



## Comparing the mechanical influence of vinculin, focal adhesion kinase and p53 in mouse embryonic fibroblasts

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### ABSTRACT

Cytoskeletal reorganization is an ongoing process when cells adhere, move or invade extracellular substrates. The cellular force generation and transmission are determined by the intactness of the actomyosin-(focal adhesion complex)-integrin connection. We investigated the intracellular course of action in mouse embryonic fibroblasts deficient in the focal adhesion proteins vinculin and focal adhesion kinase (FAK) and the nuclear matrix protein p53 using magnetic tweezer and nanoparticle tracking techniques. Results show that the lack of these proteins decrease cellular stiffness and affect cell rheological behavior. The decrease in cellular binding strength was higher in FAK- to vinculin-deficient cells, whilst p53-deficient cells showed no effect compared to wildtype cells. The intracellular cytoskeletal activity was lowest in wildtype cells, but increased in the following order when cells lacked FAK+p53 > p53 > vinculin. In summary, cell mechanical processes are differently affected by the focal adhesion proteins vinculin and FAK than by the nuclear matrix protein, p53.

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In order for cells to function properly they must obtain a variety of information which is of molecular biological, biochemical and biophysical origin. To understand their organizational principles, the field of cell mechanics has been concerned on how cells move, deform and interact with each other as well as how they sense, generate and respond to mechanical forces. Recent developments have included studies of cytoskeleton dynamics and cell–extracellular matrix (ECM) interactions which are believed to be related generally to the shape, function, deformability and mechanical properties of cells. Aspects of viscoelasticity and connectivity of cellular structures like focal adhesion protein formation and actomyosin interaction on cell adhesion, locomotion, mechanical forces and their effects of mechanical perturbations on cellular processes have been the focus of much research [1–5].

Although the importance of cellular mechanical stimuli is now clear, the field of cell mechano-transduction has remained virtually unexplored until recently, mainly due to the lack of experimental techniques to apply and to detect forces [6–8]. Understanding processes of folding/unfolding of protein domains or the strengthening of receptor–ligand bonds by force on the molecular level and the mechanism by which force detection is performed and integrated within the cell to influence cellular behavior is the reason for this work.

We attempt to characterize the molecular mechanism by which cells recognize and respond to physical forces in their local environment. The work is based on the hypothesis that cells sense mechanical stresses through their cell surface receptors, such as integrins and *via* the focal adhesion complex (FAC) and actin cytoskeleton and that the molecules such as focal adhesion kinase (FAK), vinculin and p53 are involved in mechanical regulation [5,9,10,11].

The aim of this study is to elucidate their influence in cellular mechano-regulation. Based on studies that many signal transducing and structural molecules are transiently immobilized on the cytoskeleton at the site of integrin binding and within the FAC, we will use mouse embryonic fibroblasts that are deficient of focal adhesion kinase and/or p53 as well as vinculin. Using these cells will provide a unique opportunity to describe the mechano-regulatory influence of these proteins upon mechanical stimulation by applying magnetic tweezer and nanoparticle tracking methods.

### Materials and methods

**Cell lines.** Mouse embryonic fibroblasts (MEFs) deficient in vinculin (–/–) and vinculin wildtype (+/+, wt) were a kind gift of Dr. E.D. Adamson [12]. MEFs deficient in focal adhesion kinase [(–/–) and p53 (–/–)] as well as MEFs of focal adhesion kinase [(+/+, wt) and p53 (–/–)] were purchased from ATCC (CRL-2644+2645).

**Cell culture.** All MEF cell lines were maintained in low glucose (1 g/L) Dulbecco's modified Eagle's medium supplemented with

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10% fetal calf serum (low endotoxin), 2 mM L-glutamine and 100 U/ml penicillin–streptomycin (DMEM complete medium was purchased from Biochrom, Berlin, Germany) and cultivated at 37 °C with 5% CO<sub>2</sub>.

**Magnetic tweezer.** The principle of the magnetic tweezer device used was previously described by Kollmannsberger and Fabry [13]. In detail, superparamagnetic 4.5 μm epoxytated beads (Invitrogen, Karlsruhe, Germany) were coated with fibronectin (100 μg/ml, Roche Diagnostics GmbH, Mannheim, Germany) in PBS at 4 °C for 24 h. Beads were washed in PBS and stored at 4 °C. Prior to measurements, fibronectin-coated beads were sonicated, added to cells (100,000 beads/dish) and incubated for 30 min at 5% CO<sub>2</sub> and 37 °C. A magnetic field with a high field gradient was generated by a needle-shaped tip. Bright-field images of the cell, bead and needle tip were taken by a CCD camera at a rate of 40 frames/s. The bead position was tracked on-line, using an intensity-weighted center-of-mass algorithm. To ensure that cells had not experienced any significant forces resulting from previous measurements, the needle was moved by at least 0.5 mm between two measurements. Image acquisition was triggered and synchronized with the solenoid current generator using a customized C++ program run.

**Force protocol and data analysis.** When a force step with an amplitude,  $\Delta F$  was applied to a bead, it moved with a displacement,  $d(t)$  towards the needle tip. The ratio,  $d(t)/\Delta F$  defines a creep response,  $J(t)$  that was for all force amplitudes well described by a power-law,  $J(t) = a(t/t_0)^b$ , where,  $t_0$  is the reference time. The parameter,  $a$  (units of μm/nN) characterizes the elastic cell properties and corresponds to a compliance (i.e. inverse of stiffness), and the power-law exponent,  $b$  describes the dissipative (i.e. frictional) cell properties [14].  $b$  reflects the stability of the force-bearing structures of the cell that are connected to the bead. Note, that a value for  $b = 1$  indicates Newtonian viscous or fluid-like behavior, and  $b = 0$  indicates an elastic, static behavior [15,16].

**Bead detachment.** The fractions of beads that detached during measurements were taken as a measure of the bead binding strength between the cell and the FN-coated bead.

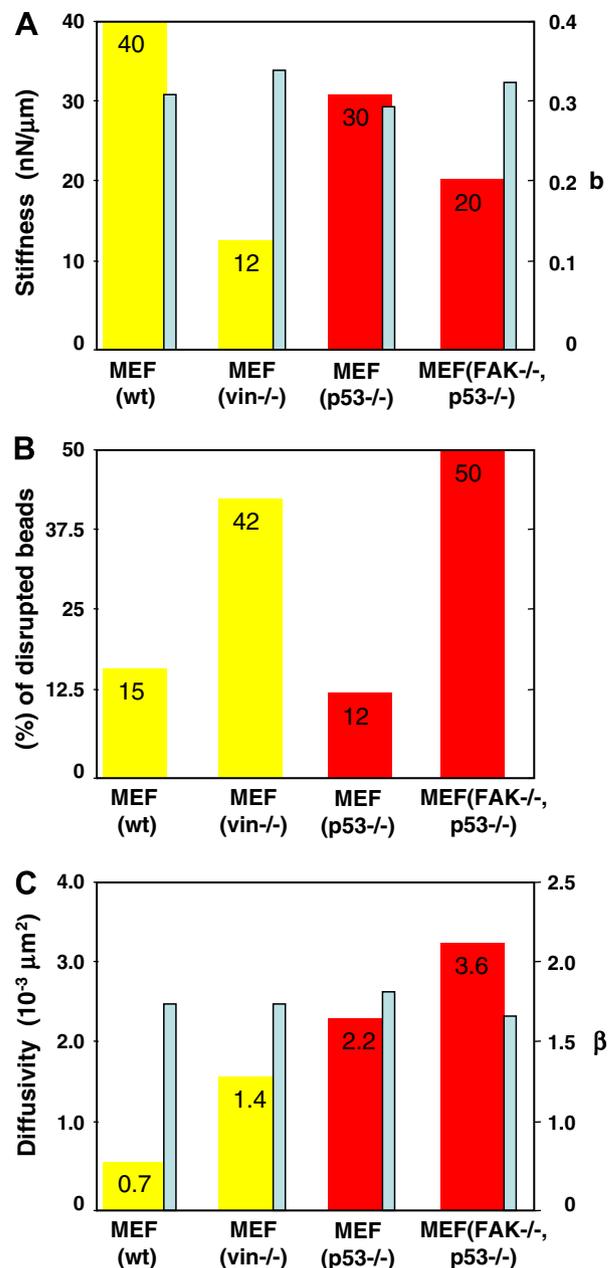
**Nanoscale particle tracking.** The position of unforced beads bound to integrin receptors was tracked over 5 min. These beads moved spontaneously with a mean square displacement (MSD) that also followed a power-law in time,  $MSD = D^*(t/t_0)^\alpha + c$ . The power-law exponent,  $\alpha$  was determined by a least-squares fit as described in [17].

## Results and discussion

### MEF cell stiffness and cytoskeletal fluidity

The cell internal forces that resist cell shape changes can be estimated from cell stiffness measurements. We used a magnetic tweezer to apply forces of up to 10 nN to fibronectin-coated superparamagnetic beads. The bead displacement after a stepwise increase in force followed a power-law as described in [5,14]. Cell stiffness was determined from the power-law prefactor,  $a$  of the relation:  $J(t) = a(t/t_0)^b$ . Fig. 1A (left, y-axes) shows the result from these measurements using various MEF cell lines. The cell stiffness,  $1/a$  determined at 10 nN was of the following order: MEF(wt) > MEF(p53<sup>-/-</sup>) > MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) > MEF(vin<sup>-/-</sup>) cells. The cell fluidity in Fig. 1A (right, y-axes) was characterized by the power-law exponent,  $b$ . MEF(vin<sup>-/-</sup>) and MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) cells displayed a higher power-law exponent compared to MEF(wt) and MEF(p53<sup>-/-</sup>) cells.

The magnetic tweezer device measured the lateral displacement of FN-coated superparamagnetic beads in response to increasing forces. In cell rheological terms MEF(wt) cells were about 3-fold stiffer and less fluid-like ( $b = 0.3$ ) than MEF(vin<sup>-/-</sup>) cells ( $b = 0.34$ ), whilst MEF(p53<sup>-/-</sup>) were 1.5-fold stiffer and less



**Fig. 1.** (A) The creep response data at 10 nN of MEF(wt), MEF(vin<sup>-/-</sup>), MEF(p53<sup>-/-</sup>) and MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) cells follow a superposition of power-laws characterized by the force-dependent stiffness,  $1/a$  and the power-law exponent,  $b$  (cell fluidity). Number of cells,  $n \sim 70$ . (B) Percentage of beads disrupted from the cells vs. pulling force at 10 nN. The adhesion strength is significantly lower in MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) and MEF(vin<sup>-/-</sup>) cells compared to MEF(wt) and MEF(p53<sup>-/-</sup>) cells. Number of cells,  $n \sim 70$ . (C) The MSD vs. time curves was fitted to a power-law:  $MSD = D \cdot (t/t_0)^\beta$ , with the apparent diffusivity,  $D$  describing the speed of cytoskeletal remodeling processes, and  $\beta$  is the persistence of bead motion.  $D$  was increased by 100% in MEF(vin<sup>-/-</sup>) cells compared to MEF(wt) cells and by 50% between MEF(p53<sup>-/-</sup>) and MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>), however,  $\beta$  remained almost unchanged. Number of beads,  $n \sim 250$ . Note, that the values (in A–C, left y-axes) are expressed as median. The yellow bar indicates MEFs obtained from E.D. Adamson, the red bars marks MEFs purchased from ATCC and the light green bar denotes y-values on the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

fluid-like ( $b = 0.28$ ) compared to MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) cells ( $b = 0.33$ ). Note, that an exponent of  $b = 0$  is indicative of a purely elastic solid, and  $b = 1$  of a purely viscous fluid. In cells, the power-law exponent usually falls in the range between 0.1 and 0.5, whereby higher values have been linked to a higher turnover rate of cytoskeletal structures. According to Fabry et al. [15,16]

higher *b*-values are often associated with reduced cell stiffness which is foremost determined by the amount of contractile prestress which is carried by the actin cytoskeleton [18].

#### Adhesion strength of MEF cells

The mechanical stability of the adhesion contacts was measured by pulling on fibronectin-coated magnetic beads attached to the cell surface receptors. The attachment of fibronectin-coated beads to cells is known to induce local actin remodeling such as the accumulation of cortical actin and the formation of stress fibers around the bead [19]. F-actin staining indicated that this process was not impaired in MEFs (data not shown). To quantify the binding strength between the bead and the cytoskeleton, stepwise increasing forces between 0.5 and 10 nN were applied to the beads using the magnetic tweezer. The bead detachment was recorded at a force value of 10 nN. Fig. 1B shows the order of bead detachment in percent compared to total bead numbers: MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) > MEF(vin<sup>-/-</sup>) > MEF(wt) > MEF(p53<sup>-/-</sup>). The adhesion strength of the integrin–matrix connection was clearly more weakened by the loss of focal adhesion kinase and vinculin than by the loss of the protein p53.

This result is consistent with previous reports that inferred reduced adhesion strength of MEF(vin<sup>-/-</sup>) [5] and MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) cells (unpublished observations) compared to MEF(wt) cells. It is, therefore, conceivable that the extracellular matrix–cytoskeleton connections are structurally impaired due to the loss of vinculin's coupling and FAK's binding function to FAC. The loss of p53 in MEFs showed little to no effect on bead binding strength. Exactly where the bond breakage occurs, when beads are disrupted from the cells, remains unclear.

#### Cytoskeletal dynamics in MEF cells

To establish whether the decreased bead binding strength was associated with increased remodeling dynamics of the cytoskeleton, nanoscale particle tracking of fibronectin-coated beads was used. Since the fibronectin-coated beads bind to integrin receptors, which connect the actin cytoskeleton via the focal adhesion protein complex, the spontaneous movement of these beads report the remodeling dynamics of the cytoskeletal structures [17,20,21]. The apparent diffusivity, *D* and the index of persistence, *β* are a measure for these activities according to [17,20]. The diffusivity values were of the following order (Fig. 1 C, left, y-axes): MEF(FAK<sup>-/-</sup>, p53<sup>-/-</sup>) > MEF(p53<sup>-/-</sup>) > MEF(vin<sup>-/-</sup>) > MEF(wt). The difference in diffusivity shows that the lack of vinculin was less detrimental to cytoskeletal dynamics than the lack of p53 or p53 and focal adhesion kinase combined. Whether the increased diffusivity was caused by increased cytoskeletal remodeling, increased focal adhesion protein or nuclear matrix protein turnover could not be distinguished by these measurements.

The speed of bead motion, i.e. the square root of the apparent diffusivity, *D* indicating cytoskeletal remodeling dynamics was much higher in MEFs lacking p53 and FAK compared to vinculin-deficient and wildtype cells. Moreover, the bead motion was highly superdiffusive in these cell lines, i.e. the MSD increased with time according to the relation: MSD  $\sim t^\beta$ . Note, that exponents of *β* > 1 have been linked to the persistence of motion, however, *β* did not differ much between the cell lines, indicating that the increased bead motion was probably due to an increased speed of cytoskeletal remodeling but not due to an increased persistence [17]. Cell remodeling dynamics measured from the spontaneous movements of unforced beads is normally related to the viscoelastic properties of the cell that are measured from the movement of beads exposed to external forces.

In conclusion, the intactness of the actin cytoskeletal connection with the extracellular matrix via FAC is paramount for cell stiffness and traction force generation and the basis for intracellular dynamic processes. The lack of cytoskeletal connecting proteins like vinculin and focal adhesion kinase increases intracellular dynamics and reduces the overall cellular binding strength more than the nuclear matrix protein p53. Therefore, focal adhesion proteins seem to have a greater influence on cell mechanical behavior.

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