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Influence of divalent cations on the cytoskeletal dynamics of K562 cells determined by nano-scale bead tracking

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ABSTRACT

Cytoskeletal reorganization processes can be analyzed by studying the nanometer-scale spontaneous motion of beads bound to the cytoskeleton. The bead motion is determined by force fluctuations within the cytoskeletal network that originates from myosin motor activity and dynamic restructuring of cytoskeletal filaments. We investigated to what extend the spontaneous bead motion is influenced by the dynamics of the link between the bead and the cytoskeleton in the presence of divalent cations. Our data show that, when K562 cells expressing constitutively (alpha 5 beta 1) integrin and when stably transfected with (alpha v beta 3) integrin, spontaneous bead motion is dramatically affected by the presence of 1 mM Mn^{2+} (integrin, activate state) compared to 1 mM Ca^{2+}/Mg^{2+} ions (integrin, inactive state). The directionality of the bead motion, which is influenced by the overall stability of the cytoskeletal network and by actomyosin-generated forces, is markedly different, whilst the persistence remained similar due to the specific binding of either Mn^{2+} or Mg^{2+}/Ca^{2+} ions.

1. Introduction

Integrins are a family of alpha and beta heterodimeric membrane-bound glycoproteins that mediate cell–matrix and cell–cell adhesions [1]. Together with their ligands, integrins play a central role in many biological processes, which include cardiovascular disease, cancer invasion, immune dysfunction, osteoporosis, and also constitute the target for therapeutics.

The fact that integrins span across cell membranes enables them to convey signals [1,2]. It also reflects a dynamic link between integrin's head and tail providing outside-in and inside-out signaling. They in turn activate intracellular signaling pathways by connecting to the cytoskeleton. Outside-in signaling refers to the extracellular ligand-binding which induces conformational changes and transmission of signals from exterior to the interior of cells that lead to changes in cell growth, cell motility, morphology, and gene expression [3]. Additionally, stimuli received by cell surface receptors initiate intracellular signals that trigger integrin activation (inside-out signaling). Activation of integrins depends on efficient ligand binding which relies on the presence of divalent metal ions [4,5]. Metal divalent cations are essential for integrin function. Their activity ranges from stabilizing the integrin structure to enhancing or suppressing its interaction with ligands (cf. Plow et al. JBC 2000, 275:21785). Early EM studies of integrins showed a globular head structure formed by two subunits and stalk-like C-terminal portions which was confirmed by Xiong et al. [6,7]. However, there is still some debate about the exact mechanism. It is possible that it involves distinct and separate events such as ligand-binding to the heterodimer, flexion at the genu to modulate integrin extension, separation of the cytoplasmic tails, and integrin clustering [8].

In our experiments, we used a non-adherent myelogenous leukemia cell line (K562) which constitutively expresses (alpha 5 beta 1) integrin, but lacks (alpha v beta 3) integrin expression, which was stably transfected to express (alpha v beta 3) wild type receptors [9]. Their activation depends on efficient ligand binding in the presence of divalent metal ions [4,5]. These researchers reported that structural changes of (alpha v beta 3) integrins induced through ligand binding to Arg–Gly–Asp (RGD) may be facilitated by Mn^{2+} ions.

Our hypothesis is that the binding of ligands in the presence of Mn^{2+} or Mg^{2+}/Ca^{2+} ions to constitutively expressed (alpha 5 beta 1) and stably transfected (alpha v beta 3) integrins can induce different mechanical behavior of the actomyosin cytoskeleton in K562 cells. The objective is to determine whether the activation of these integrins reflects different dynamics of the cytoskeleton, which is crucial for wound healing, cancer cell adhesion, and tissue migration.

2. Materials and methods

2.1. Cell lines and culture

Myelogenous leukemia K562 are non-adherent cells that serve as a model cell line. A stable K562 cell line expressing full length

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wildtype (alpha v beta 3) integrin has been described by [9]. Fig. 1 shows the flow cytometry analysis of native K562 cells expressing stably transfected (alpha v beta 3). We have screened for other integrins and have found that (alpha 5 beta 1) is constitutively expressed in this cell line. The rest of the RGD-specific integrin tested were negligible. These cells express inactive integrins until activated by external signals. As cell culture medium, we used Iscove's modified Dulbecco's medium (IMDM), supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418. Attachment of 7×10^4 K562 cells was achieved on 0.01% poly-L-lysine-coated cell-culture dishes. Preliminary tests showed that bead binding only occurred on K562 cells, expressing (alpha 5 beta 1) and (alpha v beta 3) integrin receptors, when coated with RGD. We tested these cells under non-activating (1 mM Ca^{2+}/Mg^{2+} dissolved in TBS) and activating (1 mM Mn^{2+} dissolved in TBS) ion conditions.

2.2. Beads

Epoxylated polystyrene beads (4.5 μm) were coated with RGD obtained from Peptide 2000 (Glycosan Biosystems) dissolved in 1 ml PBS per 1 mg of beads. Approximately 2×10^5 beads were added to individual wells and incubated for 20 min prior to measurements. Unbound beads were removed by washing the cells with medium. During measurements, the wells were placed in an incubation chamber at humidified air with 5% CO_2 at 37 °C mounted on the microscope.

2.3. Nano scale bead tracking

The nanometer-scale bead tracking method has been described by [10,11]. RGD-coated beads are bound to K562 cells *via* the integrin receptors and are therefore connected to focal adhesions (FAs) and the actomyosin network [12,13]. Movement of the beads reflects internal remodeling processes of the cytoskeleton (Fig. 2). To analyze the spontaneous bead motion, the position of unforced beads bound to integrin receptors was tracked for 10 min at 10 frames/s and 20x magnification with an accuracy of 5 nm. The mean square displacement (MSD) for each bead was calculated which followed a power law with time, $MSD = D(\Delta t/t_0)^{\beta} + c$ [10,11], where t_0 is the time interval of the bead recordings, the prefactor D is the apparent diffusivity, equivalent to the square of the distance traveled during the time intervals, and the power law exponent, β is a measure of the persistence, which classifies the bead motion as diffusive/Brownian for $\beta = 1$, as subdiffusive for $\beta < 1$, superdiffusive for $\beta > 1$ and ballistic for $\beta = 2$. The prefactor D describes the diffusivity of the bead motion, which is influenced by the overall stability of the cytoskeletal network and by actomyosin-generated forces. Bead positions were corrected for the effect of microscope stage drift. The stage drift was estimated from the mean motion of all beads in the field of view. Unbound beads were not evaluated.

2.4. Statistics

The data are expressed as the mean values ± S.E., if not indicated otherwise. Statistical analysis was performed using a two-tailed paired *t*-test. $p < 0.05$ was considered to be statistically significant and marked (*).

3. Results and discussion

RGD-coated beads were bound to the cell receptors for 20 min and thereafter followed by a continuous 10 min recording of the bead trajectories during spontaneous motion (Fig. 2A). Prior to measurements in the presence/absence of cations, we tested the mechanical bead-integrin-FA-cytoskeleton link by using cytochalasin D. Disrupting this link should show a change in bead motion. With increasing concentration of cytochalasin D from 0 μM to 20 μM, we observed an about 7-fold increase in bead movement, whilst the persistence β decreased by approximately 1.5-fold from superdiffusive to diffusive (Fig. 3A + B). These results confirm that, when the bead-integrin-FA-cytoskeleton link is not intact, the bead moves more freely and less directed, which is a reaction of stress fiber coupling. Measuring the influence of various cations, the diffusivity of the bead in the presence of 1 mM Mn^{+2} was smaller compared to the presence of 1 mM Mg^{+2}/Ca^{+2}, however, the persistence remained similar (superdiffusive) under both conditions (Fig. 4). These results show that the activation of integrins depends on efficient ligand (bead) binding which relies on the type of divalent metal ion coordinated to the cation-binding sites. Ajroud et al. [4] and Ligezowska et al. [14] have reported that the binding of RGD-induced structural changes in integrins is caused by the ligand and not by Mn^{2+} ions alone. When cells are in the resting state under normal physiological conditions, their integrin receptors become inactive and bind poorly to ligands. In response to stimuli, integrins undergo activation making the cells adhesive to their counter receptors. Strong traction forces are applied on the integrins during these processes which must have profound consequences on integrin conformation, cell signaling, cytoskeletal

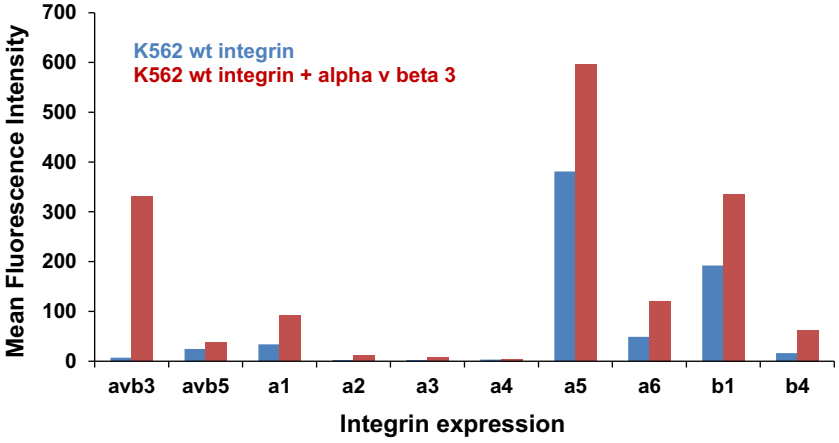

Fig. 1. Flow cytometry analysis of integrin expression on wildtype (wt) and K562 cells expressing stably transfected (alpha v beta 3) integrin. The mean fluorescence intensities (MFI) are plotted for wildtype in blue and wt + (alpha v beta 3) integrin expressing K562 cells in red. Note, that the expression level of (alpha v beta 3) integrin in K562 cells was increased by around 48-fold compared to K562 wt cells. All other integrins showed an increase between 1.5 and 5-fold compared to K562 wt cells.

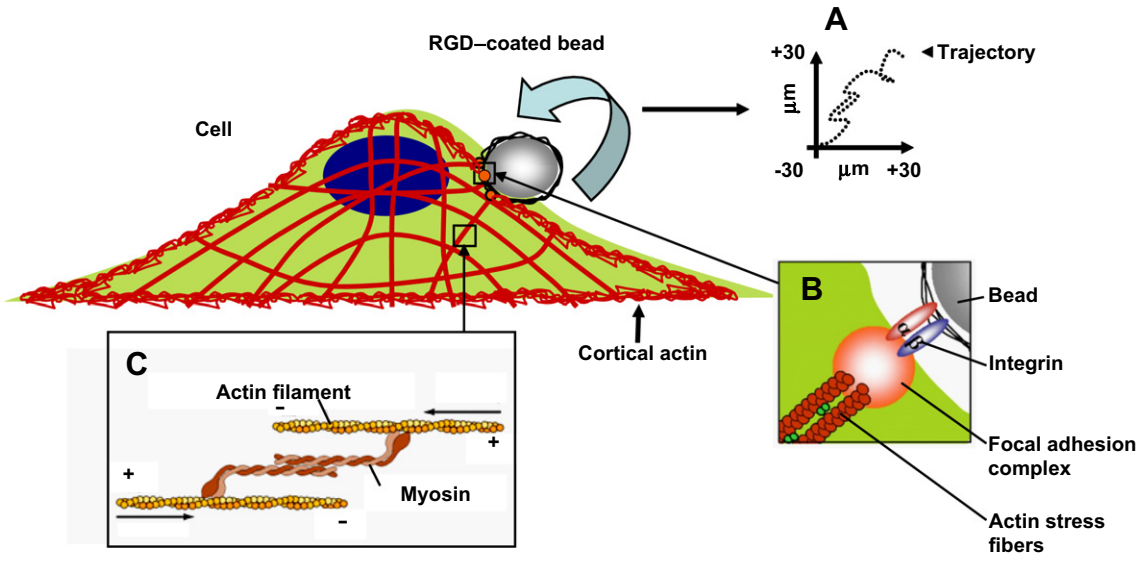

Fig. 2. Schematic representation of a 4.5 µm ∅ RGD-coated polystyrene bead attached to integrins of K562 cells that span the cell membrane. Integrins are connected *via* focal adhesions to the actin cytoskeleton. The insets show (A) the movement (trajectory) of a bead attached to the integrin receptor over 10 min, (B) the integrin-FA-cytoskeleton link, and (C) the contractile apparatus of the cell.

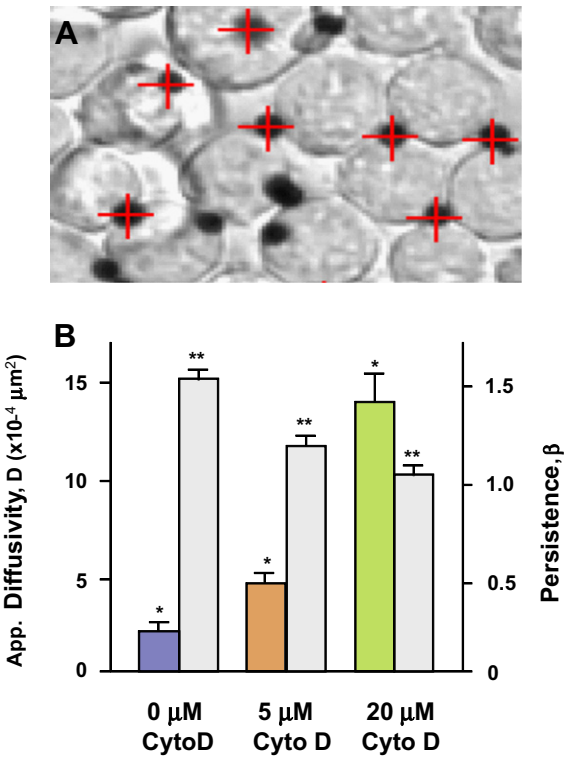

Fig. 3. (A) K562 cells are seeded on 0.01% poly-L-lysine cell culture dish. Only cells with beads that are marked by the red cross are analyzed. (B) The apparent diffusivity of bound beads increases upon treatment with cytochalasin D (0 µM violet, 5 µM brown and 20 µM green), whilst the persistence (gray) decreases, which is indicative of a disruption of the integrin-FA-cytoskeleton link. The number of cells analyzed were between 500 and 750; number of experiments $n = 5$. (*) and (**) show statistically significant data, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

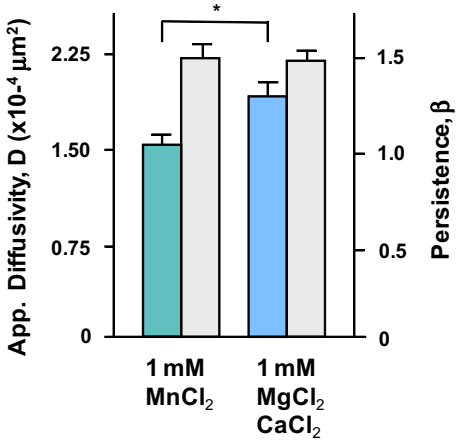

Fig. 4. The apparent diffusivity of RGD-coated beads on K562 cells upon treatment with 1 mM Mn^{2+} and Ca^{2+}/Mg^{2+} ions. Mn^{2+}-treated cells (activated integrin, green) show a tighter link of the integrin-FA-cytoskeleton connection than Ca^{2+}/Mg^{2+}-treated cells (inactive integrin, blue), whilst the persistence (gray) remained similar (superdiffusive) for both Mn^{2+}- and Mg^{2+}/Ca^{2+} ion treated cells. Note, that the bead motion only reflects the dynamics of the cytoskeleton as long as the bead is connected. The number of cells analyzed were between 750 and 950; number of experiments $n = 5$. (*) indicates statistically significant data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rearrangement, shape changes, and cell movement [13,15–17]. Although integrins can trigger signal transduction cascades and induce focal adhesion formation as a result of ligand binding and changes in receptor conformation, application of mechanical forces to bound integrins may convey distinct signals to the cell. Recently, Roca-Cusachs et al. [18] reported that the clustering of (alpha 5 beta 1) integrins determines the adhesion strength whereas (alpha v beta 3) and talin enables reinforcement and mechano-transduction in MEFs. In addition, (alpha 5 beta 1) fibronectin links form catch bonds that strengthen under force [19], and Morgan et al. [20] reviewed the similarities and differences of these important integrins with regard to cell migration. Our hypothesis is that the binding of ligands in the presence of Mn^{2+} or Mg^{2+}/Ca^{2+} ions to integrins can induce different dynamic behavior and mechanical properties of the actomyosin cytoskeleton in cells.

Finally, (i) persistent bead motion is dependent on actin cytoskeleton (as shown by the treatment with cytochalasin D), and

(ii) MSD measurements are suitable to record changes in integrin activation caused by divalent cations, i.e. beads attached to cells with activated (Mn^{2+}-treated) integrin receptors move less diffusive than cells with non-activated integrin receptors (Mg^{2+}/Ca^{2+}-treated). Recently, Ligezowska et al. [14] found that Mn^{2+} and Mg^{2+}/Ca^{2+} ions can work synergistically on (alpha 7 beta 1) integrin–invasin binding and have a strong dependence on bond affinity, which is probably contingent to the size and binding site of cations on integrins. Yin et al. [21] previously reported that ligand attachment to (alpha 5 beta 1) of BV-173 cells was cation-dependent which was of the order of $Mn^{2+} > Mg^{2+} > Ca^{2+}$ ions. All these novel findings of the influence of cations on the binding of various integrins are of interest and need to be investigated further.

The data presented here establish that nano-scale bead tracking is a robust method to measure the reorganization dynamics of cytoskeletal structures to which the beads are bound, and that bead movement is insensitive to the dynamics of the receptor-mediated bead link. In particular, our data support the conveyor-belt model [11] that regards beads as fiducial marker of the cytoskeleton. According to these authors, cytoskeletal remodeling can be followed on any bead that is tightly attached to the cytoskeleton, independent of bead size and coating [11].

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