Comparative study of the impact of the actin cytoskeleton on cell shape and membrane surface in mammalian cells in response to actin toxins

Francisco Lázaro-Diéquez and Gustavo Egea*

Dept. de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, and Instituts de Nanociències i Nanotecnologia (IN²UB) and d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, 08036 Barcelona (Spain).

We performed a correlative epifluorescence and scanning electron microscopy study of the morphological alterations, both in the actin cytoskeleton organization and in the cellular shape and surface morphology of HeLa, NRK and Vero cells, caused by a variety of actin toxins. To this end, we used actin toxins that depolymerize (cytochalasin D, latrunculin B, and Clostridium botulinum C2 toxin) or stabilize (jasplakinolide) filamentous actin. By immunofluorescence we observed that the resulting actin cytoskeleton alterations were cell type- and toxin-dependent. Analysis of the cell shape and membrane surface by scanning electron microscopy also showed that the actin disruption produced variable changes depending on the cell type and the actin toxin used. Therefore, our results indicate that actin directly participates both in the maintenance of cell shape and in membrane surface morphology, revealing significant differences depending on the actin toxin and cell type involved.

Keywords: Actin, cytoskeleton, actin toxins, cell surface, scanning electron microscopy

1. Introduction

The mechanical properties of the cytoskeleton (actin, microtubules and intermediate filaments), as well as its organization, largely determine the morphology and machinery of animal cells [1]. The most direct evidence that the cytoskeleton is necessary for the establishment and maintenance of cell morphology stems from the utilization of various pharmacological agents and natural toxins. This is particularly true for actin filaments. Thus, it has long been known that the use of cytochalasin B induces the reversible loss of cell shape in mouse salivary gland epithelial cells, with the consequent abrogation of gland morphogenesis [2]. Another pioneering example involved the same toxin in cultured neurons, wherein it halted axon elongation as growth cones rounded up [3].

Numerous new actin-disrupting agents or actin toxins have been reported in recent years. [4,5,6]. Moreover, they have been used extensively in a variety of studies examining the potential involvement of either the actin cytoskeleton organization (see below) or actin dynamics (depolymerization/polymerization cycle), or both, in various biological functions, including the formation of filopodia and lamellipodia in cell movement [7], membrane trafficking [8,9,10], podosome and invadopodium formation [11,12,13], cell adhesion [14,15,16], pathogen infection [17,18], neurite extension, and spine formation [19,20,21,22,23].

In animal cells there are several organizational levels of actin filaments or microfilaments: (i) antiparallel arrays, which occur in stress fibres, and which are homologous to the myofibrillar organization seen in skeletal and cardiac muscles; (ii) parallel arrays, which form cell surface protrusive structures such as microspikes and filopodia; (iii) dendritic arrays, which form polarized and branched short actin filaments that give rise to plasma membrane extension(s) known as lamellipodium (a); and (iv) isotropic microfilament arrays localized beneath the plasma membrane and attached to

* Tel.: (+34)93-4021909. Fax: (+34)93-4021907. E-mail: gegea@ub.edu
transmembrane proteins via actin-binding proteins. The usual and most characteristic actin cytoskeleton organization observed in a steady state in animal cells is stress fibre organization (see Fig. 1), which can be easily observed by using phalloidin tagged to tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) [24].

![Fig. 1](image)

**Fig. 1.** Fluorescence (a-c) and scanning electron microscopy (SEM) (d-i) imagery of the actin stress fibre organization stained with TRITC-phalloidin (a-c), as well as of the cell shape and cell membrane surface (d-i) in HeLa (a, d, g), NRK (b, e, h), and Vero (c, f, i) cells. Scale bar, 10 μm (a-c), 5 μm (d-f, i) 2 μm (g,h).

Whereas cell shape was long thought to be determined by the (actin) cytoskeleton crossing the cytoplasm and undergoing translocation to the underside of the cellular membrane, this hypothesis has now been complemented not only by the recent discovery of BAR- or F-BAR/EFC-domain-containing membrane-deforming proteins, for example, WASP-WIP complex [25], but also by the contribution of endocytosis, exocytosis and cytokinesis processes.

The present study comprises a comparative examination of how actin cytoskeleton organization, as well as cell shape and the membrane surface, undergo alterations in different mammalian culture cell lines as a result of various natural toxins that depolymerize or stabilize the filamentous actin. The actin-depolymerizing toxins we used include cytochalasin D (CyD), latrunculin B (LtB), and botulinum C2 toxin (C2). Cytochalasins block the barbed-end of actin filaments, thereby preventing microfilament growth [26]. However, latrunculins and botulinum C2 toxin sequester [27,28] and ADP-rybosylates [29,30] G-actin monomers, respectively, giving rise to G-actin monomers that cannot be incorporated into the growing microfilament without altering the intrinsic depolymerization activity that occurs in the pointed-end of the filament [31]. Consequently, microfilaments depolymerize and the actin cytoskeleton

©FORMATEX 2007
collapses. At the same time, we have used the actin-stabilizing toxin jasplakinolide (Jpk), which binds to the filamentous actin competing with phalloidin for the same location [32].

2. Procedures

2.1. Cell cultures

NRK, HeLa, and Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM). This was supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and MEM sodium pyruvate (1 mM). Cell cultures were maintained at 37 °C in a humidified CO₂ (5%) atmosphere.

2.2. Actin toxin treatments

Cells were grown on 10 mm diameter glass coverslips at a density of 2 x 10⁶ cell/ml. CyD was used at 1 µM for 60 min, LtB at 500 nM for 45 min, and Jpk at 500 nM for 45 min; all were diluted in supplemented DMEM at 37 ºC. The binary botulinum C2 toxin contains two compounds: the membrane-binding subunit (C2II) and the ADP-ribosylation enzyme subunit (C2I). Thus, for C2 toxin experiments, cells were first rinsed twice with DMEM without FBS, and then incubated with 200 ng/ml of C2II plus 100 ng/ml of C2I diluted in DMEM with low FBS (0.5 %) at 37 °C for several hours. At the end of each actin toxin treatment, cells were quickly rinsed, fixed, and stained for filamentous actin as described above.

2.3. TRITC-phalloidin staining

Visualization of the actin cytoskeleton was performed using TRITC-phalloidin. Control and toxin-treated cells (see above) were quickly rinsed in warm phosphate buffer saline (PBS; 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4) and fixed with 4 % paraformaldehyde in PBS for 15 min at room temperature. Thereafter, cells were washed with PBS and permeabilized for 10 min at room temperature with 0.1 % saponin in PBS containing 1 % BSA. Cells were incubated for 20 min at room temperature with 10 µg/ml TRITC-phalloidin in PBS containing 1 % BSA, extensively washed with PBS, and mounted in Mowiol. Cells were observed with a BX60 Olympus epifluorescence microscope equipped with an Orca-ER cooled CCD Hamamatsu camera.

2.4. Scanning electron microscopy

Control and actin toxin-treated NRK, HeLa, or Vero cells were quickly rinsed in cacodylate buffer (0.1 M, pH 7.4) and fixed with 2.5 % glutaraldehyde in cacodylate buffer for 60 minutes at room temperature. Cells were then thoroughly washed with cacodylate buffer and incubated for 5 min with tannic acid (1 %) in cacodylate buffer. Subsequently, cells were dehydrated in a graded series of ethanol, critical point dried with a Polaron CPD 7501 system, mounted, and coated with gold in a Bio-Rad SC510 sputter coater. All samples were observed under the same kilovolt and electron beam current conditions using an Hitachi S-2300 scanning electron microscope.

3. Results

HeLa, NRK, and Vero cells are three well-known cultured mammalian cells widely used to explore the potential contribution of both the actin cytoskeleton organization and actin dynamics in cellular functions; e.g., in our particular case, vis-a-vis membrane trafficking and organelle architecture [8]. Therefore, we first examined alterations in actin cytoskeleton organization and cell shape change using a
panel of actin toxins. To this end, we combined fluorescence and scanning electron microscopy techniques. Control HeLa, NRK, and Vero cells stained with TRITC-phalloidin showed a high density of actin stress fibres (Fig. 1, a-c), although these were thicker and more densely packed in NRK and Vero cells (Fig. 1, b and c, respectively). In the case of HeLa cells, the density and organization of stress fibres depended on the source of the cell line and on the passing number (not shown). In contrast, NRK and Vero cells were much more consistent in this respect.

We then examined cell shape and membrane surface morphology utilizing a scanning electron microscope (SEM) (Fig. 1, d-i). While the cell shape of the three cell lines was very similar (Fig. 1, d-f), cell surface morphology displayed significant differences (Fig. 1, g-i). For example, HeLa cells showed numerous long and thin filopodia-like structures, which accumulated to a significant degree in the membrane surface located just above the nucleus (Fig. 1, g). The membrane surface of NRK cells also exhibited extensions of the plasma membrane, although these were significantly shorter and more evenly distributed throughout the cell surface (Fig. 1, h). Conversely, Vero cells showed a smooth cell surface, in which the prominence of nucleus and nucleoli were easily observable (Fig. 1, f and i). Moreover, SEM also revealed that Vero cells were flatter than NRK and HeLa cells (compare panels d, e and f in Fig. 1), which correlated with recorded levels of actin stress fibre organization (Fig. 1, a, b, and c, respectively).

Thereafter, HeLa, NRK and Vero cells were treated with actin-depolymerising agents. Cytochalasin D (CyD)-treated cells (Fig. 2A) typically already exhibited dissolution of actin stress fibres after 10 min of treatment, proving even more severe over longer time periods (60 min). After 10 min of treatment, both NRK and Vero cells still contained some stress fibres but HeLa cells did not (for a comparison, see Fig. 2A; a, e and i). After 60 min, stress fibres were no longer visible, although numerous small and highly fluorescent cytoplasmic structures could be seen (Fig. 2A; b, f and j). Unlike NRK and Vero cells, the shape of HeLa cells remained relatively intact, with numerous small and variable sized globular structures or blebs appearing at the cell surface (Fig. 2A; c and d). Such a structure represents, in fact, a ballooning out of the plasma membrane when it detaches from the actin cortex [33]. Their appearance occurred concomitantly with the disappearance of short and long filopodia-like structures characteristic of untreated cells (Fig. 1; d and g). NRK cells showed blebs at the cell surface after 60 min of CyD treatment (Fig. 2A; h), although they were much smaller than those seen in HeLa cells (Fig. 2A; c, d). Strikingly, Vero cells displayed no particularly remarkable structures at the cell surface (Fig. 2A; k, l). Moreover, the cellular body retracted these leading thin and longer extensions as the toxin treatment was extended (compare at 10 and at 60 min), with Vero cells proving the more affected than NRK cells (Fig. 2A; k, l, and g, h, respectively).

Unlike the CyD treatment, the use of LtB or C2 toxin resulted in significant differences between NRK and Vero cells (Fig. 2B). Compared with the CyD treatment, LtB was more potent, since a similar depolymerized actin cytoskeleton organization, cell shape, and membrane surface alterations were already evident after 30 min, whereas CyD required 60 min (compare Fig. 2A; f and j with Fig. 2B; m and q). Strikingly, Vero cells showed several small lamellipodia (arrows in Fig. 2B; q and r, respectively). The formation of lamellipodia was practically absent in HeLa (not shown) and NRK cells. The depolymerization of the actin cytoskeleton by the C2 toxin required much longer time periods (2-4 h, depending on the toxin concentration used and the batch) (Fig. 2B; o, p, s, t), since this toxin slowly internalizes to endosomes and subsequently translocates to the cytosol, where it ADP-ribosylates G-actin. Vero cells seem to be more sensitive than NRK cells to C2 toxin, as the latter still contained some actin stress fibre after 3 h of C2 internalization (compare o and s in Fig. 2B). Moreover, numerous C2 toxin-treated NRK cells displayed a long and large lamellipodium (arrow in Fig. 2B; o). In contrast, Vero cells collapsed with no stress fibres and exhibiting a general diffuse cytoplasmic fluorescent staining (Fig. 2B; s). Moreover, some thin and long extensions were easily recognizable in the SEM images (Fig. 2B; t). The body retraction in C2 toxin-treated Vero cells was extreme compared to that seen in NRK cells (compare panels p and t in Fig. 2B).
Fig. 2. (A) Actin cytoskeleton organization after staining with TRITC-phalloidin, and SEM images of HeLa, NRK and Vero cells treated with cytochalasin D (CyD) for 10 and 60 min. (B) The same procedure described in (A) though here NRK and Vero cells were treated with latrunculin B (LtB) for 25 min, or botulinum C2 toxin (C2) for 3 h. Scale bar, 10 µm (a,b,e,f,i,j,m,o,q,s), 5 µm (c,d,g,h,k,l,n,p,r,t).
Finally, we used jasplakinolide (Jpk) as an actin-stabilizing toxin (Fig. 3). Jpk-treated HeLa cells displayed a rapid dissolution of actin stress fibres after 30 min of toxin treatment (Fig. 3; a). After 60 min, the phalloidin staining was difficult to visualize due to the fact that Jpk occupied the same binding site as phalloidin in the actin filament (Fig. 3; b). HeLa cells maintained their cell shape relatively well; in addition, they displayed dorsal ruffles when viewed by SEM (Fig. 3; c and d, respectively), which can also be seen as strong fluorescent lines corresponding to the plasma membrane (Fig. 3; a). After short incubation periods with Jpk (30 min), both NRK and Vero cells still exhibited some stress fibres (Fig. 3; e and i, respectively), as well as changes in cell shape (Fig. 3; g and k, respectively). At longer incubation times (60 min), phalloidin staining was weak (Fig. 3; f, j) while cell shape was severely affected. Moreover, there were numerous globular membrane structures or blebs of variable size in both HeLa and NRK cells (Fig. 3; c, d, g, h), though none were ever noted in Vero cells (Fig. 3; k, l).

![Fig. 3](image_url)

**Fig. 3.** Actin cytoskeleton organization after staining with TRITC-phalloidin, and SEM images of HeLa, NRK, and Vero cells treated with jasplakinolide (Jpk) for 30 or 60 min. Scale bar, 10 µm (a,b,e,f,i,j), 5 µm (c,d,g,k,l), 2 µm (h).

### 4. Concluding remarks

The experiments presented in this chapter resulted in the following findings: (i) actin poisons have different effects both on actin cytoskeleton organization and on cellular shape and membrane surface morphology; (ii) the severity of the alterations caused by actin toxins depends on the cell type used. As reported here, some mammalian cell lines are more sensitive to these actin toxins than others. We recommend the use of at least two actin-depolymerizing toxins (CyD and LtB), as well as one actin-stabilizer (Jpk) when investigating the potential contribution of actin on a determined cellular function.
Acknowledgements. We thank the Serveis Científico-Tècnics of the University of Barcelona (campus Casanova) for help with SEM. This work was supported by grants from MEC (BMC2006-00867/BMC and CONSOLIDER CSD2006-00012) to G.E.

References


©FORMATEX 2007
Modern Research and Educational Topics in Microscopy.
A. Méndez-Vilas and J. Díaz (Eds.)


