A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs

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Summary

Sand dollar eggs were microinjected with botulinum C3 exoenzyme, an ADP-ribosyltransferase from *Clostridium botulinum* that specifically ADP-ribosylates and inactivates rho proteins. C3 exoenzyme microinjected during nuclear division interfered with subsequent cleavage furrow formation. No actin filaments were detected in the equatorial cortical layer of these eggs by rhodamine–phalloidin staining. When microinjected into furrowing eggs, C3 exoenzyme rapidly disrupted the contractile ring actin filaments and caused regression of the cleavage furrows. C3 exoenzyme had no apparent effect on nuclear division, however, and multinucleated embryos developed from the microinjected eggs. By contrast, C3 exoenzyme did not affect the organisation of cortical actin filaments immediately after fertilisation. Only one protein (molecular weight 22 000) was ADP-ribosylated by C3 exoenzyme in the isolated cleavage furrow. This protein co-migrated with ADP-ribosylated rhoA derived from human platelets when analysed by two-dimensional gel electrophoresis. These results strongly suggest that a rho-like, small GTP-binding protein is selectively involved in the organisation and maintenance of the contractile ring.

Keywords: Actin filaments, C3 exoenzyme, Cytokinesis, Small G-protein

Introduction

Animal cells and some lower eukaryotic cells undergo cytokinesis by means of contraction of the contractile ring that forms in the cortex of the cleavage furrow (for a review, see Mabuchi, 1986). The contractile ring is a dynamic structure composed primarily of actin filaments that rapidly organises prior to cell cleavage and disintegrates soon after contraction (Schroeder, 1972). Although it has been established that contraction is accomplished via interactions between the actin filaments and myosin (Mabuchi & Okuno, 1977; Knecht & Loomis, 1987; De Lozanne & Spudich, 1987), many details of the structure of the contractile ring, its interaction with the plasma membrane, and mechanisms of regulation of its formation are unknown.

ADP-ribosylating enzymes have been shown to be useful tools for investigating the functions of GTP-binding proteins, since specific enzymes ADP-ribosylate and affect the function of specific target G-proteins (for a review, see Kaziro *et al.*, 1991). *Clostridium botulinum* produces exoenzyme C3, an enzyme that ADP-ribosylates and inactivates a group of ras-related, small G-proteins termed rho proteins (Aktories *et al.*, 1987; Narumiya & Morii, 1993). Recent reports show that introduction of this enzyme into cultured mammalian cells causes the disappearance of stress fibres and induces the cells to round up (Chardin *et al.*, 1989; Rubin *et al.*, 1988; Paterson *et al.*, 1990; Ridley & Hall, 1992). These data suggest that a rho protein may function in the organisation and maintenance of the actin filament cytoskeleton. Here,

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we show that microinjection of C3 exoenzyme into sand dollar eggs interferes with cellular cleavage by affecting the structure of the contractile ring.

Materials and methods

Materials

Eggs of the sand dollars *Clypeaster japonicus* and *Scaphechinus mirabilis* were obtained by injection of 1 mM acetylcholine chloride into the body cavity. They were fertilised in ordinary sea water and fertilisation membranes were removed by treatment with 1 M urea (Mabuchi & Takano-Ohmuro, 1990). The eggs were cultured at 20 °C in ordinary sea water.

Microinjection of C3 exoenzyme

Recombinant C3 exoenzyme from *Clostridium botulinum* was prepared as described previously (Nemoto *et al.*, 1991) and dissolved at a concentration of 1.06 mg/ml in 0.15 M NaCl/20 mM Hepes buffer, pH 7.5. The eggs, either fertilised or unfertilised, were microinjected using a braking micropipette (Hiramoto, 1974) with recombinant C3 exoenzyme using an Optiphot microscope equipped with a DIC apparatus (Nikon, Tokyo, Japan). A Fluor 40 objective lens was used.

Fluorescence microscopy

Eggs microinjected with C3 exoenzyme were collected, briefly washed with Ca-free sea water and processed for fluorescence microscopy as described previously (Mabuchi & Takano-Ohmuro, 1990) with minor modifications. The eggs were immobilised on protamine-coated glass slides, and fixed in medium A (0.8 M glucose/0.1 M KCl/2 mM MgCl$_2$/5 mM EGTA/10 mM Mops buffer, pH 7.4) containing 5% (v/v) formalin at 20 °C for 30 min and then in 0.2% Nonidet P-40 dissolved in 5% formalin-medium A for another 30 min. The eggs were then washed in medium B (0.8 M glucose in medium A was replaced with 0.8 M glycerol), stained in medium B containing 0.15 mM rhodamine–phalloidin (Molecular Probes, Eugene, Ore.), 0.05 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) and 0.1 M 2-mercaptoethanol, and examined with an Optiphot microscope equipped with an epifluorescence apparatus (Nikon, Tokyo). A Fluor 20 objective lens was used.

Two-dimensional electrophoresis

Cleavage furrows were isolated from *C. japonicus* eggs as described previously (Yonemura *et al.*, 1991) and incubated with recombinant C3 exoenzyme (25 μg/ml) in an ADP-ribosylation medium (Morii *et*

al., 1988) consisting of 0.1 M Tris-HCl, pH 8.0/10 mM thymidine/10 mM dithiothreitol/10 mM nicotinamide/5 mM MgCl$_2$/50 μM [^{32}P]NAD$^+$ (900 cpm/pmol) at 30 °C for 2 h. Human platelet extracts were prepared as described previously (Nemoto *et al.*, 1992) and processed as described above. These fractions were first resolved by either isoelectric focusing electrophoresis (IEF) as described previously (Mabuchi *et al.*, 1988) or non-equilibrium pH gradient gel electrophoresis (NEpHGE) according to O'Farrell *et al.* (1977). These first-dimensional tube gels were placed in tandem on a second-dimensional slab gel and electrophoresed in the presence of sodium dodecyl sulphate (SDS). The final two-dimensional gels were autoradiographed.

Results

C3 exoenzyme inhibits cleavage

Recombinant C3 exoenzyme was microinjected into fertilised eggs of *Clypeaster japonicus* and *Scaphechinus mirabilis*. Injection of more than 3 μg/ml (final intracellular concentration) into the eggs during the period between nuclear membrane breakdown and the end of anaphase interfered with cleavage furrow formation in each of 21 *C. japonicus* and 78 *S. mirabilis* eggs tested. By contrast, chromosome separation and movement towards the poles of the mitotic apparatus, as well as daughter nuclei formation, occurred normally. Consequently, the injected embryos became multi-nucleated (Fig. 1). When one blastomere at the 2-cell stage was injected, cytokinesis failed to take place in the injected cell, although the non-injected blastomere developed normally up to the blastula stage. Injection of C3 exoenzyme at final concentrations lower than 0.1 μg/ml did not interfere with cleavage. Intracellular concentrations of C3 exoenzyme between 0.1 and 2 μg/ml yielded intermediary results: 25% of the eggs underwent cleavage, 58% formed furrows but then regressed, and the remainder failed to form furrows. Injection of bovine serum albumin (final concentration 10–300 μg/ml) had no effect on furrow formation and cellular cleavage.

When C3 exoenzyme was injected into cleaving eggs at concentrations greater than 7.7 μg/ml, the cleavage furrow regressed after a lag of around 1 min in each of 22 *C. japonicus* and 74 *S. milabilis* eggs tested (Fig. 1B). These eggs developed into multi-nucleated embryos as described above.

Contractile ring is absent from C3-injected eggs

The organisation of actin filaments in eggs microinjected with C3 exoenzyme was examined by rhodamine–phalloidin staining. This analysis revealed a dark zone in the equatorial cortex indicating the
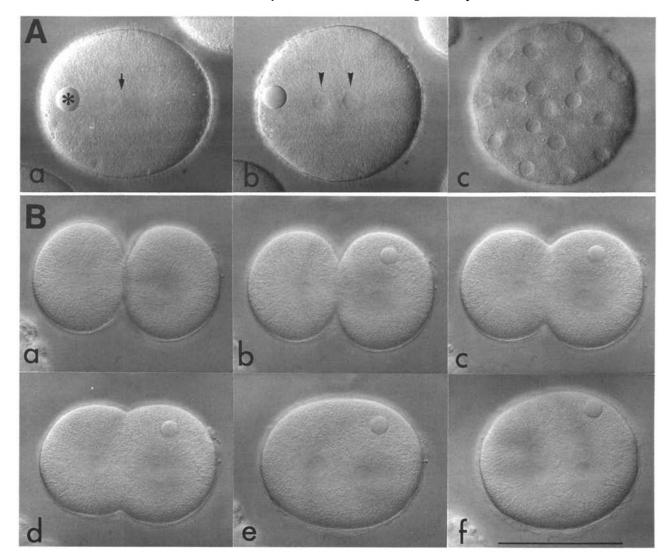
Figure 1 Microinjection of C3 exoenzyme into fertilised sand dollar eggs inhibits cell cleavage. (*A*) *C. japonicus* eggs microinjected with C3 at metaphase. (*a*) Immediately after microinjection of 20 μg/ml C3; (*b*) Same egg as (*a*), 26 min after injection; (*c*) an embryo 3.5 h after injection of 9.4 μg/ml C3. (*B*) *S. mirabilis* eggs microinjected with C3 during cleavage. (*a*) Before injection; (*b*) 1 min after injection of 12 μg/ml C3; (*c*) at 1 min 22 s; (*d*) at 5 min; (*e*) at 12 min; (*f*) at 33 min. Asterisk, an oil droplet introduced by microinjection; arrow, chromosomes; arrowheads, daughter nuclei. Scale bar represents 100 μm.

depletion of actin filaments in eggs injected between nuclear membrane breakdown and the end of anaphase as well as those injected during cleavage (Fig. 2). A similar depletion of actin filaments in the anticipated furrow region has previously been observed in sea urchin eggs cultured in the presence of ML-9, an inhibitor of myosin light chain kinase (Mabuchi & Takano-Ohmuro, 1990).

Identification of target of C3 in the isolated cleavage furrow

Proteins in isolated cleavage furrows and cortices that are ADP-ribosylated by C3 exoenzyme were investigated by electrophoresis in two-dimensional gels. One protein spot with an apparent molecular mass of 22 kDa and an isoelectric point a little more basic than that of actin was observed by IEF/SDS-PAGE or NEpHGE/SDS-PAGE to be ADP-ribosylated in the isolated cleavage furrow (Fig. 3A) and in isolated cortices (not shown). We did not estimate the amount of this protein in these preparations since it was detected only by silver staining. The ADP-ribosylated 22 kDa protein co-migrated with ADP-ribosylated rhoA in a human platelet extract (Nemoto *et al.*, 1992) in both IEF/SDS-PAGE (Fig. 3B) and NEpHGE/SDS-PAGE (not shown).

C3 does not affect actin assembly after fertilisation

In addition to the contractile ring, a meshwork of actin filaments is also present in the cortical layer of the sea
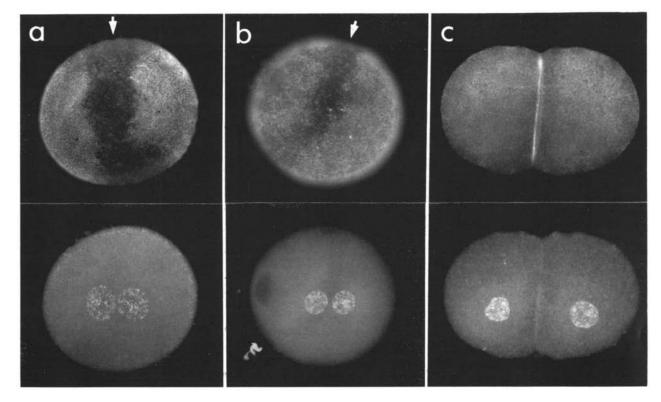
Figure 2   Organisation of actin filaments in *S. mirabilis* eggs microinjected with C3 exoenzyme. (*a*) An egg microinjected with C3 at metaphase. (*b*) An egg microinjected with C3 during cleavage. (*c*) A control egg forming the contractile ring. Upper panel, rhodamine–phalloidin staining; lower panel, DAPI staining of the corresponding eggs. Arrows, actin-depleted dark zones.
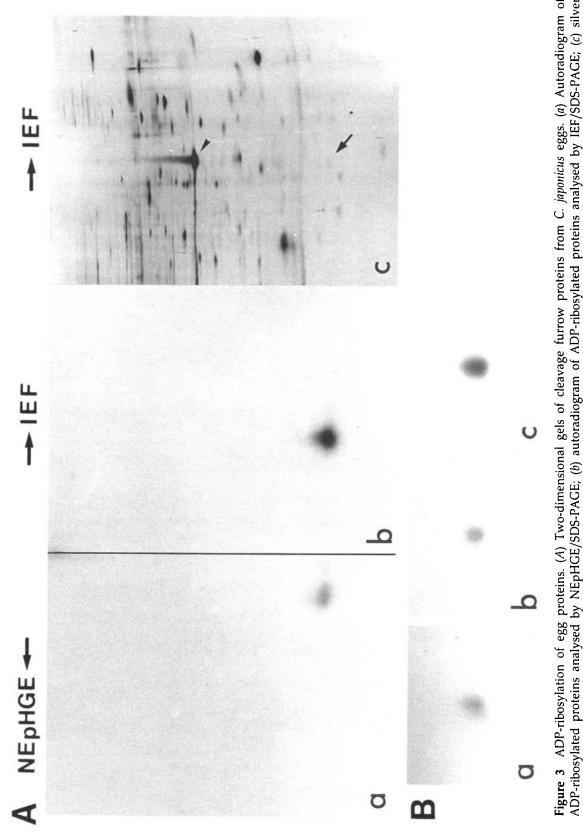
urchin and sand dollar eggs shortly after fertilisation (Yonemura & Mabuchi, 1987; Hamaguchi & Mabuchi, 1988). Microinjection of C3 exoenzyme prior to fertilisation did not affect the organisation of these filaments, however; nor did the injection of C3 exoenzyme stimulate actin filament formation (Fig. 4).
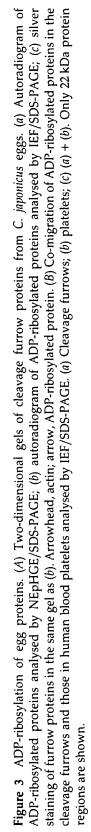
Discussion

C3 exoenzyme specifically ADP-ribosylates Asn41 in rho proteins and interferes with its interaction with downstream effectors (Sekine *et al.*, 1989; Morii *et al.*, 1991). Another group of rho-related proteins, rac proteins, have also been reported to be ADP-ribosylated by this enzyme (Didsbury *et al.*, 1989). Recent studies have shown, however, that rac proteins, are ADP-ribosylated 100 times to several thousand times less effectively than rho proteins (Menard *et al.*, 1992; Ridley & Hall, 1992). These data, in combination with our observation that the only protein in isolated cleavage furrows that is ADP-ribosylated by C3 exoenzyme co-migrates with human platelet rhoA, suggest that the effects of C3 exoenzyme observed in the present experiments can be attributed to modification of a rho-like protein. Thus, we conclude that a rho-like

protein is involved in the organisation of actin filaments in the contractile ring.

Recently, the involvement of a rho protein in the organisation of cytoskeletal actin has been reported. This protein appears to be involved in stress fibre formation in cultured mammalian cells, since cells to which C3 exoenzyme or ADP-ribosylated rho is introduced tend to round up and lose their stress fibres (Rubin *et al.*, 1988; Chardin *et al.*, 1989; Paterson *et al.*, 1990) or become unable to form stress fibres in response to serum (Ridley & Hall, 1992). Recently, it has been shown that rho is involved not in actin polymerisation *per se* but in the assembly of the integrin-based adhesion site complex to which the actin filaments bind (Morii *et al.*, 1992; Tominaga *et al.*, 1993). It is noteworthy that the enzyme concentration required to inhibit cytokinesis is several times lower than that required to inhibit stress fibre formation in cultured mammalian cells (Ridley & Hall, 1992). This may suggest that the contractile ring structure is more unstable than that of the stress fibre.

On the other hand, actin cytoskeleton formation occurring shortly after fertilisation was not affected by C3 exoenzyme. In cultured mammalian cells, membrane ruffling, another activity involving dynamic actin organisation, has been shown to be regulated by
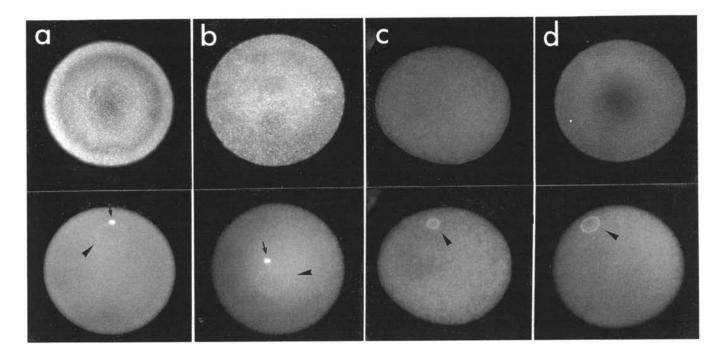
Figure 3 ADP-ribosylation of egg proteins. (*A*) Two-dimensional gels of cleavage furrow proteins from *C. japonicus* eggs. (*a*) Autoradiogram of ADP-ribosylated proteins analysed by NEpHGE/SDS-PAGE; (*b*) autoradiogram of ADP-ribosylated proteins analysed by IEF/SDS-PAGE; (*c*) silver staining of furrow proteins in the same gel as (*b*). Arrowhead, actin; arrow, ADP-ribosylated protein. (*B*) Co-migration of ADP-ribosylated proteins in the cleavage furrows and those in human blood platelets analysed by IEF/SDS-PAGE. (*a*) Cleavage furrows; (*b*) platelets; (*c*) (*a*) + (*b*). Only 22 kDa protein regions are shown.

Figure 4   C3 exoenzyme does not affect actin organisation at fertilisation of *S. mirabilis* eggs. (*a*) An egg microinjected with C3, incubated for 10 min, fertilised and fixed 15 min after fertilisation. (*b*) An egg fertilised normally and fixed 15 min after fertilisation. (*c*) An unfertilised egg microinjected with C3, incubated for 10 min and fixed. (*d*) An untreated unfertilised egg. Upper panel, rhodamine–phalloidin staining; lower panel, DAPI staining of the corresponding eggs. Arrows, sperm nuclei; arrowheads, egg nuclei.

rac proteins and not by rho (Ridley *et al.*, 1992). Thus, different types of actin cytoskeletal organisation may be controlled by distinct small GTP-binding proteins.

The effect of C3 exoenzyme microinjection was dramatic and remarkably fast, causing the disassembly of the contracting contractile ring in a very short time. This was not observed when myosin-specific antibodies, which also inhibit cleavage, were injected into cleaving starfish blastomeres (Mabuchi & Okuno, 1977). Thus, the rho-like protein appears to play a key role in contractile ring formation and stability. GTP-binding proteins serve as a molecular switches in the cell by turning on signals in transduction pathways following conversion to the GTP-bound form (Bourne *et al.*, 1991). Recent studies on the action of rho protein in mammalian cells revealed that it also works as an intracellular switch, linking external signals to integrin activation, stress fibre formation and cell adhesion (Ridley & Hall, 1992; Morii *et al.*, 1992; Tominaga *et al.*, 1993). Cytokinesis occurs following nuclear division, and there should be some signal linking these two events (for reviews, see Mabuchi, 1986; Rappaport, 1986). The present study raises the possibility that a rho protein may be such a switch linking nuclear division with cytokinesis. How such a signal is regulated is an interesting question for future investigation.

While we were preparing the manuscript, a paper appeared which reports that injection of C3 exoenzyme into *Xenopus* eggs interferes with the cleavage furrow formation (Kishi *et al.*, 1993).

Acknowledgements

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