTPγS, was purchased from Boehringer Mannheim and purified as in [17]. ^{35}S-labeled anti-rabbit Ig (SJ.434) was obtained from Amersham. The sources of all other materials used are those described in [4,18].

2.2. *Preparation of IAP substrate-rich fractions from sea urchin eggs*

Unfertilized eggs of sea urchin (*Strongylocentrotus intermedius*) were obtained by intracoelomic injection of 0.5 M KCl, washed with seawater and stored at −80°C until use. All subsequent procedures were performed at 0–4°C. Approx. 10 ml of the frozen egg pack were thawed and suspended in 30 ml of 20 mM Tris-HCl (pH 8.0)/1 mM EDTA/1 mM dithiothreitol (TED) containing 50 kallikrein inhibitory units per ml of aprotinin and 100 mM NaCl and homogenized with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 39000 × *g* for 10 min. The membrane pellets were resuspended with 50 ml of TED containing 100 mM NaCl and 1% Lubrol-PX and stirred for 1 h. The clear supernatant (extract) obtained by centrifugation at 100000 × *g* for 90 min was subjected to the purification procedures of the IAP substrate. Details of the procedures will be reported elsewhere. Briefly, the extract was applied to columns of DEAE-Toyopearl 650(S) and hydroxyapatite successively, by a modification of the procedures described in [14]. After the two chromatographies, aliquots of the fraction containing IAP substrate activity were further fractionated on either Mono Q anion exchanger (fig. 1A) or Superose 12 gel filtration (fig.1B) column using a Pharmacia FPLC system, as follows. 2 ml IAP substrate-rich fractions from the hydroxyapatite column were applied to Mono Q HR5/5 which had been equilibrated with TED/150 mM NaCl/0.1% Lubrol and eluted at the flow rate of 0.75 ml/min with the following series of NaCl gradients; 150 mM for 5 min; 150–350 mM over 25 min; 350 mM–1 M over 5 min. The eluate was collected in fractions of 0.75 ml. Other aliquots (1 ml) from hydroxyapatite were concentrated to 0.2 ml by pressure filtration using a Millipore SJGC, and the concentrate was fractionated on Superose 12 HR10/30 in TED/100 mM NaCl/0.1% Lubrol at the flow rate of 0.3 ml/min. The eluate was collected in fractions of 0.45 ml.

2.3. *Assay of activities*

IAP substrate protein was identified by its abilities to be ^{32}P-labeled ADP-ribosylated in the presence of IAP and ^{32}P-labeled NAD and to bind ^{35}S-labeled GTPγS as in [4,18]. Electrophoresis of polypeptides through SDS-polyacrylamide gels and the treatment of the samples with *N*-ethylmaleimide are described in [2,4,18]. The procedures employed to prepare affinity-purified antibodies against the α_{41}-, α_{39}- or $\beta\gamma$-subunits of rat brain IAP substrates will be described elsewhere. Western blots were performed by transferring proteins from a slab gel to a Durapore GVHP sheet (Millipore) by electroelution at 0–4°C with constant voltage (8 V/cm) for 4 h. After transfer, the sheet was incubated with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) for 1 h to block nonspecific protein binding. The sheet was then incubated with PBS/0.1% BSA containing affinity-purified antibodies at the final concentration of 5–10 μg/ml for 2 h. After being washed with ice-cold PBS, the sheet was finally incubated with a second antibody (1 μCi/ml of ^{35}S-labeled anti-rabbit donkey Ig) in PBS/0.1% BSA for 2 h. These incubation procedures of the sheet were carried out at room temperature. The sheet

was washed extensively with ice-cold PBS and dried, and autoradiography was then performed with Kodax X-AR film with an intensifying screen at −80°C.

3. RESULTS AND DISCUSSION

Lubrol extracts of sea urchin egg membranes were prepared as the source of the GTP-binding protein serving as a specific substrate of IAP-catalyzed ADP-ribosylation. The IAP substrate-rich fractions, eluted from DEAE-Toyopearl 650(S) and hydroxyapatite chromatographic columns, were then applied to either the Mono Q anion exchanger or the Superose 12 gel filtration column (fig.1A,B). In either of the two chromatographies, one symmetrical peak of IAP substrate activity was eluted with the same specific molar activity of GTPγS binding. Both peaks were analyzed for their polypeptide compositions by SDS-polyacrylamide gel electrophoresis as shown in fig.1C and D. A 39 kDa polypeptide which was the target of IAP-catalyzed ADP-ribosylation (see fig.2) was readily visualized in the fractions from both columns. An additional protein with a molecular mass of 37 kDa was also observed in the IAP substrate-rich fractions and co-migrated with the 39 kDa polypeptide in a similar intensity.

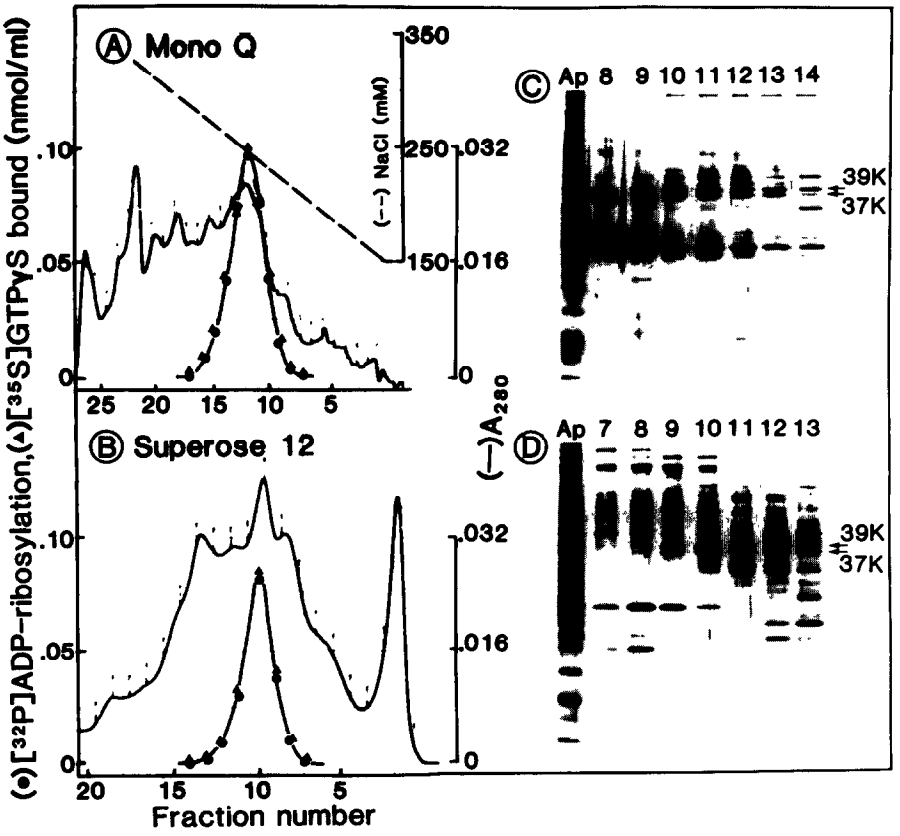

Fig.1. Mono Q and Superose 12 chromatography of IAP substrate proteins from sea urchin eggs. IAP substrate-rich fractions from the hydroxyapatite column were applied to either Mono Q (A) or Superose 12 (B) and eluted as described in section 2. Aliquots (20 μl) of fractions were assayed for IAP substrate (•) and ^{35}S-labeled GTPγS-binding (▲) activities. The absorbance at 280 nm of the eluted protein was also monitored (——). The polypeptide composition of the protein eluted was analyzed by SDS-polyacrylamide gel (12.5%) electrophoresis, and the silver stain of protein on the gel is illustrated in C and D for Mono Q and Superose 12, respectively; Ap shows the original fraction applied, and the number of each lane corresponds to the fraction number.

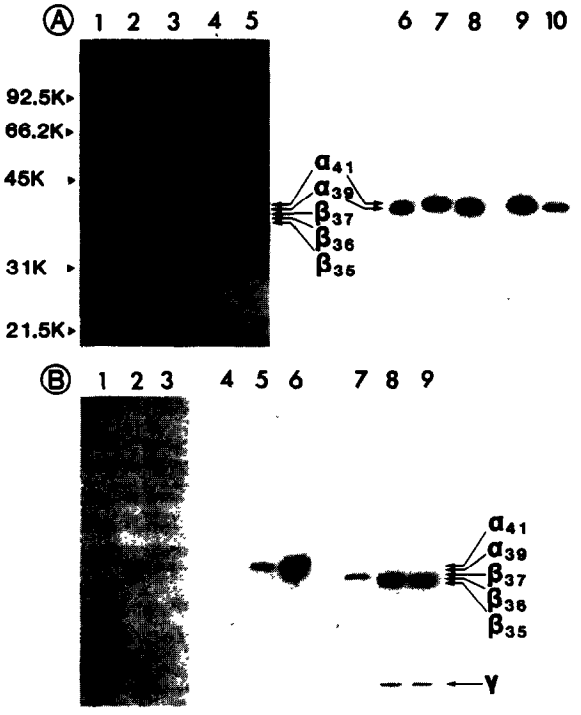

Fig.2. (A) SDS-polyacrylamide gel electrophoresis of IAP substrate proteins from sea urchin eggs and rat brain membranes, and α-subunits of the proteins radiolabeled by IAP and ^{32}P-labeled NAD. Lanes 1–5, Coomassie blue-stained electrophoretic gels (11% polyacrylamide); lanes 6–10, autoradiogram of ^{32}P-labeled ADP-ribosylated α-subunits. 1, Molecular mass standard (Bio-Rad); 2, sample buffer; 3 and 6, sea urchin IAP substrate from Mono Q column; 4 and 7, and 5 and 8, purified rat brain G_i and G_o, respectively; 9 and 10, the proteins contained in the chromatographic peaks in fig.3A and B, respectively. (B) The cross-reaction of the sea urchin IAP substrate protein with affinity-purified antibodies specific for the α- and $\beta\gamma$-subunits of rat brain IAP substrates. Sea urchin IAP substrate-rich fractions from hydroxyapatite (lanes 1, 4 and 7) and purified rat brain G_i (lanes 2, 5 and 8) or G_o (lanes 3, 6 and 9) were subjected to SDS-polyacrylamide gel (11%) electrophoresis and analyzed for immunoblot as described in section 2. Lanes 1–3, anti-α_{41} antibody; lanes 4–6, anti-α_{39} antibody; lanes 7–9, anti-$\beta\gamma$ antibody.

Protein stain of the sea urchin IAP substrate obtained from the Mono Q column and purified rat brain IAP substrates is illustrated in fig.2A, together with the autoradiogram of the same proteins radiolabeled by IAP and ^{32}P-labeled NAD. The proteins from sea urchin eggs predominantly exhibited 39 and 37 kDa bands, although there were still several contaminants. The 39 kDa polypeptide was the α-subunit of the sea urchin IAP substrate on the basis of the finding that the polypeptide contains the site for IAP-catalyzed ADP-ribosylation (lane 6) and probably the binding site for ^{35}S-labeled GTPγS. The molecular mass of the sea urchin α (lanes 3 and 6) was slightly smaller than that of rat brain G_i-α (α_{41}; lanes 4 and 7) and similar to that of rat brain G_o-α (α_{39}; lanes 5 and 8). The 37 kDa protein seemed to be the β-subunit of the sea urchin IAP substrate, since it co-migrated with the sea urchin α (see fig.1). The more clear evidence will be described below (see figs 2B and 3). The β (β_{37}) was apparently larger than the β-subunits of the 36/35 kDa doublet from rat brain GTP-binding protein. The existence of a γ-subunit in the sea urchin IAP substrate is unclear here. The specific activity was about 3 nmol of ^{32}P-labeled ADP-ribosylation or ^{35}S-labeled GTPγS bound per mg for the protein eluted as a peak from Mono Q or Superose 12. The purity of the protein is 20–30%, if an assumption is made that the sea urchin protein having one modification and one binding site per molecule is a heterotrimer ($\alpha\beta\gamma$) or heterodimer ($\alpha\beta$) as are rat brain IAP substrates [4]. The similar purity was suggested by the relative intensities of the α_{39}- and β_{37}-bands in the protein-stained gels. Probably, the assumption is correct; there appears to be one site for ADP-ribosylation and one site for GTP binding per molecule of the sea urchin IAP substrate as observed in mammalian IAP substrates from rat brain [4] and rabbit liver [19].

It has been reported that incubation of rat brain IAP substrates ($\alpha\beta\gamma$) with GTPγS and Mg^{2+} results in its dissociation into GTPγS-bound α and $\beta\gamma$ [4,18]. This property implies that an apparent molecular size of $\alpha\beta\gamma$ heterotrimer is larger than that of GTPγS-bound α. Such was the case with the sea urchin IAP substrate, as shown in fig.3. When IAP substrate-rich fractions from sea urchin eggs were treated with ^{35}S-labeled GTPγS and Mg^{2+} and then applied to Superose 12, the ^{35}S-labeled GTPγS-prebound α-subunit appeared as a smaller molecular species (fig.3B) than did the untreated IAP substrate (fig.3A). The polypeptide compositions of the treated and untreated fractions thus obtained are also illustrated in fig.3D

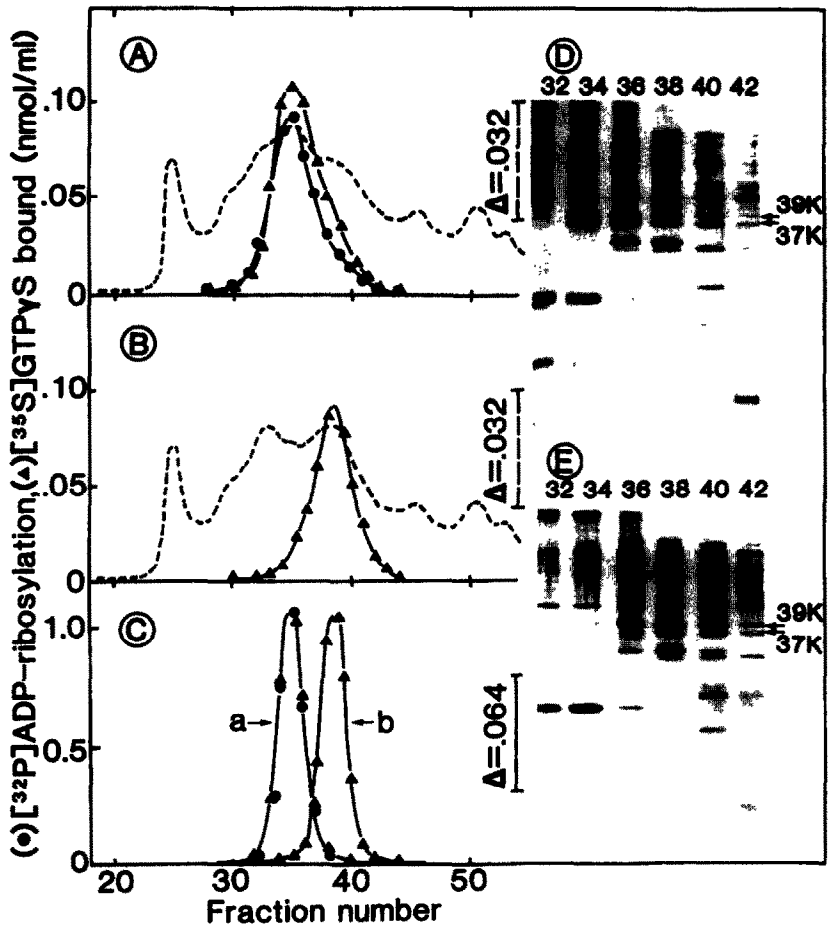

Fig.3. Elution profile of GTPγS-treated IAP substrate proteins from gel exclusion chromatography. (A) Aliquots (500 μl) of IAP substrate-rich fractions from the hydroxyapatite column were fractionated on Superose 12 as in fig.1B and assayed for IAP substrate (●) and ^{35}S-labeled GTPγS-binding (▲) activities. (B) Additional 500 μl of the fractions was incubated with 50 μl of 10 μM ^{35}S-labeled GTPγS (2500 cpm/pmol)/250 mM $MgCl_2$ for 45 min at 30°C and then fractionated on the same column in the presence of 5 mM $MgCl_2$. Aliquots (10 μl) of the eluted fractions were counted for ^{35}S, and nmol of ^{35}S-labeled GTPγS bound/ml is shown (▲). The absorbance at 280 nm of the eluted protein was also monitored (– – –). The polypeptide composition of the protein eluted was analyzed as shown in fig.1 and illustrated in D and E for A and B, respectively; the number of each lane corresponds to the fraction number. (C) a; 200 μl of purified rat brain G_o (200 μg) was fractionated as in A; b, additional 200 μl of the G_o which had been incubated with ^{35}S-labeled GTPγS and Mg^{2+} was fractionated as in B. The absorbance at 280 nm of the eluted G_o was monitored (——).

and E, respectively. Both the α- (39 kDa) and the β- (37 kDa) subunits of the sea urchin IAP substrate were eluted at a slower rate after being treated with GTPγS and Mg^{2+}, without any significant change in the elution profile of other contaminating proteins. As expected, similar retardation of elution was observed when purified rat brain G_o was similarly fortified with GTPγS (fig.3C); the steeper elution profile of the purified G_o was due to a smaller volume applied to the column.

ADP-ribosylation of mammalian IAP substrates has been reported to be feasible only when the α-subunits are associated with the βγ-subunits [4]. This property was also true for the sea urchin IAP substrate; the extent of ADP-ribosylation of the

GTPγS-treated sea urchin protein (fig.2A, lane 10) was much lower than that of the untreated one (lane 9).

Because of these structural similarities between the sea urchin and vertebrate IAP substrates, further studies were carried out for immunological cross-reactivity between the two proteins. Purified α_{41}-, α_{39}- and $\beta\gamma$-subunits of rat brain IAP substrates were used to prepare affinity-purified antibodies for immunoblot analysis. The IAP substrate-rich fraction of sea urchin eggs and purified rat brain G_i and G_o were electrophoresed in SDS-polyacrylamide gels, and then blotted onto a Durapore sheet. Individual lanes were cut out and treated with the antibodies raised against the α_{41}- (Ab-α_{41}), α_{39}- (Ab-α_{39}) or $\beta\gamma$- (Ab-$\beta\gamma$) subunits of rat brain IAP substrates (fig.2B). Ab-α_{41} reacted with the α_{41}, but not with the α_{39} or the $\beta\gamma$ of the rat brain IAP substrates (lanes 2 and 3), whereas Ab-α_{39} strongly reacted with the α_{39}, weakly with the α_{41}, but not with the $\beta\gamma$ (lanes 5 and 6). Ab-$\beta\gamma$ reacted with the β- (36/35 kDa doublet) and the γ- ($\sim$10 kDa) subunits of both G_i and G_o from rat brain, but not with α_{41} or α_{39} of either source (lanes 8 and 9). Ab-α_{41} did not react with any of the polypeptides present in sea urchin preparations (lane 1). In contrast, Ab-α_{39} cross-reacted very weakly with a polypeptide of 39 kDa (lane 4) and Ab-$\beta\gamma$ also cross-reacted weakly with a polypeptide of 37 kDa (lane 7) in the sea urchin fractions. The 39 and the 37 kDa polypeptides detected by the immunoblot analysis showed the same electrophoretic mobility as assumed α- and β-subunits of the sea urchin IAP substrate, respectively. The cross-reactivity observed using Ab-$\beta\gamma$ or α_{39} thus indicated that the α- and β-subunits of the sea urchin IAP substrate share a common antigenic determinant to those from rat brain G_o.

Properties of the partially purified IAP substrate from sea urchin eggs and the immunological cross-reactivities show that invertebrates contain a mammalian protein-like GTP-binding protein serving as the specific substrate of IAP, although there are significant differences in molecular size and immunological reactivities between these proteins. Since it has been reported that octopus photoreceptors also contain a protein which cross-reacts with antibody raised against $\beta\gamma$-subunits of T from bovine retina [13], the β-subunit of GTP-binding protein seems to have a broad distribution in nature and to be highly conserved. In contrast, each α-subunit of the GTP-binding proteins may interact with a specific effector system owing to different active centers [1]. For example, the α-subunit of rat brain G_i (α_{41}) can inhibit the catalytic activity of adenylate cyclase in a manner competitive with the α of G_s, whereas the α of G_o (α_{39}) cannot [18]. The α-subunits of the GTP-binding protein family must be more diverse.

Finally, what is the physiological role of the IAP substrate in sea urchin eggs? Recent work has revealed that a GTP-binding protein is involved in the process between sperm-egg interaction of sea urchin as described in section 1. Thus, it is tempting to speculate that the IAP substrate identified in the present communication is capable of mediating such signal transduction in sea urchin eggs. The validity of this speculation is currently under study in our laboratories.

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