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Evidence for the presence of the $G_{q/11}$ α-subunit in unfertilized and fertilized sea urchin eggs

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Heterotrimeric GTP-binding proteins (G-proteins) are a class of signal transducing molecules that couple transmembrane receptors to effector molecules inside the cell. They consist of one α, one β, and one γ subunit. The α-subunit has the GTP-binding and hydrolyzing activity. Heterotrimeric G-proteins act as molecular switching devices in a variety of signalling pathways. A role for heterotrimeric G-proteins at egg activation during fertilization has been proposed. The experimental evidence so far confirms the presence of G-proteins in eggs of a variety of species but the role of G-proteins in eggs remains unclear.

Recently two novel pertussis-toxin-insensitive α-subunits of the Gq subclass of G-proteins, G_q and G_{11}, have been cloned and characterized. They are widely distributed among cell types and show a high degree of evolutionary conservation. An antibody directed against the common carboxyterminal decapeptide of the α-subunits of G_q and G_{11} was shown to recognize distinct proteins of 43 and 44 kDa molecular weight in unfertilized and fertilized eggs of the sea urchin species Lytechinus pictus and Strongylocentrotus purpuratus. This study provides evidence for the presence of members of the Gq subclass of heterotrimeric G-proteins in sea urchin eggs.

Key words: *Egg activation, G-proteins, $G_{q/11}$ α-subunit.*

INTRODUCTION

Prior to fertilization, the sea urchin egg is arrested in interphase. The fertilizing sperm triggers the initiation of the cell cycle and the beginning of development. Many of the events at fertilization have been well described but the molecular mechanisms of the inter- and intracellular signals that activate the egg still remain elusive. The sperm triggers a transient increase in intracellular free calcium that starts at the point of sperm-egg interaction and propagates as a wave throughout the egg cytoplasm (reviewed by Jaffe, 1983; Swann and Whitaker, 1986). The subsequent exocytosis of cortical vesicles results in the elevation of the fertilization envelope and constitutes the permanent block to polyspermy. The calcium wave is thought to propagate in a positive feedback loop: the second messenger inositol 1,4,5-triphosphate (IP_3) releases calcium from intracellular stores. Increased calcium stimulates phospholipase C to hydrolyze phosphatidylinositol bisphosphate to IP_3 and diacylglycerol, thus completing the loop (Swann and Whitaker, 1986; Whitaker and Crossley, 1990; Swann and Whitaker, 1990).

Several models have been proposed of how the sperm activates the egg based on experimental observations (reviewed by Nuccitelli, 1991). One hypothesis suggests that the calcium wave is triggered by a diffusible factor that the sperm introduces into the egg when the sperm-egg membranes fuse (Loeb 1913; Swann and Whitaker, 1990; Whitaker and Crossley, 1990; Whitaker and

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Swann, 1993). Another model is the receptor G-protein hypothesis (reviewed by Jaffe, 1989; Jaffe, 1990). The sperm binds to a receptor in the egg plasma membrane which activates a heterotrimeric GTP-binding protein (G-protein) that in turn couples to phospholipase C (PLC). According to this model, the sperm-egg interaction may be analogous to the interaction between a neurotransmitter or a hormone and its target cell. In mammalian cells phosphatidylinositol second messengers are generated by a signal transduction mechanism that involves heterotrimeric G-proteins (Gilman, 1987). Heterotrimeric G-proteins are a class of proteins that couple specific transmembrane receptors, such as hormone receptors, to effector molecules. They function as molecular switching devices in many signal transduction pathways (for review see Stryer and Bourne, 1986). They are heterotrimers of one α, one β, and one γ-subunit, with the α-subunit responsible for receptor interaction, GTP-binding and GTPase activity. So far, at least fifteen α subunits have been cloned and grouped into subclasses according to their amino acid sequence homology (Spiegel, 1992).

Recently two members of the G_q subclass of α-subunits, G_q and G_{11} have been identified using molecular cloning techniques (Strathmann and Simon, 1990) and biochemical methods (Pang and Sternweis, 1990). They show structural differences to α-subunits of other subclasses (Strathmann and Simon, 1990) and are not substrates for ADP-ribosylation by pertussis toxin (PTX) (Pang and Sternweis, 1990). Members of the G_q subclass are found in vertebrates and invertebrates and are highly conserved through evolution (Strathmann and Simon, 1990). Reconstitution experiments have indicated that G_q proteins couple to a PLC β1 isoform and are likely responsible for PTX insensitive pathways of phosphatidylinositol metabolism in mammalian cells (Smrcka *et al*, 1991).

In a recent study, Hsieh and Martin (1992) showed that G_q and G_{11} couple the receptors for thyrotropin-releasing hormone and gonadotropin-releasing hormone to PLC. They generated a rabbit polyclonal antibody against the carboxyterminal sequence that is identical in the α-subunits of G_q and G_{11}. The antibody has been shown to recognize unique 42 kDa and 43 kDa proteins in membranes of GH_3 cells and αT3-1 pituitary cells (Hsieh and Martin, 1992).

The $G_{q/11}$ peptide antibody generated by Hsieh and Martin (1992) was used in this study to investigate the presence of members of the Gq subclass of a heterotrimeric G-proteins in sea urchin eggs. In Western blot experiments the antibody specifically recognized proteins of approximately 43 and 44 kDa in membrane preparations of eggs of two different species of sea urchins. Immunocytochemistry was used to study the localization of the antigen in fertilized and unfertilized sea urchin eggs. These results demonstrate the presence of a $G_{q/11}$ α-subunit in sea urchin eggs.

METHODS

Gamete preparation

Gametes from sea urchins, *Lytechinus pictus* and *Strongylocentrotus purpuratus*, were obtained by injection of 0.55 M KCl solution into the intracoelomic cavity. Eggs were collected directly into artificial sea water (ASW) and maintained at 16° C for *L. pictus* and at 12° C for *S. purpuratus*. Sperm was collected and maintained "dry" on ice until use. Dejellied eggs were denuded of the vitelline layer by treatment with 6 mM Dithiothreitol (DTT) and 0.01% trypsin in calcium-free sea water Ca^{2+}FSW 2 min after insemination. Fertilized eggs were then washed in ASW and cultured in ASW until used. (Chemicals are from SIGMA Chemical company, St. Louis, USA, unless otherwise specified).

Cell surface complex isolation of sea urchin eggs and preparation of samples for SDS Polyacrylamide Gel Electrophoresis.

To isolate the cell surface complex, a modified protocol of Kinsey (1986) was used. Unfertilized eggs were collected into ASW and processed as described above. The eggs were diluted in ice cold calcium and magnesium free sea water containing

aprotinin (1 μg/ml; Sigma) as protease inhibitor and homogenized in a prechilled conical homogenizer until all eggs were lysed and sheets of cell surface complexes could be seen under the microscope. Three centrifugation steps resulted in a pellet of cell surface complex. It was diluted 1:4 with 5x reducing SDS-sample buffer (4.0 ml distilled water; 1.0 ml 0.5M Tris-HCl, pH 6.8; 0.80 ml Glycerol; 1.6 ml 10% (w/v) SDS; 0.4 ml β-mercaptoethanol; 0.2 ml 0.05% (w/v) Bromphenol blue) and boiled for 5 min. These preparations are referred to as membrane preparations in the following descriptions. The Pierce BCA* Protein Assay was used to determine protein concentrations.

SDS Polyacrylamide Gel Electrophoresis and Western Blot

A Bio-Rad Mini-PROTEAN II system was used for 7 cm mini-gels. Membrane preparations were subjected to electrophoresis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) on 12% polyacrylamide gels prepared according to the protocol of Laemmli (1970). Proteins were transferred to nitrocellulose (Bio-Rad) using the Mini Trans Blot Electrophoretic Transfer Cell from Bio-Rad. The transblot buffer contained 25 mM Tris, 192 mM glycine, 10% (v/v) methanol and 0.0075% SDS, pH 8.3. The transfers were blocked by incubation in Tris-buffered saline (TBS; 0.137 M NaCl, 0.02 M Tris-base, 0.05% TWEEN-20, pH 8.0) containing 5% nonfat dry milk (BLOTTO) for 1 h at 37° C. Antibodies were diluted in TBS containing either 5% BLOTTO or 5% BSA. Determination of immunoreactive proteins was accomplished using a horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy and light chain) polyclonal antibody (Zymed, #62-6120). The developing solution for the horseradish peroxidase reaction contained diaminobenzidine and ammonium nickel (II) sulfate hexahydrate (98%; Aldrich, Milwaukee, WI). Instead of TBS/TWEEN-20 (0.05%), PBS with 0.05% TWEEN-20 was used without changing the results (PBS: 24 g NaCl, 0.6 g KCl, 0.78 g KH_2PO_4, 3.3 g Na_2HPO_4 for 3 l, pH 7.2). The $G_{q/11}$ peptide antibody was an affinity purified rabbit polyclonal antibody raised against the carboxyterminal $G_{q/11}$ α-subunit decapeptide (CQLNLKEYNLV) with an amino-terminal cysteine extension for conjugation to the carrier maleimide-activated bovine serum albumin (Hsieh and Martin, 1992).

Immunocytochemistry on eggs and fluorescence microscopy

Sea urchin eggs were collected, processed as described above and attached to cover slips coated with 1 mg/ml poly-L-lysine (Mazia *et al* 1975). The eggs were fixed in −10° C methanol and blocked in 5% Normal Goat Serum in PBS before immunostaining with a dilution of the $G_{q/11}$ peptide antibody. The secondary antibody used was a fluorescein conjugated goat anti-rabbit polyclonal (Zymed, South San Francisco, CA, USA). Cells were also stained with diamino phenylindole (Dapi). Cover slips were mounted on slides in SlowFade mounting medium (S-2828, Molecular probes, Inc. Eugene, OR) and viewed with a Zeiss Axiophot microscope through 63 x oil immersion Plan-Apochromat (N. A. 1.4) objective or a 100 x oil immersion Plan Neofluar (N. A. 1.3) objective. Micrographs were taken on Kodak Tri-X film and developed in Diafine Developer in Accufine.

RESULTS

Western Blot experiments

Hsieh and Martin (1992) generated a rabbit polyclonal antibody against the carboxyterminal peptide (CQLNLKEYNLV) of the α-subunit of both G_q and G_{11}, members of the Gq subfamily of the heterotrimeric G proteins. This antibody is referred to as $G_{q/11}$ antibody.

Initially we investigated if the $G_{q/11}$ antibody would recognize proteins in sea urchin eggs. On Western blots of membrane preparations of unfertilized eggs of the sea urchin species *L. pictus* the antibody showed cross reactivity with protein bands of

approximately 43, 52 and 54 kDa (Fig 1, lane 3). In fertilized eggs of *L. pictus* (30 min post insemination) two bands of approximately 43 and 54 kDa were seen on blots (Fig 1, lane 2). The rabbit preimmune serum at the same dilution as the immune serum does not recognize any proteins on Western blots of these membrane preparations (data not shown). In membrane preparations of *S. purpuratus* the $G_{q/11}$ immune serum recognized a single band of slightly higher molecular weight (approximately 44 kDa, Fig 1, lane 1). Preimmune serum did not show immunoreactivity in *S. purpuratus* membrane preparations (data not shown). Cholate extract of GH_3 cells was used as positive control on Western blots. The 42 kDa band that corresponds to the $G_{q/11}$ protein could be reproduced. Also a band of higher molecular weight (63 kDa) appeared

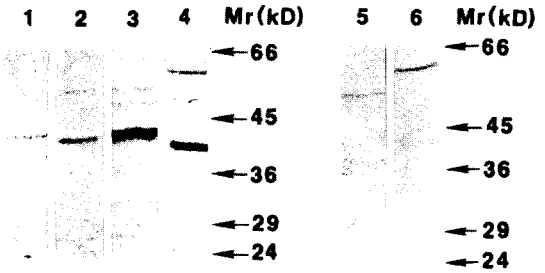

Fig 1: Western blot of membrane preparations of sea urchin eggs. Membrane preparations of unfertilized eggs of *S. purpuratus* (lane 1), of fertilized eggs of *L. pictus* (lane 2), of unfertilized eggs of *L. pictus* (lane 3), cholate extract of pituitary cells (lane 4) subjected to 12% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose-membrane and stained with anti-$G_{q/11}$ rabbit immuneserum followed by a horseradish peroxidase-conjugated secondary antibody, as described in Methods. In lanes 5 and 6, anti-$G_{q/11}$ immuneserum incubated with the peptide (CQLNLKEYNLV), against which the antibody was raised, prior to immunostaining of membrane preparation of unfertilized eggs of *L. pictus* (lane 5) and cholate extract of GH_3 pituitary cells (lane 6). Molecular weight of markers indicated in kDa (arrows). Anti-$G_{q/11}$ rabbit immuneserum recognizes one band of 44 kDa in membrane preparations of unfertilized eggs of *S. purpuratus* (lane 1), three bands of 43, 52 and 54 kDa in membrane preparations of unfertilized eggs of *L. pictus* and two bands of 43 and 54 kDa in membrane preparations of fertilized eggs of *L. pictus*. Lane 2 slightly overloaded. In cholate extract of GH_3 pituitary cells, the 42 kDa band corresponding to $G_{q/11}$ and a band of 60 kDa are present. The bands at 54 kDa in lane 3 and at 60 kDa in lane 4 could not be competed by the presence of the peptide the antibody was raised against (lanes 5 and 6, respectively). Rabbit preimmuneserum did not recognize any bands in either of the preparations (data not shown).

(Fig 1, lane 4). Staining of the transfers with immune serum that was preincubated with the peptide against which the immune serum was raised revealed that antibody binding to the higher molecular weight band in the GH_3 cell cholate extract was not blocked and is presumably due to nonspecific staining (Fig 1, lane 6). Also one higher molecular weight band present in blots of membrane preparations of *L. pictus* unfertilized eggs is present in the blot stained with immuneserum preincubated with peptide and thus probably due to nonspecific staining (Fig 1, lane 5). Antibody binding to both the 42 kDa band in GH_3 membrane preparations and the 43 kDa band in membrane preparations of *L. pictus* could be blocked by the peptide and are probably due to specific antibody antigen recognition.

Immunocytochemistry

The intracellular distribution of the antigen was investigated using the $G_{q/11}$ antibody in indirect immunofluorescence. Immunostaining in methanol-fixed unfertilized and fertilized eggs of *L. pictus* was observed (Fig 2a). The possibility that the secondary antibody binds to the cells in a nonspecific manner was excluded in controls with secondary antibody only (data not shown). Evidence for specificity of the antibody binding was obtained in controls where the fluorescence was not detected when immune serum was replaced by preimmune serum (Fig 2b). The antigen seemed to be distributed throughout the egg, no specific subcellular localization could be distinguished with epifluorescence microscopy.

DISCUSSION

In this study, biochemical methods and immunofluorescence microscopy were used to show that a peptide-antibody against the α-subunit of the heterotrimeric G-proteins G_q and G_{11} specifically recognizes 43 and 44 kDa proteins in sea urchin eggs. In GH_3 cells the apparent molecular weight of G_q is 42 kDa and of G_{11} 43 kDa (Hsieh and Martin, 1992). These mobilities correspond well to

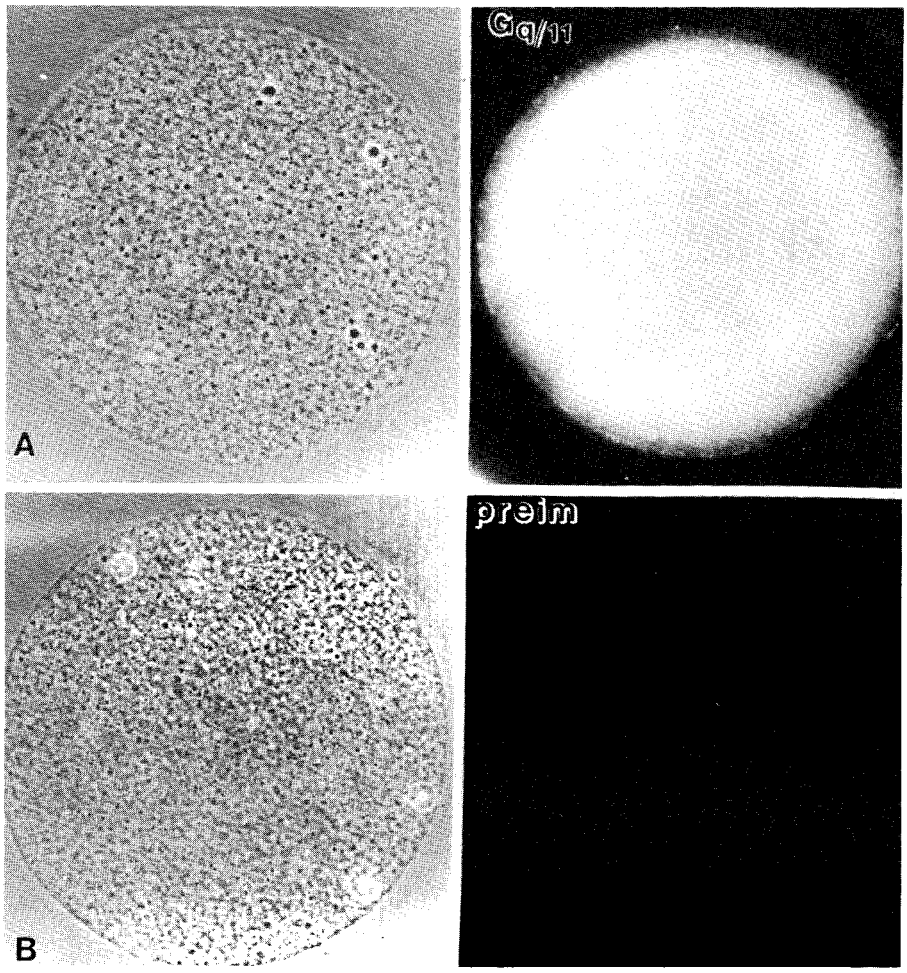

Fig 2: Immunocytochemical staining of *L. pictus* eggs using the $G_{q/11}$ peptide antibody. Eggs fixed in methanol and processed for immunocytochemical staining, as described in Methods. Left panel: phase contrast microscopy; right panel: fluorescence microscopy of the same fields. A: Eggs stained with $G_{q/11}$ peptide antibody (1:200 dilution). B: Egg stained with rabbit preimmune serum (1:200 dilution). The secondary antibody used was Fluorescein-conjugated goat anti-rabbit IgG. The exposure time of the fluorescence micrograph in B was the same as in A. Also the processing of the negative into the print was done in exactly the same way for print B as for A.

those found in the sea urchin egg membrane preparations. This provides evidence that a member of the G_q subfamily is present in sea urchin eggs. The antigen is present in low abundance in sea urchin eggs or shows minor cross reactivity to the antibody compared to GH_3 cells. The bands on the Western blots in egg membrane preparations are weaker although equal amounts of protein were loaded on the lanes of the polyacrylamide gels. On Mini-gels the antigen in sea urchin eggs and the $G_{q/11}$ in GH_3 cells show a slight difference in molecular weight of approximately 1 kDa. Interesting is also the slight difference in molecular weight between the antigen in *L. pictus* and *S. purpuratus* egg membrane preparations. This could be due to modifications of the antigen in sea urchin eggs or presence of different members of the G_q subclass in sea urchins. The *Drosophila*-homologue of G_q has been reported to lack six amino acids (Strathmann and Simon, 1990).

Heterotrimeric G-proteins are membrane associated. So it was expected that immunocytochemical staining of unfertilized eggs would reveal a cortical localization of the antigen. The immunocytochemical studies though do not show cortical staining but rather staining of the whole egg. This

staining pattern was consistent regardless of antibody dilution and fixation protocol (data not shown). Immunoelectron microscopy of sections would be useful to identify the exact distribution of the antigen in sea urchin eggs.

It has been suggested that members of the G_q subclass of heterotrimeric G-proteins couple to specific isotypes of PLC (Simon *et al*, 1991). G_q and G_{11} have been shown to couple to the β-1 isoform of PLC (Smrcka et al, 1991). If there is a member of the G_q subclass in sea urchin eggs, one could speculate a possible role in a pathway linked to phosphatidylinositol turnover. So it would be of interest to study the role of G_q proteins at cortical granule exocytosis and egg activation at fertilization.

The question of the involvement of G-proteins at egg activation during fertilization is as yet unanswered and the G-protein hypothesis has been challenged by recent findings. G-proteins have been shown to be present in gametes not only in sea urchins (Oinuma *et al*, 1986; Turner *et al*, 1987) but also in mammals (Garty *et al*, 1988) and *Xenopus* (Olate *et al*, 1989; Kline *et al*, 1991). The first evidence for the involvement of G-proteins at egg activation came from experiments where the non-hydrolyzable GTP-analogue, GTPγS, was microinjected in sea urchin eggs and shown to initiate cortical granule exocytosis. GDPβS was found to inhibit cortical granule exocytosis in response to sperm but not to microinjection of IP3 (Turner *et al*, 1986). This evidence in favor of the involvement of a G-protein have been challenged by Crossley *et al* (1991), who instead of looking at cortical granule exocytosis, which is a consequence of the calcium increase, looked at the calcium rise itself. According to their findings GDPS does not affect the fertilization calcium transient and GTPγS generates a calcium transient in sea urchin eggs and activates them. The fertilizing sperm seems to use a different pathway bypassing the egg's G-proteins.

A second approach to study the presence and function of G-protein in eggs uses the bacterial toxins, pertussis toxin (PTX) and cholera toxin (CTX). Microinjection of CTX in sea urchin eggs caused cortical granule exocytosis but solubility difficulties with PTX caused problems (Turner *et al*, 1987). The effects of PTX (or non-effects) would be of special interest in respect of the presence of members of the PTX-insensitive G_q subfamily.

The third way of studying the possible involvement of G-proteins at egg activation at fertilization was done in *Xenopus* (Kline *et al*, 1988) and starfish (Shilling *et al*, 1990) eggs. The mRNA of an exogenous hormone receptor, which is known to signal via G-protein, was injected in oocytes. The receptor was expressed and integrated into the egg plasma membrane. Addition of the agonists caused fertilization-like activation in the eggs. The conclusion of these experiments was that frog and starfish eggs contain the machinery to stimulate the phosphatidylinositol messenger system via a G-protein-mediated pathway. Whether this pathway is involved at fertilization remains to be resolved.

The missing link is obviously the sperm receptor. Recently, a highly glycosylated 350 kDa membrane protein has been characterized in sea urchin egg membranes (Foltz and Lennarz, 1990, 1992). The sperm protein binding that coats the acrosomal process binds to this glycoprotein in a species-specific manner (Foltz and Lennarz, 1990, 1992). The extracellular domain of this sea urchin egg-sperm receptor shows aminoacid homology to the heat shock protein 70 (Foltz *et al*, 1993) but its role in egg activation remains to be investigated.

The question if heterotrimeric G-proteins are involved in egg activation during fertilization has obviously no simple answer. Clearly, they are present in eggs and may play a role in egg maturation (for review see Turner and Jaffe, 1989). The presence in mature eggs could thus be residual. They could also be prestored in the egg membrane for function later in development. It is also possible that the egg has multiple pathways for activation to assure that the fertilization process occurs in an accurate and timely fashion. These pathways may be arranged in parallel, in series, or in some other, more complex network which is yet to be unraveled.

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