Piezotolerance of the cytoskeletal structure in cultured deep-sea fish cells using DNA transfection and protein introduction techniques

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Abstract We used DNA transfection and protein introduction techniques to investigate the pressure tolerance of cytoskeletal structures in pectoral fin cells derived from the deep-sea fish *Simenchelys parasiticus* (habitat depth, 366–2,630 m). The deep-sea fish cells have G418 resistance. The cell number increased until day 6 of cultivation and all cells had died by day 35 when cultured in 35-mm Petri dishes in medium containing G418. Enhanced yellow fluorescent protein-tagged human β -actin (EYFP-actin) was stably expressed by 1 in 100,000 deep-sea fish cells. Because almost none of the EYFP-actin was incorporated into actin filaments of the cells, we replaced the relatively large EYFP tag with a chemical fluorescent compound and succeeded in incorporating fluorescently labeled rabbit actins into the deep-sea fish actin filaments. Most of the filament structure in the cells with rabbit actin inserted underwent depolymerization when subjected to pressure of 100 MPa for 20 min, in contrast to control cells. There were no differences in the tubulin filament structure between control cells and deep-sea fish cells with fluorescein-labeled bovine tubulin

inserted after the application of pressure ranging from 40 to 100 MPa for 20 min.

Keywords Deep-sea fish · Hydrostatic pressure · Actin · Tubulin · DNA transfection · Protein introduction · Cell culture

Introduction

The deep sea represents a large portion of the total volume of the oceans and this extreme environment is characterized by the absence of light, low temperature, and high hydrostatic pressure. After the advent of deep-sea vessels opened the door to research on deep-sea organisms, many deep-sea microorganisms have been isolated and characterized (Horikoshi and Tsujii 1999). Although increasing attention has been paid to deep-sea animals, no group has succeeded in culturing them. There are three major obstacles in molecular and cellular biological studies of deep-sea multicellular organisms under atmospheric pressure. The first is the development of devices for capturing and maintaining them, because most succumb as a result of decompression and exposure to the high-temperature surface seawater (Koyama and Aizawa 2000; Koyama et al. 2002, 2005b). The second is acclimation of the captured organisms to atmospheric conditions. The third is cells from deep-sea animals and the cryopreservation of these cells under atmospheric conditions.

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In our previous studies, we solved those problems by developing a novel piezostat aquarium system to capture and maintain deep-sea organisms (Koyama et al. 2002, 2003a, 2003b, 2005b). Using this system, the deep-sea fish *Simenchelys parasiticus* (habitat depth, 366–2,630 m; Nakabo 2000) captured from an ocean depth of 1,162 m survived under atmospheric pressure for 5 days after gradual, slow decompression (Koyama et al. 2003a, 2003b). Furthermore, we succeeded in the cultivation and freeze-storage of pectoral fin cells from the living deep-sea fish *S. parasiticus* in salt-enriched L-15 medium supplemented with 20% (v/v) fetal bovine serum (FBS) at atmospheric pressure (Koyama et al. 2003a, 2003b, 2005a).

S. parasiticus-derived pectoral fin cells have greater pressure tolerance than conger eel and mouse 3T3-L1 cells (Koyama et al. 2005a). Although no mouse and conger eel cells remained alive after being subjected to pressures of 100 and 130 MPa (1 bar = 0.1 MPa) for 20 min, all of the deep-sea fish cells remained alive after being subjected to pressure of 150 MPa (Koyama et al. 2005a). Surface-dwelling organism-derived cells including human, mouse, and conger eel cells undergo morphologic changes accompanied by cytoskeletal depolymerization under hydrostatic pressure of greater than 30 MPa for 20 min (Bourns et al. 1988; Crenshaw et al. 1996; Koyama et al. 2001, 2005a). In contrast, deep-sea fish cells undergo reversible actin and tubulin depolymerization when subjected to hydrostatic pressure of 100 MPa (Koyama et al. 2005a). It is not known whether the binding force between deep-sea cytoskeletal proteins contributes to the pressure resistance of the cytoskeletal structure of deep-sea fish cells. Actin and tubulin amino acid sequences are extraordinarily well conserved among eukaryotes (Alberts et al. 2002). We used DNA transfection and protein introduction techniques to investigate the pressure tolerance of the cytoskeletal structure of deep-sea fish cells into which terrestrial animal-derived actin and tubulin were inserted.

Materials and methods

Materials

Alexa Fluor 594 phalloidin, Alexa Fluor 488 phalloidin, actin from rabbit muscle-Alexa Fluor 594

conjugate, Alexa Fluor 594 goat anti-mouse IgG (H + L), and Alexa Fluor 488 rabbit anti-mouse IgG (H + L) were purchased from Molecular Probes Inc. (Eugene, Oregon, USA). Fluorescein-labeled bovine brain tubulin was from Cytoskeleton (Denver, CO, USA). Anti-tubulin (Ab-4 cocktail, clone DM1A + DM1B) was from Neo Markers (Fremont, CA, USA). Block Ace powder was from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Vectashield mounting medium was obtained from Vector Laboratories (Burlingame, CA, USA). Ten percent formalin neutral buffer solution (pH 7.4) and phosphate-buffered saline (PBS) powder were purchased from Wako Pure Chemical Industries (Osaka, Japan). Triton X-100 was from Sigma Chemical (T-9284, St. Louis, MO, USA). Lab-Tek II chamber slides with covers (RS sterile glass slides) and 25-cm² culture flasks were obtained from Nalgene Nunc International (Rochester, NY, USA). Petri dishes (35 mm) were from Becton Dickinson Labware (Falcon 3001 tissue culture dish, Franklin Lakes, NJ, USA). L-15 medium, a mixture of penicillin (5,000 IU/mL) and streptomycin (5,000 µg/mL), and fetal bovine serum (FBS) were obtained from MP Biomedicals (Aurora, OH, USA). Plasmid DNA enhanced yellow fluorescent protein (pEYFP)-actin and plasmid green fluorescent protein (pAcGFP1)-tubulin were from Clontech (Mountain View, CA, USA). Lipofectamine 2000, Plus reagent, opti-MEM I reduced-serum medium, and the elongation inhibitor of ribosomes G418 (Bar-Num et al. 1983) were purchased from Invitrogen (Carlsbad, CA, USA). The lipofection reagent Chariot was from Active Motif (Carlsbad, CA, USA).

Cell culture

The procedures for cell culture of pectoral fin tissues from the deep-sea fish *S. parasiticus* were described previously (Koyama et al. 2003a, 2003b, 2005a). Deep-sea fish cells were plated at a density of $4\text{--}7 \times 10^3$ cells/cm² on Lab-Tek II chamber slides or in 25-cm² culture flasks in L-15 medium containing excess NaCl 4 g/L (final concentration of 12 g/L), 1% antibiotic solution consisting of penicillin and streptomycin, and 20% (v/v) FBS. The salt-enriched L-15 medium was adjusted to pH 7.3. The deep-sea fish cells were grown at 15 °C in a humidified atmosphere.

DNA transfection and protein introduction into deep-sea fish cells

Insertion of pEYFP-actin or pAcGFP1-tubulin into deep-sea fish cells was performed using Lipofectamine 2000 and Plus reagent according to the manufacturer's instructions. To obtain stable expression in the transformed deep-sea fish cells, they were cultured in L-15 medium containing excess NaCl 4 g/L, 1% antibiotic solution consisting of penicillin and streptomycin, 20% (v/v) FBS, and G418 1–4 mg/mL.

Insertion of Alexa Fluor 594-conjugated rabbit muscle actin or fluorescein-labeled bovine brain tubulin into the deep-sea fish cells was performed using the lipofection reagent Chariot according to the manufacturer's instruction. The amount of actin and tubulin proteins inserted was set at 1 µg protein/ 6×10^4 cells. Transfection efficiency was calculated by counting the percentage of fluorescent cells observed under a confocal laser scanning microscope system (Fluoview FV500 system, Olympus, Tokyo, Japan). More than 100 cells were counted in each experiment.

Application of hydrostatic pressure to deep-sea fish cells

Cultured deep-sea fish cells at a density of $4\text{--}7 \times 10^3$ cells/cm² on Lab-Tek II chamber slides were inserted into flat-bottomed glass tubes with an inside diameter of 26 mm and height of 118 mm. The cells on the chamber slides were then immersed in serum-free salt-enriched fresh L-15 medium containing the same concentrations of penicillin and streptomycin. After the air bubbles had been removed, the glass tubes were tightly sealed with parafilm (American National Can, Greenwich, CT, USA). The medium volume in the glass tube was 64 mL. The sealed glass tubes were placed in a stainless steel pressurization vessel (Teramecs, Kyoto, Japan) connected to a hydrostatic pressure apparatus (TP200L, Teramecs). Compression and decompression rates were about 3–5 MPa/s and between about –20 and –40 MPa/s, respectively. The temperature was maintained at 15 °C with a water bath during pressurization. We confirmed that there were no changes in the pH of the culture medium prior to or following exposure to high hydrostatic pressure. The dissolved oxygen concentration in each medium was

measured with a portable dissolved oxygen sensor (DO-21P, Toa Electronics, Tokyo, Japan) before and after pressurization, and little or no change was detected.

Actin and tubulin staining

Cells on the chamber slides were lightly rinsed with PBS and fixed in 10% formalin neutral buffer solution for 15 min. After fixation, the cells were washed three times in PBS for 3 min each using a slide washer (SW-4, Juuji Field Co. Ltd., Tokyo, Japan). The cells were then placed in 0.1% (v/v) Triton X-100 in PBS and incubated for 5 min at room temperature. Then the cells were placed in PBS and incubated for another 15 min at room temperature. The cells were further incubated in 10 g/L of Block Ace in distilled water for 60 min and washed three times with PBS for 3 min each. For tubulin staining, the cells were reacted with anti-tubulin antibody (Ab-4 cocktail solution) for 60 min at 37 °C and washed three times with PBS for 3 min each. The cells were further reacted with a 1:200 dilution of Alexa Fluor 488 rabbit anti-mouse IgG or Alexa Fluor 594 goat anti-mouse IgG for 30 min at 37 °C and then washed three times with PBS for 3 min each. For actin staining, the cells were reacted with a 1:20 dilution of Alexa Fluor 594 phalloidin or Alexa Fluor 488 phalloidin for 20 min at room temperature and then washed three times with PBS for 3 min each. After these procedures, the cells on chamber slides were mounted on Vectashield mounting medium and observed under a confocal laser scanning microscope system (Fluoview FV500 system, Olympus).

Results

Resistance of deep-sea fish cells to G418 under atmospheric pressure

Twenty-four hours after plasmid DNA, composed of human β -actin with EYFP-actin genes, was introduced into the cells with a commercial lipofection reagent, intracellular EYFP-actin fluorescence was observed with a confocal laser scanning microscope (Fig. 1). Figure 2 shows the intracellular distribution of EYFP-actin in the deep-sea fish cells 24 h after

Fig. 1 Schematic illustration of the pEYFP-actin transfected deep-sea fish cell. In this report, we don't confirm introduction of the plasmid DNA into chromosomal DNA

Fig. 2 Intracellular distribution of EYFP-actin in deep-sea fish cells 24 h after transfection. Transfection efficiency of plasmid DNA into the cells was 96% when they continually grew with passage at the logarithmic growth phase. When the cells reached 100% confluence, transfection efficiency decreased to 76% even if passaged after trypsinization

transfection. The transfection efficiency of the plasmid DNA was 96% when the cells grew continually with passage at the logarithmic growth phase. When the cells reached confluence, transfection efficiency decreased to 76% even when passaged with trypsinization.

Fig. 3 Inhibitory effects of G418 on deep-sea fish cell growth. G418 4 mg/mL (circles) and G418 1 mg/mL (squares) in salt-enriched L-15 medium containing 20% FBS and 1% antibiotics. Values are the mean $\pm$ S.D. of eight independent experiments (* $p < 0.05$ compared with day 0, Student's t -test). The deep-sea fish cell density was examined in random 1-mm² areas

Figure 3 shows the effects of G418 on cell growth. In medium containing 1 mg/mL of G418, the number of deep-sea fish cells in 35-mm Petri dishes increased until day 6 of cultivation and then decreased, and none remained alive on day 35 (Fig. 3). When the cells were cultured in medium containing G418 4 mg/mL, all cells died by day 14 (Fig. 3). Table 1 shows the results of treatment with G418 after the

Table 1 Effects of G418 on deep-sea fish cells

	Plasmid DNA inserted	G418 in medium (mg/mL)			
		1	2	3	4
Day by which all 2×10^4 cells were dead	No	Day 35	Day 25	Day 21	Day 14
Adhesion to culture basement after passage	Yes	++	+	–	–
Proliferation after passage	Yes	++	–	–	–

++, Always; +, rarely; –, seldom or never

deep-sea fish cells had been transfected with the EYFP-actin plasmid DNA. Cell adhesion to the culture dish was inhibited by the addition of G418 at a dose of 2 mg/mL or greater to the medium. Figure 4a shows stable EYFP-actin expression in the deep-sea fish cell line. After culture with G418 4 mg/mL for 14 days and then replacement with G418 1 mg/mL containing medium, where red and green fluorescence indicate actin filaments and EYFP-actin, respectively. The ratio of cells stably expressing EYFP-actin was approximately 1 in 100,000. Although we attempted to incorporate EYFP-actin protein with actin filaments in the cells, most of the proteins were not inserted into the filaments (Fig. 4a). EYFP-actin was integrated into the actin filaments of approximately 1 in 30,000 cells with transient expression (Fig. 4b). Most AcGFP1-tagged tubulin was not incorporated into the tubulin filaments in the deep-sea fish cells (data not shown). Therefore, we

replaced the relatively large (27-kDa) EYFP tag with a chemical fluorescent compound and reinvestigated whether the fluorescent compound-labeled actin and tubulin proteins were incorporated into the cytoskeletal structures of the cells.

Piezotolerance of actin and tubulin filaments in deep-sea fish cells

Alexa Fluor 594-conjugated rabbit muscle actins were directly introduced into the deep-sea fish cells using the lipofection reagent. After 1 day of incubation, the cells were subjected to pressure of 40–100 MPa for 20 min. Figure 5 shows the piezotolerance of actin filaments in the cells, where red and green fluorescence indicate rabbit actin and deep-sea fish actin filaments, respectively. Yellow fluorescence was observed in some actin filaments in the control

Fig. 4 EYFP-actin expression in deep-sea fish cells. Red and green fluorescence indicate actin filaments and EYFP-actin, respectively, in the deep-sea fish cells. **(a)** Stable expression of EYFP-actin in the cells. Stable EYFP-actin expression was seen in cells treated with 4 mg/mL of G418 for 14 days and then further cultured in medium containing 1 mg/mL of G418.

Approximately 1 in 100,000 deep-sea fish cells stably expressed EYFP-actin. **(b)** EYFP-actin inserted into actin filaments in the cells. Approximately 1 in 30,000 cells exhibiting transient expression incorporated EYFP-actin into the actin filaments.

Fig. 5 Piezotolerance of actin filaments in deep-sea fish cells. Red and green fluorescence indicates Alexa Fluor 594-conjugated rabbit muscle actin and deep-sea fish actin filaments, respectively, in deep-sea fish cells. (a) Rabbit actin

was inserted into deep-sea fish cells under atmospheric conditions and (b) after being subjected to pressure of 100 MPa for 20 min. (c) Deep-sea fish cells after being subjected to pressure of 100 MPa for 20 min

cells (Fig. 5). The results in Fig. 5 demonstrate that the fluorescent compound-labeled rabbit actin was incorporated into the deep-sea fish actin filaments. The transfection efficiency of rabbit actin into the cells was 96% when they grew continually with passage during the logarithmic growth phase.

Because the pressure sensitivity of the cytoskeletal structure can be altered by the intracellular distribution of cytoskeletal proteins that depend on cell cycle and cell motility, we compared the best-preserved actin filament structure of control cells with that of cells into which rabbit actin was inserted after 20-min application of hydrostatic pressure of 40–100 MPa. At 100 MPa, most of the filament structure in the cells into which rabbit actin was inserted depolymerized, in contrast to control cells (Fig. 5). The results in Fig. 5 indicate that weak binding between actin proteins causes depolymerization of filaments in cells with rabbit actin inserted after the application of hydrostatic pressure of 100 MPa for 20 min.

There were no differences in the tubulin filament structure between fluorescein-labeled bovine tubulin inserted cells and control cells, even after being subjected to pressure of 40, 60, 80, and 100 MPa for 20 min (data not shown).

Discussion

High hydrostatic pressure of 30–60 MPa for 20 min induces reversible morphologic changes accompanied by cytoskeletal depolymerization in mammalian tissue cells (Landau 1960, 1961; Bourns et al. 1988; Crenshaw et al. 1996; Wilson Jr. et al. 2001; Koyama

et al. 2005a). In contrast, deep-sea fish cells undergo reversible actin and tubulin depolymerization when subjected to hydrostatic pressure of 100 MPa for 20 min (Koyama et al. 2005a). After the pressure is released, mammalian and deep-sea fish cells respread and progress normally through the cell cycle (Landau, 1960, 1961; Koyama et al. 2005a). Increased hydrostatic pressure shifts the self-assembly equilibrium of cytoskeletal proteins toward depolymerization and promotes the disassembly of actin (Swezey and Somero 1985; Morita 2003), myosin II (Davis 1981, 1985), and tubulin (Salmon 1975). The cytoskeletons of surface-dwelling organisms are much more pressure sensitive than those of deep-sea organisms because polymerization of their cytoskeletal proteins in vitro entails large increases in volume (~ 100 mL/mol). Among deep-sea species, the association constants for the polymerization of actin from *Coryphaenoides armatus* and *Coryphaenoides yaquinae*, rattail fishes occurring at depths of 2,700–5,000 m and 4,000–6,400 m, respectively, are relatively unaffected by pressure, with a small volume change associated with polymerization of less than 20 mL/mol (Swezey and Somero 1985; Morita 2003). Actin is extraordinarily well conserved among eukaryotes. The amino acid sequences of actins from different species are usually about 90% identical (Alberts et al. 2002). Based on the results of amino acid sequence analysis of actin species, only three substitutions, V54A or L67P, Q137K, and A155S, distinguish abyssal and nonabyssal actins of *Coryphaenoides* sp. (Morita 2003).

We used DNA transfection and protein introduction techniques to examine the pressure tolerance of

cytoskeletal structures in deep-sea fish cells, which induced and expressed terrestrial animal-derived actin proteins. Most of the actin filament structure depolymerized in the cells into which rabbit actin was inserted, in contrast to control cells. These results indicate that a few amino acid substitutions in key functional positions of the actin protein reduce the volume change associated with polymerization and thereby alter the pressure sensitivity of actin filaments in deep-sea fish cells. However, there were no differences in pressure sensitivity between control cells and deep-sea fish cells labeled with fluorescein into which bovine tubulin was inserted. The amount of tubulin proteins directly introduced into the deep-sea fish cells appeared to be small and therefore had little effect on the pressure sensitivity of tubulin filaments.

Most of the EYFP-actin proteins did not bind to the actin filaments in the deep-sea fish cells. One reason is that EYFP is a relatively large fluorescent tag of 27 kDa, compared with the actin protein of 42 kDa. The large EYFP tag interferes with the biological function and structural dynamics of the actin molecule. In addition, modern chemical fluorophores are usually brighter than EYFP because they have higher extinction coefficients and better quantum yields. Therefore, we directly transfected Alexa Fluor 594-conjugated rabbit actin into the deep-sea fish cells.

An additional discovery of the present study is that the deep-sea fish cells can tolerate for a time G418. Cell number increased until day 6 of cultivation in medium containing 1 mg/mL of G418. By day 35 of cultivation, all cells had died. G418 exhibits a wide spectrum of activity and inhibits protein synthesis in bacteria, yeast, fungi, algae, and plant and animal cells (Davies and Jimenez 1980). G418 blocks polypeptide synthesis in eukaryotic cells by binding irreversibly to 80S ribosomes and therefore disrupting their proofreading ability (Bar-Num et al. 1983). Because G418 had little effect on the deep-sea fish cells, the ribosomal structure of the deep-sea fish *S. parasiticus* may differ from that of typical eukaryotic cells.

In a future study, we will replace a few amino acids in key functional positions of the mammalian actin protein and investigate the differences in pressure tolerance between control and amino acid-substituted mammalian cells.

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