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RESEARCH HIGHLIGHT

A biomimetic system to reconstitute and test the actomyosin-dependent mechanosensitive protein complexes

Corina Ciobanasu

Department of Interdisciplinary Research, Alexandru I. Cuza University, Blvd. Carol I, no. 11, Iaşi, RO-700506, Romania

Correspondence: Corina Ciobanasu
E-mail: vasilica.ciobanasu@uaic.ro
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Many biological processes are mechanically controlled. For example, when a cell migrates, the extracellular matrix (ECM) controls its shape, dynamics and adhesion as a response to the mechanical stress of the environment. Focal adhesions (FAs) are dynamic structures which connect the actomyosin cytoskeleton with the extracellular matrix. Up to date, several mechanosensitive pathways have been discovered, but with the currently available techniques is quite difficult to investigate single mechanosensitive proteins and to observe the mechanosensitive complexes in real time. We developed a microscopy assay based on a glass coverslip micropatterned with discs where the actin binding protein (ABP) talin was immobilized and proteins like actin, vinculin, myosin or actinin were added in solution. The self-organized actomyosin network exerts force on ABP and in response talin exposes hidden vinculin-binding sites (VBSs). We also observed that the activation of vinculin by talin determines a mechanosensitive reinforcement in the actin-talin-vinculin association. The biomimetic system and the activity of the fluorescently labeled proteins are followed with total internal refection fluorescence (TIRF) microscopy. This technique can be used to study the actin binding proteins that sense and respond to the force generated by the actomyosin network in various physiological and pathological processes.

Keywords: focal adhesions; mechanosensitive proteins; talin; vinculin; TIRF microscopy

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Cell migration is essential for physiological processes such as embryogenesis, tissue formation and regeneration, wound healing or immune response in multicellular organisms. Also, the malfunction of cell migration is responsible for many pathological processes including cancer and immune disorders [1]. During cell migration, focal adhesions sense and respond to the force generated by various types of actin networks by recruiting regulatory proteins [2, 3]. This compositional and morphological maturation strengthen the attachment with the actin cytoskeleton and the connection to the extracellular matrix [4, 5, 6].

Although multiple mechanosensitive pathways have been identified, the ABPs talin and vinculin play a fundamental role for the formation of stable focal adhesions. Talin is one of the first actin binding proteins recruited in focal adhesions. Talin is a large protein and consists of a head domain with a globular FERM (4.1/ezrin/radixin/moesin) N-terminal domain, a central rod domain and the C-terminal domain [7]. The FERM domain binds actin filaments (F-actin) and also binds and activates integrins [8]. The rod domain contains a central actin binding region and eleven vinculin binding sites (VBSs) [9]. The C-terminal part of talin interacts with the actin and integrin [10].

Vinculin contains a head domain (Vh) that interacts with

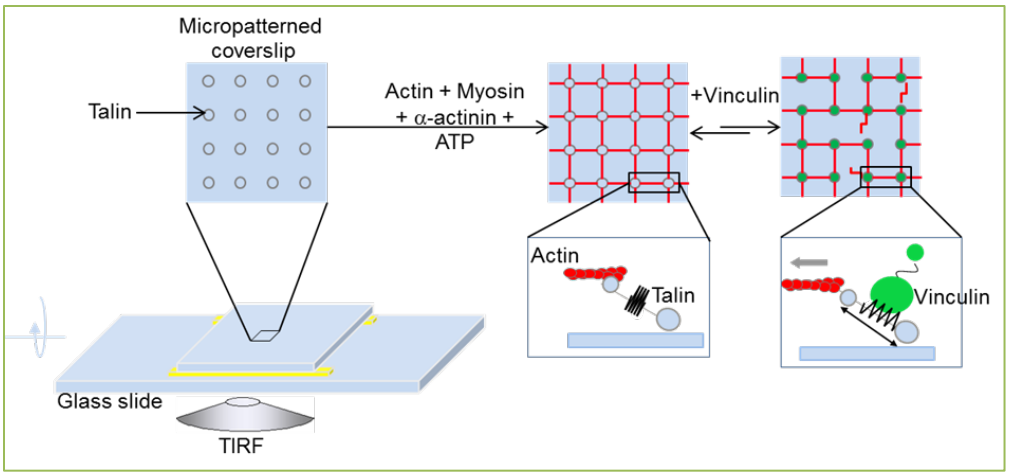

Figure 1. The experimental setup. On the left side: the reaction chamber. The unlabelled ABP talin is immobilized on the micropatterned glass surface in 5 µm discs separated by a distance of 35 µm. Actin, vinculin, myosin, actinin and ATP regenerating mix are added in solution. On the right side, cycles of attachment and detachment of dynamic actomyosin cables crosslinked with α-actinin exerts force on talin and exposes the cryptic vinculin-binding sites (VBSs).

talin and a tail domain (Vt) that interacts with actin filaments. In the inactive state, the intramolecular interaction between head and tail restrains the binding of Vt to the actin filaments [11]. Vinculin can be activated by the simultaneous binding to talin and actin and application of a mechanical stress to talin or other binding partners favors its recruitment to FAs [12]. It was predicted by molecular dynamics simulations that force-dependent unfolding of the talin rod induces the sequential exposure of VBSs [13]. *In vitro*, application of a physiologically relevant mechanical stimulus by means of magnetic tweezers and atomic force microscopy (AFM) demonstrated that the stretching of the talin rod reveals the cryptic VBSs, followed the binding of Vh [14].

Recently, we developed an *in vitro* system with purified human recombinant proteins to determine whether the actomyosin cytoskeleton induces force to stretch talin and then to bind vinculin [15, 16]. In this microscopy assay, actin, myosin II and/or α-actinin self-assemble into a contractile network and applies force on talin immobilized on a micropatterned glass coverslip, in 5 µm discs separated by a distance of 35 µm (Fig. 1). The force-dependent binding of the fluorescently labeled vinculin to the stretched talin is observed in real time by Total Internal Reflection Fluorescence (TIRF) microscopy.

The intensity and duration of the mechanical stimulus can be tuned by choosing the appropriate concentrations of the crosslinking protein α-actinin-1 and the motor protein myosin II.

We demonstrated with this microscopy assay that the binding of a contractile actomyosin network to the talin is a sufficient mechanical stimulus to stretch the mechanosensitive domains and drive the reversible binding of vinculin. First, we determined that the N-terminal domain of talin is bound to the surface and its C-terminal and/or its central actin-binding domain is associated with actin filaments, an orientation comparable to that observed in FAs [17]. Then, we used the head domain of vinculin (Vh) labeled with EGFP to observe the actomyosin-dependent talin-vinculin interaction because, Vh interacts directly with talin VBSs, unlike full-length vinculin. We used a contractile isotropic network of prepolymerized short actin filaments and myosin II filaments to apply force on talin and to obtain an instantaneous and homogeneous mechanosensitive response. We observed that the fluorescence intensity corresponding to Vh bound to talin in the discs was significantly higher in the presence of both actin and myosin than in the control experiments, without actin or myosin, demonstrating a mechanical stimulus -dependent binding of Vh to talin. We demonstrated also that the stretching of talin is a rate-limiting reaction since the association rate of Vh to talin reached a plateau and did not show a linear dependence on the Vh concentration [15].

Furthermore, we reconstituted the assembly of dynamic stress fibers (SF)-like cables by adding in solution myosin II, α-actinin and monomeric actin in polymerizing conditions. This situation is characterized by cycles of formation, detachment and retraction of actomyosin cables, reflecting cycles of tension applied on talin. We found that the crosslinking of actin filaments by mCherry-α-actinin at different concentrations did not influence the force applied on the talin discs but reduced the frequency of cables detachment [15, 16].

Then, we observed that the recruitment of Vh was directly connected to the association of actomyosin cables with talin, while the dissociation of Vh occurred after their detachment, suggesting the stretching and refolding of talin, respectively (Fig. 1). Also, with the described biomimetic system, we were able to show that dissociation of Vh kinetically limited the talin refolding [15].

Moreover, using an auto-inhibited EGFP-labeled full-length vinculin instead of Vh, we observed that the activation of vinculin by talin also activated by the actomyosin cables is followed by a mechanosensitive reinforcement in the actin-talin-vinculin complex. This observation was in agreement with some recent studies showing that the actin binding Vt plays a role in the reorientation of focal adhesions under mechanical stimulus and slows the actin retrograde flow and so determines the extension of the lamellipodial network [18, 19].

In conclusion, this microscopy assay offers a new biomimetic approach to test in real time the mechanosensitivity of actin binding proteins. The most common techniques used to study the force-dependent activation of proteins are magnetic or optical tweezers and AFM, but it difficult to observe the mechanosensitive complexes in real time with these techniques. Therefore, our microscopy assay in which the stretched actin binding proteins are attached in equilibrium to the actomyosin cables is more close to physiological processes than other experimental approaches. This technique can be used to study the actin binding proteins that sense and respond to the mechanical stimulus generated by the actomyosin network in various physiological and pathological processes. Tumor cells, for example, exhibit aberrant behaviors in mechanical response to changes in their environment and targeting proteins in the mechanosensitive interactions could be a valuable therapeutic strategy for cancer. This model system can also be used for the biochemical and biophysical characterization of individual mechanosensitive proteins and for screening pharmacological inhibitors of mechanosensitive protein pathways.

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