Journal of Biomechanics ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Differential effects of LifeAct-GFP and actin-GFP on cell mechanics assessed using micropipette aspiration

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ARTICLE INFO

Article history:
Accepted 16 December 2015

Keywords:
Actin cytoskeleton
Micropipette aspiration
Cell mechanics
Bleb
Mesenchymal stem cell

ABSTRACT

The actin cytoskeleton forms a dynamic structure involved in many fundamental cellular processes including the control of cell morphology, migration and biomechanics. Recently LifeAct-GFP (green fluorescent protein) has been proposed for visualising actin structure and dynamics in live cells as an alternative to actin-GFP which has been shown to affect cell mechanics. Here we compare the two approaches in terms of their effect on cellular mechanical behaviour. Human mesenchymal stem cells (hMSCs) were analysed using micropipette aspiration and the effective cellular equilibrium and instantaneous moduli calculated using the standard linear solid model. We show that LifeAct-GFP provides clearer visualisation of F-actin organisation and dynamics. Furthermore, LifeAct-GFP does not alter effective cellular mechanical properties whereas actin-GFP expression causes an increase in the cell modulus. Interestingly, LifeAct-GFP expression did produce a small (~10%) increase in the percentage of cells exhibiting aspiration-induced membrane bleb formation, whilst actin-GFP expression reduced blebbing. Further studies examined the influence of LifeAct-GFP in other cell types, namely chondrogenically differentiated hMSCs and murine chondrocytes. LifeAct-GFP also had no effect on the moduli of these non-blebbing cells for which mechanical properties are largely dependent on the actin cortex. In conclusion we show that LifeAct-GFP enables clearer visualisation of actin organisation and dynamics without disruption of the biomechanical properties of either the whole cell or the actin cortex. Thus the study provides new evidence supporting the use of LifeAct-GFP rather than actin-GFP for live cell microscopy and the study of cellular mechanobiology.

1. Introduction

The actin cytoskeleton plays a key role in many cellular processes such as mechanotransduction (Janmey and Weitz, 2004), motility (Pollard and Cooper, 2009) and differentiation (Titushkin and Cho, 2011). The organisation and dynamic remodelling of cortical actin also influence the structure and biomechanics of cells (Tan et al., 2008; Yourek et al., 2007). The actin cortex is connected to the cell membrane via the family of ezrin, radixin and moesin (ERM) linker proteins (Charras et al., 2006). Mechanical rupture or physiological disassembly of these linker proteins or the underlying actin cytoskeleton results in membrane detachment from the cortex and the formation of a membrane bleb with important consequences for cell biomechanics and migration (Fackler and Grosse, 2008; Sliogeryte et al., 2014). Cell biomechanical properties are therefore associated with actin structure and dynamics and membrane bleb formation, and play a role in dictating the cellular response to the extracellular mechanical environment (Ingber, 2006; Zhelev et al., 1994).

Actin monomers exist in a globular G-actin form that polymerises into fibrous F-actin microfilaments. There is constant turnover between the two states, known as actin treadmilling, such that F-actin is able to form dynamic intracellular structures such as lamellipodia, filopodia, stress fibre bundles and cortical actin. With the increasing interest in understanding actin dynamics and its diverse roles within cell biology, the visualisation of actin in living cells has become an important and powerful technique. Live cell imaging of actin remodelling and dynamics has been widely reported through the transfection of cells with a plasmid expressing actin coupled to a fluorescent protein such as GFP (Endlich et al., 2007). This approach labels both F-and G-actin, which can be useful for assessing the relative dynamics (Engelke et al., 2010) but also reduces the signal to noise ratio when visualising F-actin structures (Lee et al., 2013). Importantly, studies have reported that actin-GFP expression directly influences actin dynamics during cell cytokinesis and migration (Aizawa et al.,

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http://dx.doi.org/10.1016/j.jbiomech.2015.12.034
0021-9290/

1997), cell-matrix adhesion (Feng et al., 2005), and mechanically induced cell deformation (Deibler et al., 2011; Pravincumar et al., 2012).

Alternatively, actin can be labelled through fusion of a fluorescent protein to the actin-binding domain of a known actin binding protein. Such tools include Utrophin (Burkel et al., 2007), F-tractin (Johnson and Schell, 2009) and LifeAct (Riedl et al., 2008), while more recently, a far-red small molecule probe incorporating silicone-rhodamine and an actin binding domain, SiR-actin, has been developed with potential applications in live cell super-resolution microscopy (Lukinavicius et al., 2014). While each of these probes is subject to some bias in cellular distribution when compared to phalloidin, LifeAct provides a balanced choice with good definition of actin structure and no observed side effects (Belin et al., 2014); and remains widely used. Riedl et al. (2008) first described the use of LifeAct tagged to a fluorescent protein as a means of labelling F-actin with reduced artefacts. LifeAct is a peptide consisting of 17-amino-acids comprising the actin-binding domain from yeast actin binding protein 140 (ABP140), which because of its small size and absence from mammalian cells, is ideal for binding F-actin with minimal disruption. Furthermore, no effects on cell migration or polarisation have been observed with its use (Riedl et al., 2008). However, little is known about how LifeAct influences actin dynamics and remodelling during cell deformation. The aim of this paper is to assess the effects of both LifeAct-GFP and actin-GFP on cellular mechanical properties and bleb formation assessed via micropipette aspiration.

2. Methods

2.1. Cell sources and culturing conditions

Human bone marrow derived mesenchymal stem cells (hMSCs) were purchased from a commercial source (STEMCELL Technologies, Cambridge, UK). For passage culture, cells were seeded at a density of 5×10^3 cells/cm^2 and cultured in media consisting of low glucose Dulbecco's Modified Eagle Media (DMEM; Gibco, Paisley, UK) with 10% foetal bovine serum (FBS), penicillin (100 U/mL)-streptomycin (100 μg/mL; all Sigma-Aldrich, Dorset, UK) and 1 ng/mL fibroblast growth factor-2 (FGF-2; PeproTech, London, UK) at 37 °C and 5% CO_2 until confluence of 70–80% was reached as previously described (Pattappa et al., 2013). Cells between passages 2 and 8 were used for experiments and were cultured in 24-well plates at an initial density of 5×10^3 cells/cm^2 for seven days before transfection.

For chondrogenic differentiation, hMSCs were cultured in medium consisting of high glucose DMEM, (1 ×) Insulin-Transferrin-Selenium-G supplement (both Gibco), penicillin (100 U/mL)-streptomycin (100 μg/mL), 1 mM sodium pyruvate, 1.5 mg/mL bovine serum albumin (BSA), 40 μg/mL L-proline, 4.7 μg/mL linoleic acid, 50 μg/mL L-ascorbic acid, 100 nM dexamethasone (all Sigma-Aldrich) and 10 ng/mL transforming growth factor-β3 (TGF-β3; PromoKine, Heidelberg, Germany) as described previously (Sliogeryte et al., 2014).

A conditionally immortalised wild-type mouse chondrocyte cell line was also used. In this case, cells were cultured in DMEM (Gibco) supplemented with 10% FBS, penicillin (100 U/mL)-streptomycin (100 μg/mL), and 2.5 mM L-glutamine (all Sigma-Aldrich). Immortalised cells were maintained under permissive conditions at 33 °C, 5% CO_2 in the presence of 10 nM interferon-γ (IFN-γ; R&D Systems, Abingdon, UK) (Thompson et al., 2014; Wann et al., 2012). Cells were then cultured under non-permissive conditions at 37 °C in the absence of IFN-γ for 3 days followed by seeding in 24 well plates 24 h before experiments.

For micropipette aspiration experiments, all cell types were detached with 0.25% Trypsin/EDTA (Sigma-Aldrich) for 3–5 min, pelleted and suspended in pre-warmed imaging medium consisting of low glucose DMEM (no Phenol Red; Gibco), penicillin (100 U/mL)-streptomycin (100 μg/mL), 10% FBS, 4 mM L-Glutamine and 25 mM HEPES (all Sigma-Aldrich). Following detachment, the cell suspension was incubated in a water bath for 10–15 min prior to micropipette aspiration.

2.2. LifeAct-GFP and actin-GFP transfections

For actin-GFP transfection, undifferentiated hMSCs were transfected with a plasmid driving expression of actin-GFP. Prior to transfection cells were cultured in antibiotic free media (low glucose DMEM with 10% FBS) for 30 min to 1 h. Plasmid transfection was performed using Lipofectamine LTX Plus (Invitrogen, Paisley, UK). For 2×10^4 cells, 0.5 μg of cDNA was used. Cells were cultured for 6 h in transfection media according to the manufacturer's instructions. Undifferentiated hMSCs, hMSCs differentiated toward the chondrogenic lineage and an immortalised chondrocyte cell line were transfected with an adeno-virus containing LifeAct-TagGFP2 (Ibidi, Martinsried, Germany) at a pre-optimised multiplicity of infection (MOI) according to the manufacturer's protocol. Two days prior to experimental observation, the reagent was directly added to the cells cultured in monolayer. The cells were incubated for two days at 37 °C, 5% CO_2. After incubation with either virus or plasmid the media was replaced. Cell viability remained high after introduction of either actin-GFP or LifeAct-GFP to cells. Control cells were cultured in parallel without subjection to transduction or transfection procedures. Prior to micropipette aspiration, cells were treated with trypsin and suspended in imaging media. For imaging of monolayer cells, both groups were seeded and transfected on coverslips.

2.3. Visualisation of actin structure in fixed cells

For visualisation of F-actin structure in cell monolayer, cells cultured on cover slips were transfected with LifeAct-GFP or actin-GFP, fixed in 4% paraformaldehyde (PFA) for 10 min, permeabilised for 5 min in 0.5% Triton X-100/phosphate buffered saline (PBS) and stained with Alexa Fluor 555-phalloidin (1:40; Invitrogen) at 25 μl/ml in PBS+0.1% bovine serum albumin (BSA; Sigma-Aldrich) for 20 min. Coverslips with cells were then washed in PBS and mounted with ProLong Gold (Invitrogen).

For visualisation of F-actin structure in rounded cells, the following procedure was performed. Transfected cells with LifeAct-GFP or actin-GFP were detached using trypsin, suspended in imaging media and fixed in 4% PFA for 10 min, followed by permeabilisation in 0.5% Triton X-100/PBS (Sigma-Aldrich) for 5 min prior to staining with Alexa Fluor 555-phalloidin (1:40; Invitrogen) in PBS+0.1% BSA for 20 min. Cells were then washed in PBS and suspended in distilled water. A drop of stained cells in suspension was placed on a coverslip and allowed to dry. Coverslips with cells were mounted using ProLong Gold and imaged using a laser scanning confocal microscope (Leica TCS SP2) with a ×40/1.25 NA oil immersion objective lens. The plane of focus was made to bisect the centre of individual cells.

2.4. Micropipette aspiration

The micropipette aspiration system controlled by a peristaltic pump (MCD standard, Ismatec, Cole-Parmer, London, UK) was used as previously described (Pravincumar et al., 2012). The pump was used to provide precise temporal control of aspiration pressure. Micropipettes were made from borosilicate glass capillary tubes (1.0 mm outer diameter and 0.58 mm inner diameter, Narishige, London, UK). The micropipettes were drawn with a programmable Flaming/Brown micropipette puller, (Model P-97, Sutter Instruments Co., Novato, CA, USA). To obtain an inner diameter of 7–8 μm, the micropipettes were fractured on a microforge (MF-900, Narishige) and coated with Sigmacote (Sigma-Aldrich) to prevent cell adhesion. Before starting an experiment, the reservoir, tubing and pump were filled with distilled water taking care to exclude all air bubbles. The micropipettes were filled with imaging media and mounted on a holder controlled by a micromanipulator (Patchman NP2, Eppendorf, Germany). The cell suspension at room temperature was placed in a chamber on the microscope and a tare pressure of 50 Pa was applied to attach an individual cell to the micropipette. The cell was then partially aspirated inside the micropipette by applying a step negative pressure of 0.76 kPa at a rate of 0.38 kPa/s. Brightfield and fluorescence images were captured every 2 s over 3 min using a confocal microscope (Leica, SP2) with a ×63/1.4 NA oil immersion objective lens. Cell elongation into the micropipette was measured from brightfield images using a Matlab routine. Micropipette aspiration was performed within 1 h following cell detachment from monolayer.

2.5. Estimation of viscoelastic properties

Viscoelastic parameters such as the equilibrium modulus, the instantaneous modulus and the viscosity of cells were estimated by fitting the theoretical standard linear solid (SLS) model to the obtained aspirated length versus time data using a Matlab routine as described in previous studies (Sato et al., 1990; Theret et al., 1988; Trickey et al., 2000). In this model the cell is assumed to be homogeneous and incompressible with a Poisson's ratio of 0.5. The model is presented as two parallel connected springs with elastic constants k_1 and k_2, and a dashpot with viscosity μ in series with spring k_2. Applying a negative pressure, the cell elongation into the micropipette is calculated as a function of time, as follows:

$$L(t) = \frac{\Phi(\eta) R_p \Delta p}{\pi E} \times \left[1 + \left(\frac{k_1}{k_1 + k_2} - 1\right) \exp\left(-\frac{t}{\tau}\right)\right] \quad (1)$$

where $L(t)$ is the aspirated length at time t, ΔP is applied pressure, R_p is the inner radius of the micropipette and $\Phi(\eta)$ is a wall function which in a wide range of experiments was assumed to be 2.0–2.1 (Theret et al., 1988). The cell viscosity can be estimated as follows:

$$\mu = \frac{\tau k_1 k_2}{k_1 + k_2} \quad (2)$$

Please cite this article as: Sliogeryte, K., et al., Differential effects of LifeAct-GFP and actin-GFP on cell mechanics assessed using micropipette aspiration. Journal of Biomechanics (2016), http://dx.doi.org/10.1016/j.jbiomech.2015.12.034