Localization of Rho GTPase in sea urchin eggs

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Received 2 October 1998; received in revised form 27 October 1998

Abstract We isolated the *urho1* (urchin rho in English or uni rho in Japanese) gene from the sea urchin cDNA library which encodes a Rho GTPase. Anti-URho1 antibodies specifically recognized a 22 kDa protein in the extracts of echinoderm eggs. URho1 was concentrated in the cortices from both unfertilized and fertilized eggs as judged by immunoblot analysis. URho1 may bind directly to the cell membrane but not be a component of the cortical layer. Immunofluorescence microscopy revealed that URho1 is localized to the cleavage furrow and the midbody during cytokinesis.

Key words: Sea urchin egg; Cytokinesis; Rho GTPase; Cleavage furrow; Contractile ring; Midbody

1. Introduction

During cytokinesis of animal cells, the contractile ring is formed in the cortex of the cleavage furrow and contracts through interaction between actin filaments and myosin to divide the cell into two [1,2]. However, little is known about components of the contractile ring and the mechanism of its formation and its contraction.

The Rho family small GTPases including Rho, Rac and Cdc42 are known to be involved in regulation of the actin cytoskeleton [3,4]. It has recently been revealed that the Rho family small GTPases also play an important role during cytokinesis. It has been shown that the *Clostridium botulinum* C3 exoenzyme specifically inhibits the activity of Rho proteins through ADP-ribosylation of the effector domain [5]. Microinjection of C3 into sand dollar eggs [6] or *Xenopus* eggs [7] interferes with the formation of the contractile ring. The microinjection of RhoGDI (GDP dissociation inhibitor) into *Xenopus* eggs also inhibits furrow formation and that of RhoA rescues the inhibition [7]. In *Xenopus* eggs into which constitutively active or dominant negative forms of Cdc42Hs are microinjected, a furrow first ingresses, but it subsequently regresses [8]. In HeLa cells, overexpression of a constitutively active form of Cdc42Hs causes formation of multi-nucleated cells [9]. In *Dictyostelium discoideum*, RacE is required for cytokinesis in suspension culture [10].

It is important to study the localization of the Rho family proteins in dividing cells in order to clarify how these proteins are involved in cytokinesis. We have previously demonstrated that Rho is a component of the cleavage furrow using a biochemical analysis [6]. In addition, RhoA seems to be concentrated in the cleavage furrow during cytokinesis in Swiss 3T3 cells [11]. However, information on the localization of Rho in dividing cells has been insufficient. Therefore, we studied the localization of Rho in detail in sea urchin eggs. We isolated the *urho1* (urchin rho in English or uni rho in Japanese) gene from a sea urchin cDNA library. Anti-URho1 antibodies which were raised against bacterially expressed URho1 protein specifically recognized a 22 kDa protein in the extracts of eggs of echinoderms including sea urchins and a starfish. Immunofluorescence microscopy using the anti-URho1 antibodies revealed that URho1 is localized to the cleavage furrow and the midbody during cytokinesis.

2. Materials and methods

2.1. DNA manipulation and cloning of urho1 cDNA

Standard methods were used for DNA manipulations [12]. The *urho1* cDNA was isolated from a cDNA library prepared from blastula of a sea urchin, *Hemicentrotus pulcherrimus*, by Dr. Koji Akasaka. The human RhoA was used as a probe as described previously [13]. Hybridization and washing of filters were performed at 50°C according to the manufacturer's instructions (Amersham International, Buckinghamshire, UK). After plaque purification, the insert was subcloned into pBluescript II SK$^-$ with in vivo excision. Sequencing was carried out using an SQ-5500 DNA sequencer (Hitachi Co., Tokyo, Japan).

2.2. Expression and purification of URho1 protein

The *urho1* cDNA was amplified using oligonucleotides (5′-gggaattcatggctgctataaggaaaaag-3′ and 5′-gggtcgacttacaagaggctgcattttgatg-3′), which contained *Eco*RI or *Sal*I sites at their 5′ ends. The PCR-amplified fragments were ligated to *Eco*RI- and *Sal*I-treated pGEX-5X-1 (Pharmacia Biotech, Uppsala, Sweden) or pET-32a(+) (Novagen, Madison, WI, USA). The fusion proteins, GST-URho1, was produced in XL1-Blue containing pGEX-URho1, and purified by a glutathione-Sepharose 4B column (Pharmacia Biotech). The fusion protein, His-URho1, was expressed in BL21 (DE3) containing pET-32URho1, and purified using a His-bind resin (Novagen).

2.3. Production of the anti-URho1 antibodies

The purified His-URho1 was subjected to SDS-PAGE, and the gel containing His-URho1 was excised. The gel piece was washed with distilled water and homogenized in 0.15 M KCl, 20 mM Na-phosphate buffer, pH 7.2. Then, it was emulsified with Freund's complete adjuvant (Difco Co., Detroit, MI, USA). 100 μg of His-URho1 was injected subcutaneously into a Japanese white rabbit. After 14 days, His-URho1 emulsified with Freund's incomplete adjuvant (Difco Co.) was injected into the rabbit, followed by boost injections at 10 day intervals until anti-URho1 antibodies were raised. The antibodies were affinity-purified using GST-URho1 immobilized on a CNBr-Sepharose 4B column (Pharmacia Biotech).

2.4. Preparation of cell extract

Eggs of sea urchins, *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus*, *Clypeaster japonicus* and *Anthocidaris crassispina*, and of a starfish, *Asterias amurensis*, were used. Extracts of the eggs were prepared as described previously [14]. In brief, dejellied unfertilized eggs were suspended in an equal volume of F-buffer (0.1 M KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 10 mM MOPS, pH 7.4) containing 0.8 M glucose, and homogenized on ice

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; Bodipy, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene

0014-5793/98/$19.00
PII: S0014-5793(98)01531-2

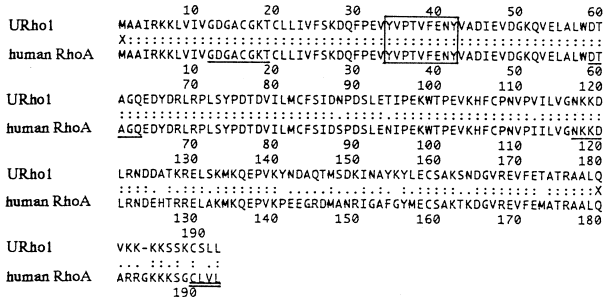

Fig. 1. Amino acid sequence of URho1. *urho1* cDNA was screened in the *H. pulcherrimus* cDNA library. The predicted amino acid sequence of URho1 is compared with that of human RhoA. Identical amino acids between the two proteins are indicated by double dots, and conserved amino acids are indicated by single dots. Sequences encoding GTP binding motifs are underscored. An effector domain is indicated by a box. The CXXL motif at the C-terminal is indicated by a double underline.

with a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 10 000×*g* for 20 min at 4°C. The supernatant was further centrifuged at 100 000×*g* for 1 h, and was stored at −80°C.

2.5. Fractionation of cortex and cytosol

Fractionation of cortex and cytosol was carried out as described previously [15]. The cortex is defined as a complex of the cell membrane and the cortical layer that lines the membrane. In brief, unfertilized or fertilized eggs were suspended in 10 volumes of F-buffer and homogenized with a hand-driven Teflon-glass homogenizer on ice. Disruption of the eggs was checked under a phase contrast microscope (Nikon, Tokyo, Japan). When all eggs were disrupted leaving the cortical hulls, the suspension was centrifuged at 220×*g* for 1 min. The pellet (the cortex fraction) was washed repeatedly with F-buffer until the supernatant became clear. The first supernatant was clarified by centrifugation at 100 000×*g* for 1 h at 4°C, and stored at −80°C as the cytosol fraction.

2.6. Solubilization of URho1 from the cell cortices

The cortex fraction was resuspended in 5 volumes of 0.6 M KI [15] or 1% Triton X-100 in F-buffer and incubated for 30 min on ice with occasional pipetting. Then the suspension was centrifuged at 10 000×*g* for 20 min at 4°C to obtain supernatant and pellet. The URho1 in these fractions was detected by immunoblot analysis.

2.7. Immunoblotting

Proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was blocked with 5% skim milk in T-TBS (0.15 M NaCl, 0.05% Tween 20, 20 mM Tris-HCl, pH 7.6). After a wash with T-TBS, the membrane was incubated in T-TBS containing the antibodies for 1 h at room temperature. After three washes with T-TBS, it was incubated with HRP-conjugated anti-rabbit IgG (Amersham International) at a dilution of 1/5000 to 1/10 000 for 1 h at room temperature. Detection of the primary antibodies was carried out using the ECL system (Amersham International).

2.8. Immunofluorescence microscopy

Indirect immunofluorescence microscopy of eggs with anti-URho1 antibodies was performed according to Mabuchi [16] except that Bodipy-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) was used as the second antibody. After immunostaining, the eggs were stained with 1 U/ml rhodamine-phalloidin (Molecular Probes) and 1 μg/ml DAPI. Immunofluorescent staining for tubulin was carried out according to Mabuchi and Ohmuro [17]. The mouse monoclonal anti-α-tubulin antibody (Amersham International) was used as the first antibody and rhodamine-conjugated anti-mouse IgG (ICN Pharmaceuticals, Inc., Aurora, OH, USA) was used as the second antibody. Stained eggs were examined with an Axioskop fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and photographed on T-MAX 400 films (Kodak, New York, USA).

3. Results and discussion

3.1. Cloning of urho1 cDNA

In order to characterize Rho proteins in sea urchin eggs, we screened a sea urchin blastula cDNA library using the human

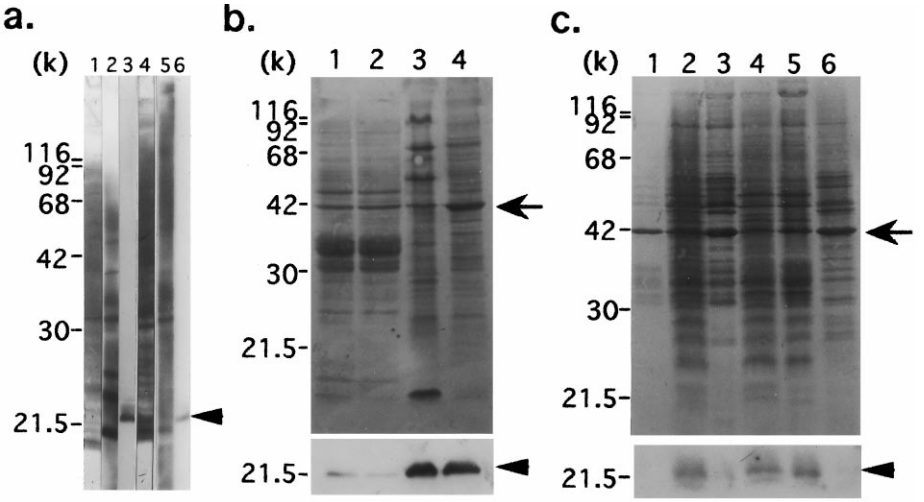

Fig. 2. Localization of URho1 in sea urchin eggs as studied by immunoblotting. a: Egg extracts of sea urchins. Lanes 1–3, *H. pulcherrimus* egg extract. Lanes 4–6, *P. depressus* egg extract. Lanes 1 and 3, CBB staining. Lanes 2 and 4, immunoblots with pre-immune serum. Lanes 3 and 6, immunoblots with anti-URho1 antibodies. Anti-URho1 antibodies reacted with a 22 kDa protein (arrowhead). b: The cortex and the cytosol fractions from *H. pulcherrimus* eggs. Lane 1, cytosol from unfertilized eggs. Lane 2, cytosol from fertilized eggs. Lane 3, cortices from unfertilized eggs. Lane 4, cortices from fertilized eggs. The upper panel shows a CBB-stained membrane and the bottom panel shows immunoblotting of the same membrane. Arrowhead indicates anti-URho1 reactions. Note that the amount of actin in cortex fractions increases after fertilization (compare lane 3 and lane 4, arrow). c: Solubilization of URho1 from the cell cortices. The cortex fraction from fertilized eggs was treated with 0.6 M KI (lanes 3 and 4) or 1% Triton X-100 (lanes 5 and 6). Lanes 1 and 2, control treatment with F-buffer. Lanes 1, 3 and 5, supernatant fractions. Lanes 2, 4 and 6, pellet fractions. Upper panel, CBB-stained membrane. Bottom panel, immunoblotting of the same membrane. URho1 (arrowhead) is solubilized by 1% Triton X-100 but not by 0.6 M KI, while a major part of actin is solubilized by 0.6 M KI (compare lane 3 and lane 5, arrow).

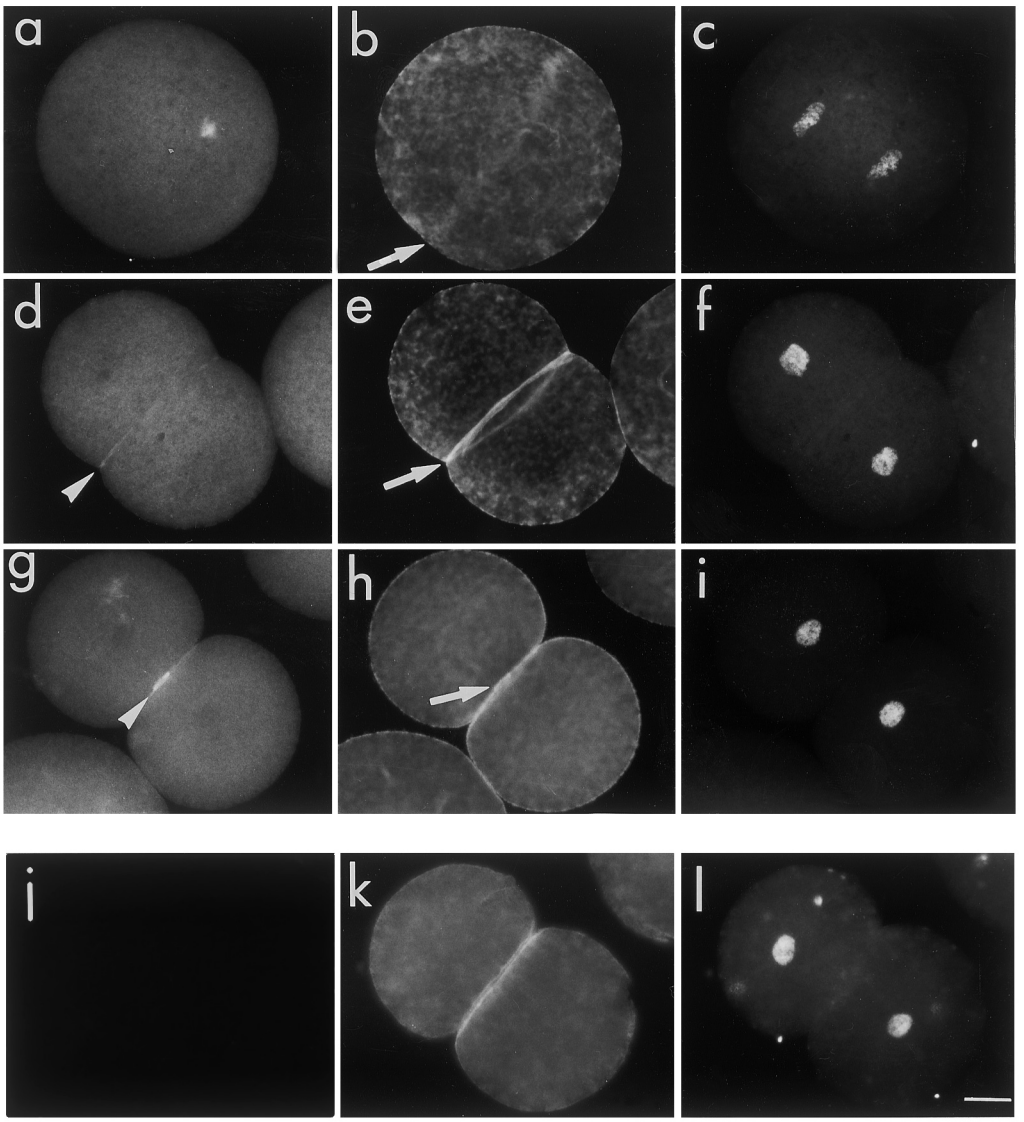

Fig. 3. Localization of URho1 in the cleavage furrow by immunofluorescence microscopy during first division of *P. depressus* eggs. The eggs were triple-stained with the anti-URho antibodies (a, d, g), rhodamine-phalloidin (b, e, h, k) and DAPI (c, f, i, l). j: Normal IgG. Arrowheads, localization of URho1 in the cleavage furrow. Arrows, the contractile ring. Bar, 50 μm.

rhoA gene as a probe. Out of 500 000 clones screened, we obtained 16 clones which hybridized strongly with the *rhoA* probe. All these clones contained a same open reading frame encoding a 192 amino acid protein. We called this gene *urho1*. The predicted amino acid sequence of URho1 was 83.9% identical to human RhoA and was perfectly conserved in the GTP binding/hydrolysis domains and the effector domains (Fig. 1).

3.2. Immunoblot analysis using anti-URho1 antibodies

Fig. 2a shows that anti-URho1 antibodies recognized a single protein band in extracts of eggs of *H. pulcherrimus* and *P. depressus*, which migrated at a 22 kDa position on SDS-PAGE. The same band was recognized by the antibodies in extracts from eggs of sea urchins, *C. japonicus* and *A. crassispina*, and a starfish, *A. amurensis*, albeit the reaction was a little weaker. On the other hand, no reaction was detected with an extract of *Xenopus* eggs or pig brain (data not shown). These results may indicate that the anti-URho1 antibodies can specifically detect echinoderm Rho proteins.

We first studied the localization of URho1 by cell fractionation (Fig. 2b). URho1 was concentrated in the cortices of both the unfertilized and the fertilized eggs. The total amounts of URho1 in the cortices and the cytosol were roughly estimated to be 60% and 40% of whole URho1, respectively. It has been reported that an organized array of actin filaments is dynamically assembled in the cortical layer after fertilization in the sea urchin egg [15,18–21]. It has also been observed that the amount of actin is increased in the cortical fraction after fertilization [15,22] (see also Fig. 2b). In contrast, the amount of URho1 in the cortex did not change after fertilization. In addition, it did not significantly change during the course of cell division (data not shown).

To determine whether the URho1 in the cortex binds to the cell membrane or is a component of the cortical layer that lines the cell membrane along with the actin filaments, we

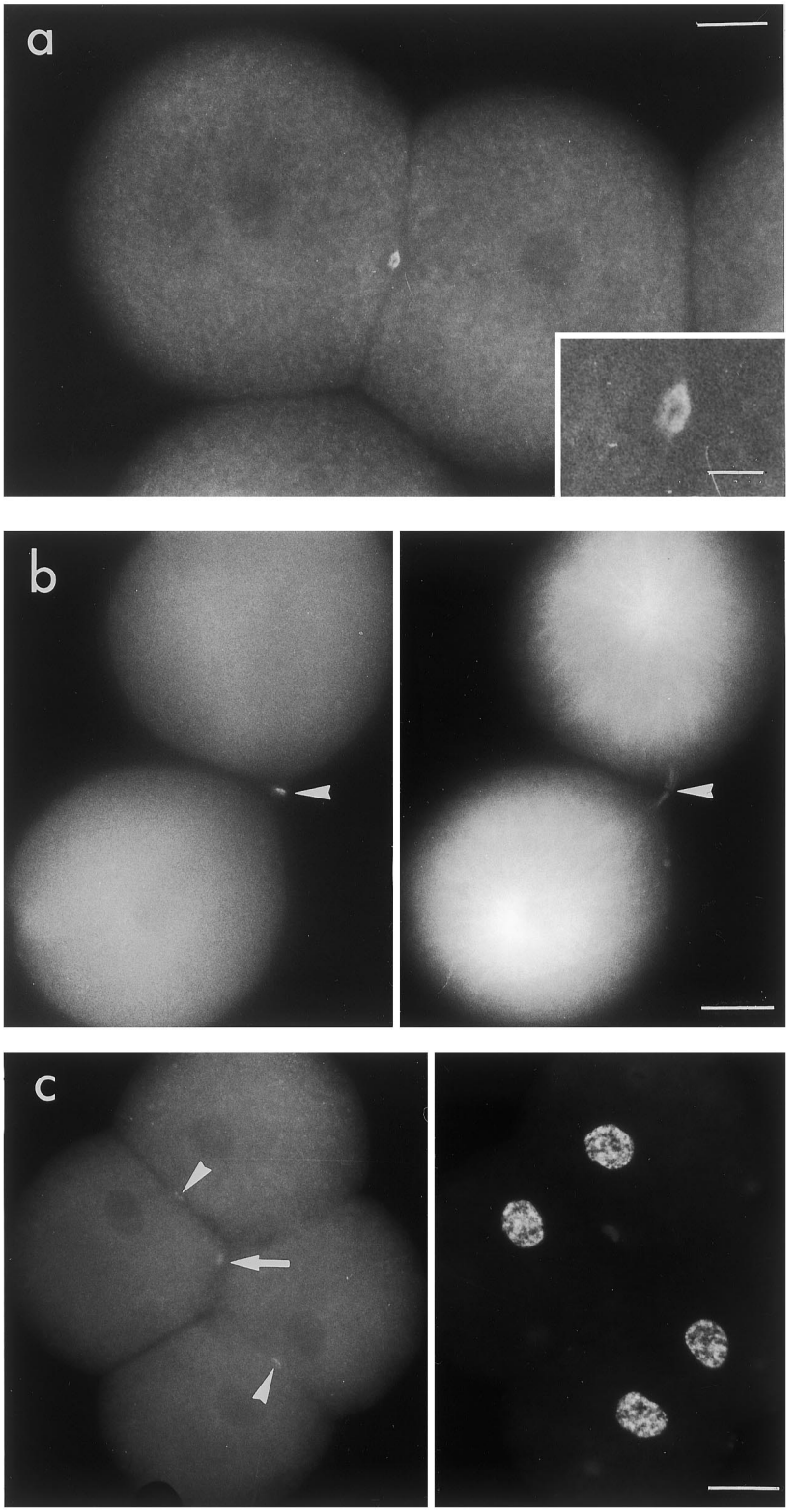

Fig. 4. URho1 ring structure at late cytokinesis. a: URho1 ring in an obliquely compressed embryo. Bar, 20 μm. Inset, the ring at a higher magnification. Bar, 5 μm. b: Two-cell embryo showing the intercellular bridge. The embryo was double-stained with the anti-URho antibodies (left) and anti-tubulin antibodies (right). Arrowheads, the midbody. Bar, 20 μm. c: Four-cell embryo. Left, staining with the anti-URho1 antibodies. Arrow, the URho1 ring formed during the first cell division. Arrowheads, the rings formed during the second cell division. Right, staining with DAPI. Bar, 20 μm.

treated the cell cortices with 0.6 M KI to dissolve the cortical layer [15], which weakens hydrophobic interaction, or 1% Triton X-100 to dissolve the membrane proteins (Fig. 2c). Most of the actin which was considered to be a marker protein of the cortical layer was solubilized by 0.6 M KI, but only a small amount of URho1 was solubilized (Fig. 2c, lane 3). In contrast, a major part of URho1 was solubilized by 1% Triton X-100 (Fig. 2c, lane 5), while actin was solubilized only to an extent similar to that of the F-buffer treatment. These results suggest that the URho1 in the cortex binds to the phospholipid bilayer of the cell membrane, probably through an isoprenyl moiety at the C-terminus [23].

3.3. Immunofluorescent localization of URho1 during cytokinesis

Eggs of *P. depressus* or *H. pulcherrimus* were fixed and stained with the anti-URho1 antibodies, rhodamine-phalloidin and DAPI. It seemed that URho1 was evenly distributed in the *P. depressus* eggs until the end of anaphase. At the initiation of telophase when actin filaments became concentrated in the equatorial cortex (Fig. 3b), URho1 still did not show particular localization (Fig. 3a). URho1 became concentrated in the cleavage furrow when it was apparent (Fig. 3d), and formed a tight ring structure as the furrow deepened (Fig. 3g). The same behavior of URho1 was observed in *H. pulcherrimus* eggs. Fig. 4a shows the URho1 ring structure of an *H. pulcherrimus* egg in a stage a little later than in Fig. 3g. This egg had been compressed obliquely by the coverslip. In order to examine the ring structure of URho1 during the last stage of cytokinesis, *H. pulcherrimus* eggs were double-stained with the anti-URho1 antibodies and the anti-tubulin antibodies (Fig. 4b). The ring structure of URho1 was localized in the middle of the intercellular bridge between the two blastomeres, the region known as the midbody, where tubulin staining is not seen (arrowheads). Interestingly, the URho1 ring formed during the first cell division remained throughout the second cell cycle (Fig. 4c, arrow). On the other hand, no distinct structure was stained with normal rabbit IgG (Fig. 3f).

In this study, we could observe the sequential appearance of actin and URho1 forming the ring structures in the cleavage furrow. This may suggest that URho1 is accumulated in the cleavage furrow after the formation of the actin contractile ring. We have shown that the microinjection of C3 exoenzyme into dividing sea urchin eggs disrupts the contractile rings [6], indicating that Rho somehow stabilizes the contractile ring structure. The present observation may support this idea; actin filaments may accumulate first in the equatorial cell cortex, and then they may be stabilized by URho1 to form the functional contractile ring. In the previous study, it had also been shown that C3 injected before accumulation of the actin filaments in the equatorial cortex interferes with contractile ring formation. This may imply that the actin filaments once accumulated in the equatorial cortex would be dispersed again caused by the loss of function of URho1 by C3.

The second possibility is that URho1 is already concentrated in the equatorial cortex at an early stage of cytokinesis along with the actin filaments or even prior to the appearance of the actin filaments, but not enough to be detected by immunofluorescence microscopy because URho1 is a minor component of the egg. The concentrated URho1 may play an important role in the assembly of the contractile actin ring. In order to assemble much actin into the tight contractile ring as the furrowing progresses [16], much URho1 may be necessary. Therefore, URho1 may have been able to be recognized by immunofluorescence microscopy when the contractile ring structure is established.

We showed that URho1 binds directly to the cell membrane and that the amount of membrane-bound URho1 does not change during division. Thus, URho1 may accumulate in the cleavage furrow by a movement of the cell membrane. It has previously been reported that surface lectin binding proteins are concentrated in the cleavage furrow [16,24–27] and distribution of these proteins coincided with that of actin filaments in the contractile ring [16]. These proteins are considered to be transferred to the equatorial region by a movement of the cell membrane [25]. It is possible that URho1 is also transported to the equatorial region by the membrane movement.

URho1 accumulated in the equatorial region would be in the active form, that is the GTP-bound form, since several proteins that bind the GTP-Rho have recently been shown to localize to the cleavage furrow. These proteins, including p140mDia and citron kinase, are involved in the regulation of cytokinesis in mammalian cells [28,29]. Therefore, URho1 would become active by an upstream signal before or during cytokinesis, bind such target proteins and mediate a signal to downstream to organize the contractile ring. However, further studies are necessary to elucidate the mechanism by which URho1 facilitates the cytokinesis.

It is noteworthy that URho1 is concentrated in the midbody during late cytokinesis. It has been reported that citron kinase is also concentrated in the midbody [29]. The role of the midbody has been unclear, but the fact that both Rho and the Rho-activated protein kinase are concentrated in this region would suggest a contribution of a Rho-mediated signaling pathway to a function of the midbody.

Acknowledgements: We are grateful to Dr. Koji Akasaka for the gift of a sea urchin blastula cDNA library and Dr. S. Narumiya for GST-fusion GTPases. We also thank A. Morimatsu for help in the preparation of antibodies.

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