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Involvement of GTP-Binding Proteins in Stage-Specific Receptor-Mediated Endocytosis of Coelomic Fluid Proteins into Oocytes of *Pseudopotamilla occelata*

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Receptor-mediated endocytosis of coelomic fluid proteins (CP), yolk precursor proteins, appears to be regulated by multiple GTP-binding proteins during oogenesis of a polychaete, *Pseudopotamilla occelata*. Transport of ^{125}I-CP into the oocytes of intermediate size class, at which CP is the most actively transported, is enhanced by GTP but inhibited by GTP analogues, either GTPγS or GDPβS. The effects of GTP and GTPγS on the transport were also confirmed by tracing internalization of gold-labeled CP with transmission electron microscope. Internalization of gold-labeled CP into the yolk granules was enhanced by GTP but inhibited by GTPγS.

KEY WORDS: GTP-Binding Protein, Endocytosis, Coelomic Fluid Proteins, Oocyte

Yolk precursor proteins have been known to be transported into oocytes at specific stages by receptor-mediated endocytosis in *Xenopus* and chicken (Stifani *et al.*, 1990). Coelomic fluid proteins, which are components in the coelomic fluid and assumed to be yolk precursor proteins, have been strongly suggested to be transferred into oocytes by receptor-mediated endocytosis in a tubiculous polychaete, *Pseudopotamilla occelata*, since the transport was observed to be specific to the intermediate stage and to the coelomic fluid proteins (Lee and Kim, 1993). Receptor-mediated endocytosis was also supported by the observation of specific binding property of the membrane proteins with coelomic fluid proteins (Nam, 1995).

Involvement of GTP-binding proteins in transferring coelomic fluid proteins into oocytes at specific stage was suggested in the previous reports that receptor-mediated endocytosis is regulated by GTP-binding proteins (Serafini, 1991). The idea was further supported by the facts that small monomeric GTP-binding proteins control vesicular transport (Balch, 1990; Goud and McCaffrey, 1991) and heterotrimeric GTP-binding proteins are also involved in endocytosis as well as in exocytosis (Donaldson *et al.*, 1991; Stow *et al.*, 1991; Barr *et al.*, 1991; Colombo *et al.*, 1992 and 1994). GTP-binding proteins were found in reproductive cells as well as in somatic cells. Heterotrimeric GTP-binding protiens were found in oocytes and spermatids of mouse (Garty *et al.*, 1988; Jones *et al.*, 1989). Allworth *et al.* (1990) also reported that GTP-binding proteins exist in oocytes, fertilized egg and cleaving eggs in stage-specific forms, probably regulating the early embryogenesis. Heterotrimeric GTP-binding proteins are correlated with fertilization in amphibia (Turner *et al.*, 1987; Steinhardt and Alderton, 1988; Kline *et al.*, 1988; Miyazaki, 1988; Kurasawa *et al.*, 1989; Turner and Jaffe, 1989). In sea urchin maturation of oocytes is also known to be induced by heterotrimeric GTP-binding proteins (Chiba *et al.*, 1992; Jaffe *et al.*, 1993). In this paper we tested whether GTP-

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binding proteins are involved in endocytosis of yolk precusor protein into oocytes.

Materials and Methods

Collection and size separation of oocytes

Pseudopotamilla occelata Moore was collected in the east coast of Korea and kept in natural sea water at 10°C in a cold room. Oocytes were collected by dissecting the female worms longitudinally along the lateral side with razor blade. Oocytes of intermediate size class ranging from 80 μm to 140 μm in diameter were separated from the small (30 μm-80 μm diameter) and large size class (140 μm-180 μm diameter) by collecting them on Nitex cloth of 70 μm pore size, while straining the mixture of oocytes ranging from 30um to 180 μm in diameter. While oocytes were sedimenting at 1xg, oocytes smaller than the large size class were transferred into another container and strained through the Nitex cloth.

Iodination of Coelomic fluid proteins

Coelomic fluid was collected by rinsing the dissected area with artificial sea water, centrifuged at 10,000 rpm for 10 minutes at 2°C to remove small oocytes and tissue fragments, and concentrated by placing the fluid-contained dialysis tubing on polyethylene glycol-6000 (PEG-6000).

For iodination coelomic fluid proteins, resuspended in 0.4 M phosphate buffer, was incubated with 1mCi of ^{125}I with chloramine-T for 5 minutes. Iodination was terminated by introducing sodium metasulfate (0.125 g/10 ml of 50mM phosphate buffer, pH 7.4) and 10 % KI solution. ^{125}I-labeled protein was separated from free ^{125}I using Sephadex G-100 column, which had been equilibrated with hydroxymethyl-aminomethane (Tris)-bovine serum albumin buffer (0.1 M Tris-HCl, pH 7.4, 2 mg/ml BSA). The radioactivity of each fraction was determined on a relative basis using a γ-specific monitor. Fractions showing radioactivity were collected in dialysis tubing, concentrated on PEG-6000 and dialyzed against blotting buffer (25mM Tris-HCl, pH 8.0, 50mM NaCl, 2mM $CaCl_2$).

Preparation of colloidal gold solution and tagging to coelomic fluid proteins

Colloidal gold particles of 15nm was prepared by adding 4 ml of 1% sodium citrate to 100 ml of 0.01% chloroauric solution with a rapid agitation when the acid solution was heated to a boiling point for 8 to 10 minutes until clear red color appears, and the solution was rapidly chilled on ice (Slot and Geuze, 1985).

Coelomic fluid proteins were labeled with gold particles. One ml of 15 nm gold solution was mixed with 100 ml of 100 μg/ml coelomic fluid proteins in phosphate saline buffer (0.02M NaH_2PO_4, 0.02M Na_2HPO_4, 0.015M NaCl; PBS). The mixture was centrifuged in a step gradient consisting of 1 ml of each 17.5%, 35% and 70% sucrose solution at 30,300 rpm for 30 minutes at 4°C. After centrifugation approximately 1 ml of centrifuged solution was decanted from the bottom, where the solution shows red color, indicating that the protein is labeled with gold particles. To the solution 1 ml of 2% PEG 20,000 was added and dialyzed against Millipore-filtered sea water (MFSW) at 4°C.

Transmission electron microscopy

Oocytes incubated with gold particles were washed several times with MFSW. The oocytes were prefixed with a mixed solution of 2.5% glutaraldehyde buffered with 0.1M phosphate buffer, pH 7.2 and 2% paraformaldehyde at 4°C for 2 hours and postfixed with 2% osmium tetraoxide in 0.1M phosphate buffer, pH 7.2 at 4°C for one hour. Fixed ooyctes were dehydrated, infiltrated in series with Epon 812 mixtures with acetone at different ratios of 1:2, 1:1 and 2:1, embedded in Epon mixture and polymerized at 60°C for 72 hours. The embedded oocytes were sectioned at 1 μm thickness and stained with 1% toluidine blue. Ultrathin section was prepared from the oocyte showing nucleolus in the section, stained with uranyl acetate and observed with a JEM-1200 EX transmission electron microscope.

Results

The effects of GTP and GTP analogues on the

transport of coelomic fluid proteins into oocytes were examined by biochemical assay and electron microscopy. For the purpose oocytes of intemediate size class, ranging from 80 μm to 140 3 μm in diameter, were employed, since these oocytes showed the highest transport activity of coelomic fluid proteins compared to that of small or large size class (Lee and Kim, 1993).

For the control experiment the oocytes were incubated with ^{125}I-labeled coelomic fluid proteins (^{125}I-CP) with a specific activity of 2.8-2.9 $\times$ 10^3 cpm/μg of the protein and the radioactivity of ^{125}I-CP transported into the oocytes was counted. The experiments was repeated several times (Fig. 1, 2). The transport increases rapidly for the first 5-10 hours and rather slowly for the next 10-15 hours but did not reach a plateau by the end of incubation. The amount of coelomic fluid proteins transported for 20 hours, when calculated on the basis of the specific acitivity, ranges from 121 ng/100 oocytes to 140 ng/100 oocytes, indicating that the assay is reproducible.

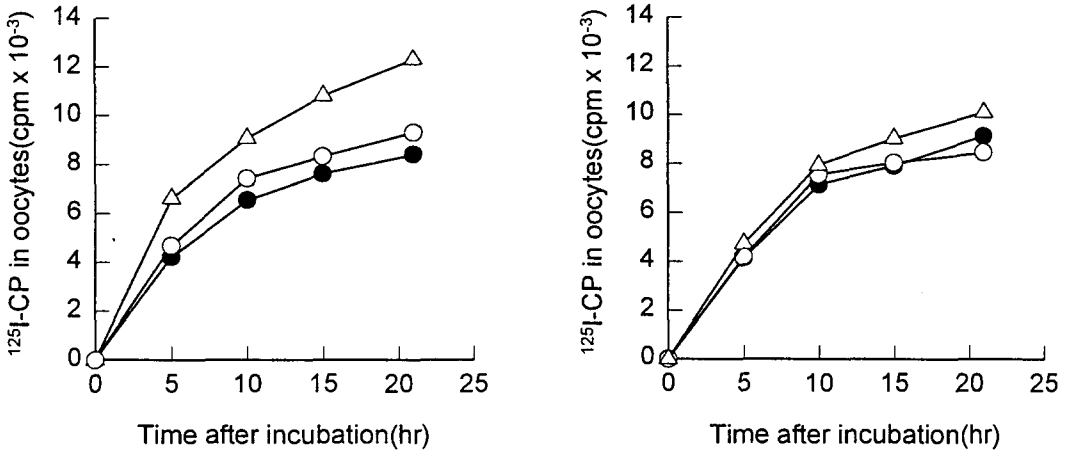

Fig. 1. Effects of GTP and related nucleotides on ^{125}I-CP transport into oocytes of intermediate size class. (A) Oocytes were incubated with ^{125}I-CP in the presence of 100 μM (○) and 500 μM GTP (△) and in the absence (●). (B) Oocytes were treated with 500 μM ATP(△), CTP (○) and without nucleotides (●).

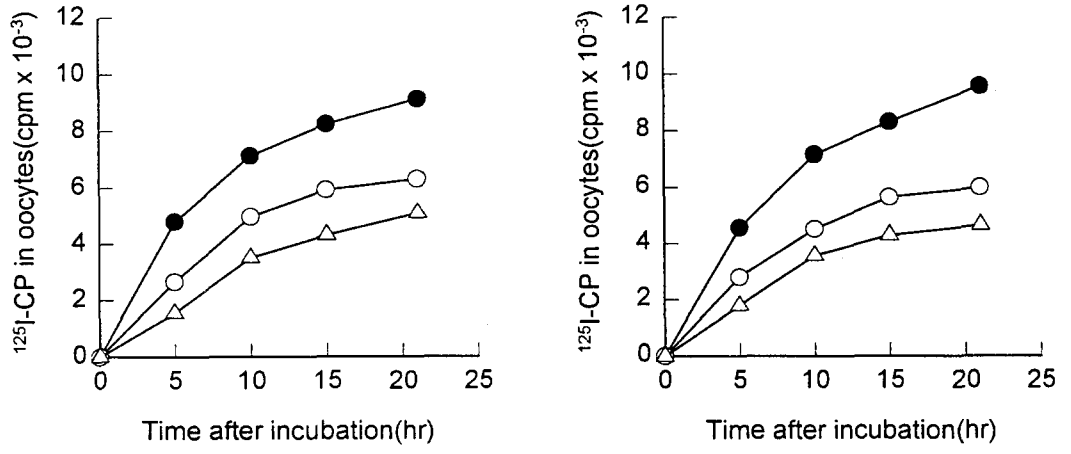

Fig. 2. Effects of GTPγS (A) and GTPβS (B) on ^{125}I-CP transport into oocytes of intermediate size class. Oocytes were incubated with ^{125}I-CP in the presence of 100 μM (○) and 500 μM GTP analogue (△) or in the absence (●).

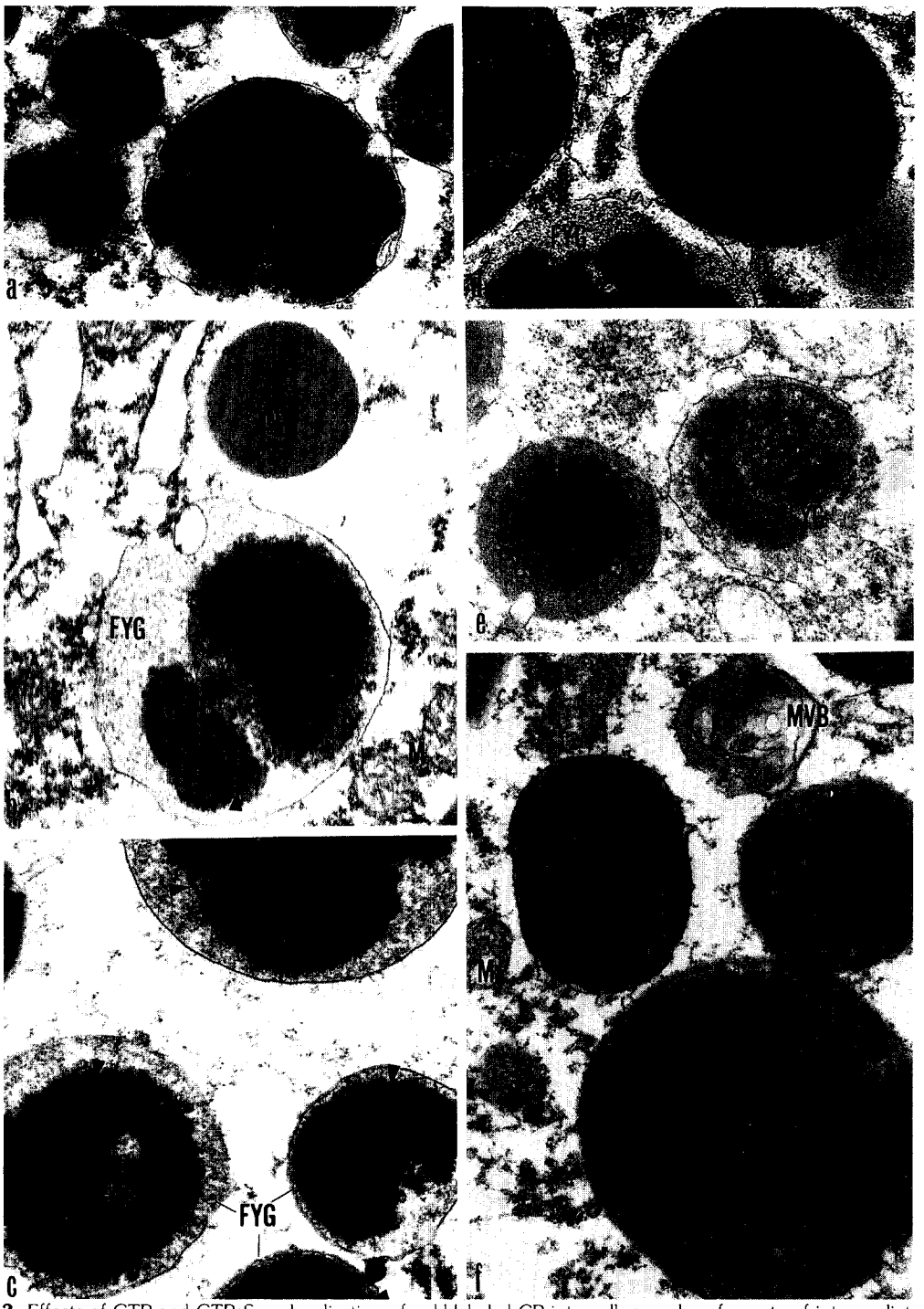

Fig. 3. Effects of GTP and GTPγS on localization of gold-labeled CP into yolk granules of oocyte of intermediate size class. Gold particles in the newly forming yolk granules (FYG) and yolk granules (YG) were indicated by arrow head. The density of gold particle increases by GTP (b, c) and decreases by GTPγS (e, f). Gold particles were not shown in the yolk granules of untreated oocytes (a,d).

The effect of GTP on the transport is dependent on the concentration; ^{125}I-CP transport is increased by 10% at 100 M and by 40% at 500 M GTP, at which the amount of coelomic fluid proteins transported for 20 hours is 170 ng/100 oocytes. The increase of CP transport is specific to GTP, since ATP or CTP did not affect the ^{125}I-CP transport at all (Fig. 1).

Transport of ^{125}I-CP was seriously affected by GTPγS, being reduced by 39% at 100 μM and by 53% at 500 μM, at which the amount of coelomic fluid proteins transported for 20 hours is 66 ng/100 oocytes (Fig. 2A). The effect of GDPβS in ^{125}I-CP is similar to that of GTPγS; the transport was reduced by 34% at 100 μM and by 44% at 500 μM, at which the amount of coelomic fluid proteins transported for 20 hours is 73 ng/100 oocytes (Fig. 2B). The results indicate that transport of coelomic fluid proteins into the oocytes of *Pseudopotamilla occelata* could be regulated by GTP-binding proteins.

Enhancement of CP transport by GTP and inhibition by GTPγS were visually confirmed by electron microscopy. When oocytes were incubated with gold-labeled CP, gold particles were localized in the newly forming and mature yolk granules, but the density of gold particles in the yolk granules varies depending on the treatment. The number of gold particles within the area of 1 μm^2 of yolk granule was approximately 10 when treated with 500 μM GTP and 2 when treated with 500 μM GTPγS, compared to 5 in the untreated one. (Fig. 3). The observations also strongly support that the transport of coelomic fluid proteins into oocytes is controlled by GTP-binding proteins.

Discussion

It has been recently reported that endocytosis as well as vesicular transport is regulated by small G proteins such as ADP-ribosylation factor and rab family G proteins (Balch, 1990; Serafini *et al.*, 1991; Stow *et al.*, 1991; Donaldson *et al.*, 1991; Colombo *et al.*, 1992; Carter *et al.*, 1993; Barbieri *et al.*, 1994; Lenhard *et al.*, 1994; Mantyh *et al.*, 1995). Heterotrimeric G proteins are also involved in hormonal signal transduction across the plasma membrane and in vesicular protein transport as well (Colombo *et al.*, 1994). The results demonstrated that GTP-binding proteins are involved in transferring coelomic fluid proteins, a yolk precursor proteins, into oocytes at specific stages across the plasma membrane and throughout the process of yolk granule formation in the membranous structures. It has been already known in our previous studies that coelomic fluid proteins are internalized by receptor-mediated endocytosis specifically into the oocytes of intermediate size class and the vesicles containing CP are assembled to form tubular endosomes, which are fused into multivesicular bodies (Kang and Lee, 1991). Yolk nucleus formed by aggregation of the precursor proteins in the multivesicular bodies grows into large yolk granules.

GTP-binding proteins appear to participate in the process of assembling yolk granules during oogenesis of *Pseudopotamilla occelata*, since G protein agonist such as GTP stimulates the internalization and localization of CP into yolk granules, whereas G protein analogues such as GDPβS inhibited the uptake of CP, as GTPγS inhibits fusion activity at high concentrations of cytosolic protein (Mayorga *et al.*, 1989).

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안점의 꽃갯지렁이 난모세포내로 체강액 단백질의 단계특이적 유입을 위한 GTP-Binding Protein의 개입

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안점의 꽃갯지렁이(*Pseudopotamilla occelata*)의 난자형성중 난황단백질의 전구체인 체강액 단백질(CP)은 수용체에 의해 중개되는데 이러한 수용체 중개에 의한 난모세포내로의 유입은 GTP-binding protein에 의해 조절되는 것으로 확인되었다. 체강액 단백질(CP)을 가장 활발히 투과시키는 중기 난모세포내로의 ^{125}I-CP의 유입은 GTP에 의해 촉진되었고, GTP 유사체인 GTPγS나 GTPβS에 의해서는 억제되었다. 체강액 단백질 유입에 미치는 GTP와 GTPγS의 효과를 금입자로 표지된 체강액 단백질을 이용하여 전자현미경으로 확인해 본 결과, gold-labeled CP는 난황립에 집중되었고, 이러한 현상도 GTP에 의해서는 촉진되었고 GTPγS에 의해서는 억제되었다.