Comparing mechano-transduction in fibroblasts deficient of focal adhesion proteins

Tuli Dey, Melanie C. Mann, Wolfgang H. Goldmann*

Department of Physics, Friedrich-Alexander-University of Erlangen-Nuremberg, Erlangen, Germany

Abstract

Mechano-transduction was studied in wildtype and focal adhesion (FA) protein-deficient mouse embryonic fibroblasts (MEFs). Using a cell stretcher, we determined the effect of stretch on cell morphology, apoptosis, and phosphorylation of ERK1/2. After 20% cyclic, uniaxial stretch, FA-deficient MEFs showed morphological changes and levels of apoptosis of the order: focal adhesion kinase > p130Cas > vinculin compared to wildtype cells. ERK1/2 phosphorylation peaked in wildtype cells at around 10 min, and in all FA-deficient cells at around 5 min. The relative change in strain energy of FA-deficient cells compared to wildtype cells was of the order: vinculin > FAK > p130Cas. Taken together, FAK and p130Cas are more important in the stretch-mediated downstream signaling and cell survival pathway, while vinculin is more critical in maintaining cell contractility.

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1. Introduction

After adhesion to the extracellular matrix, cells form a highly complex cytoskeleton whose major components are actomyosin fibers connecting with focal adhesions (FAs). The highly regulated interplay of actin stress fibers and FAs essentially defines the mechanics of cells, and thus their motility, morphology, and function [1,2]. Focal adhesion proteins including vinculin, focal adhesion kinase (FAK), and Crk associated substrate (p130Cas) are components of the mechano-chemical transduction pathway in FAs which are known to have various roles on the cytoskeletal proteins. Among these are integrin activation, the linkage of FAs to actin, FA turnover, and actomyosin contractility [3–7]. The mechanical stability is dependent on their proper integration and regulation of all these cellular processes [8,9]. To date, it has not been unambiguous as to how these FA proteins influence the cellular mechano-chemical signaling, which is defined by the interaction of different cytoskeletal components.

It has been reported that (i) FAK affects the cytoskeleton via various pathways and protein–protein interactions [10]. However, the effects of FAK deletion on the structure and stability of the cytoskeleton implied that a rho-kinase-dependent pathway is involved [11,12], whilst (ii) vinculin acts predominantly as a mechano-coupling protein, which is regulated by c-src-kinase phosphorylation [13], and (iii) p130Cas is a scaffolding protein, which is the substrate of v-src family kinases that plays an important role in cell proliferation and motility [14].

Based on this information, we investigated to what extent the three focal adhesion proteins contribute to mechano-chemical changes in mouse embryonic fibroblasts (MEFs). To test this, we used a cell stretcher and applied external forces to adherent cells and determined in viability tests the mechanical influence. 2D Traction microscopy confirmed that the various FA proteins contribute differently to mechanical changes and biochemical signaling compared to wildtype cells.

2. Materials and methods

2.1. Cells and cell culture

Mouse embryonic fibroblasts (MEFs) were purchased from ATCC (#CRL-2644, FAK-deficient and #CRL-2645, FAK wildtype cells), and other wildtype MEFs and vinculin- and pCas130-deficient cells were a kind gift from Dr. W.H. Ziegler, University of Leipzig and Dr. J. Brabek, University of Prague, respectively. All cell lines were maintained in low glucose (1 g/L) Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 100 U/ml penicillin–streptomycin (DMEM = complete medium).

2.2. Cell stretcher

A cartoon of the cell stretcher is shown in Fig. 1. This purposely built stretcher consists of a linear stage for uniaxial stretch and is...
driven by a direct current motor with an integrated gearbox. Experiments were carried out on a flexible PDMS (Polydimethylsiloxan) substrate, which was coated with extracellular matrix proteins and stretched for mechanical stimulation. The cell stretcher has a flexible stretch inset of 2.5 cm² internal surface. To seed the cells, the insets were coated with 5 μg/mL fibronectin in PBS overnight at 4 °C. Uniaxial, cyclic stretch was performed in an incubator under normal cell culture conditions (37 °C, 5% CO₂, 95% humidity) for 1 h at 0.25 Hz and 20% stretch.

2.3. Viability tests

To determine the exact number of pre-stretched cells, bright field and fluorescent images (nucleus stained with Hoechst) were taken, and the positions were stored for later analysis. After stretching in the incubator, live/dead staining was used as a measure for the viability of cells. For this purpose, the cells were stained with calcein and propidium iodide and previously stored cell positions were recalled. Fluorescent images of calcein and propidium iodide were then taken. The ratio of live/dead cells was analyzed using Image J. Note, that in addition to the number of dead cells observed in the propidium iodide channel, differences in the number of nuclei were added to the number of dead cells as we assumed the detached cells were dead.

2.4. 2D Traction microscopy

Traction measurements were performed on 6.1% acrylamide/ bisacrylamide (19:1) gels with 0.5 μm green fluorescent beads embedded. The Young’s modulus of the gels was ~12.8 kPa. Gels were coated with 50 μg bovine collagen G (Biochrom AG) diluted in 50 mM HEPES or with 5 μg/ml fibronectin diluted in PBS overnight at 4 °C. Ten thousand cells per milliliter (ml) were seeded on the gels and incubated under normal growth conditions overnight. During the measurements, the cells were maintained at 37 °C and 5% CO₂ in a humidified atmosphere. Cell tractions were computed from an unconstrained deconvolution of the gel surface displacement field [15,16] measured before and after the cells were treated with 80 μM cytochalasin D.

3. Results and discussion

Focal adhesions (FAs) form after cell adhesion to the ECM, connecting integrins with the actin cytoskeleton. The regulation and spatial organization of the cytoskeleton is central for cellular mechanics, morphology, motility, and function [2,17,18]. It is well established that FAK, vinculin, and p130Cas, which are extensively studied proteins of the FAs, directly interact with and biochemically affect many components of the cytoskeleton including talin, alpha-actinin, Arp2/3, paxillin, and small GTPases [19–21]. These proteins, which perform various functions, are especially important concerning the formation and regulation of the cytoskeleton. Although the biochemical role of single components of the cytoskeleton is well established, it is still unknown how these FA proteins affect mechano-chemical signaling in cells.

First, we investigated the mechanical signal transduction in MEFs. We used the cell stretcher shown in Fig. 1 to determine the influence of 20% stretch at 0.25 Hz for 1 h on wildtype and FAK-, vinculin-, and p130Cas-deficient cells. In viability tests, i.e. calcein and propidium iodide staining of cells after stretch, we ascertained the number of dead cells for wildtype and FA-deficient cells (Fig. 2). We found that 20% stretch had no effect on vinculin-deficient cells, whilst FAK-deficient cells showed 3.9 times and p130Cas-deficient cells 1.6 times the number of dead cells compared to wildtype (Fig. 3A). These results support earlier reports that FAK and p130Cas influence the apoptotic pathway in cells under stressed conditions [22,23], while vinculin has no direct influence on the stretch-mediated apoptotic pathway. The relative strain energy established from traction microscopic measurements was reduced to ~37% in vinculin-deficient, ~55% in FAK-deficient, and ~67% in p130Cas-deficient cells compared to wildtype (100%) (Fig. 3B). The drastic reduction in vinculin-deficient cells is probably due to its reported coupling function to the actin cytoskeleton [24,25]. Previous stiffness measurements of FAK-, vinculin-, and p130Cas-deficient cells compared to wildtype confirm these results [13,14,26].

Second, investigating the cellular mechano-chemical signal transduction, we noted that ERK1/2 phosphorylation in wildtype MEFs peaked at around 10 min stretch compared to FAK-, p130Cas-, and vinculin-deficient cells, which was at around 5 min. Note, that the signal in p130Cas-deficient was always higher compared to the other deficient cells (data not shown). We explain these differences of mechano-chemical signaling in a model (Fig. 4), which displays that src-kinase phosphorylation of p130Cas increases downstream pERK1/2 [14,27,28], whilst src-kinase phosphorylation of vinculin allows the molecule to open up and modulate ERK1/2 phosphorylation by controlling the accessibility of rho-kinase for paxillin and FAK interaction [5,29]. These observations indicate that in a wildtype cell system src-kinase influences ERK1/2 phosphorylation in a balanced measure that influences myosin light-chain kinase (MLCK) and regulates tension, whilst in the absence of FAK, vinculin, or p130Cas ERK1/2 phosphorylation peaks earlier but at a lower level and affects MLCK and the tension-generating cellular apparatus less, which leads to lower cell stiffness.

Central to resisting externally applied forces is a stable and well-structured network of FAs and actomyosin fibers. The spatial distribution of the actin cytoskeleton and FAs in wildtype MEFs has been described in [7,13,30,31]. It was reported that wildtype cells form generally well-organized, parallelly aligned actin fibers and equally distributed FAs of elongated shape, whilst FAK-, vinculin-, and p130Cas-deficient cells showed more cortical actin bundles and an increase in FA size and density compared to wildtype cells. The influence of the cytoskeleton on the stability of FA-deficient and wildtype cells was distinguished between the generation of forces (prestress) and their contribution to the overall contractility (tractions) of the cell. In conclusion, forces generated by actomyosin fibers inside the cell only contribute to the overall cellular contractility and stability, if they are transmitted via FAs to the ECM which act as an anchor.
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References


Fig. 2. Cell viability test. (A) Bright-field image of wildtype MEF cells; (B) live cell staining with calcein; (C) dead cell staining with propidium iodide; (D) cell nucleus staining with Hoechst dye. Conditions: 750 nM Propidiumiodide (PI) and 2 µM Calcein–acetoxymethlester (Calcein–AM) in 600 µl PBS are added to MEFs at 37 °C for 15 min after 20% stretching at 0.25 Hz for 1 h. Excitation and emission of (PI) = 488 nm and 535 nm and (Calcein–AM) = 495 and 1517 nm, respectively.

Fig. 3. Cell viability and tractions. (A) Dead FAK-, vinculin-, and p130Cas-deficient MEFs after 1 h of 20% cyclic stretch at 0.25 Hz relative to wildtype cells. Number of experiments per cell line, n = 3. (B) Strain energy of FAK-, p130Cas-, and vinculin-deficient MEFs relative to wildtype cells. Number of cells, n = 30 – 50. 2D Traction images of wildtype and p130Cas−/− MEFs indicating forces ranging from 0 to 8 kPa. Note, that the white dotted lines show the cell perimeter.

Fig. 4. Cellular Signaling. A schematic diagram of the influence of src-kinase phosphorylation on vinculin and p130Cas and the effect on signaling of ERK1/2, MLCK as well as cellular tension regulation [5,12,14,20,29,33].


