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Heparin-dependent regulation of fibronectin matrix conformation

Brant Hubbard [a], Jo Ann Buczek-Thomas [b], Matthew A. Nugent [b,c,d,*], Michael L. Smith [c,**]

[a] Molecular Biology, Cell Biology & Biochemistry Program, Boston University, Boston, MA 02215, United States
[b] Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118, United States
[c] Department of Biomedical Engineering, Boston University, Boston, MA 02215, United States
[d] Department of Biological Sciences, University of Massachusetts Lowell, Lowell, MA 01854, United States

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ABSTRACT

Extracellular matrix (ECM) conformation is regulated by a variety of stimuli in vivo, including mechanical forces and allosteric binding partners, and these conformational changes contribute to the regulation of cell behavior. Heparin and heparan sulfate, for example, have been shown to regulate the sequestration and presentation of numerous growth factors, including vascular endothelial growth factor, on the heparin 2 binding domain in fibronectin (Fn). However, mechanical force also alters Fn conformation, indicating that the growth factor binding region may be co-regulated by both heparin and mechanical force. Herein, we describe a simple antibody-based method for evaluating the conformation of the heparin 2 binding domain in Fn, and use it to determine the relative contributions of heparin and mechanical strain to the regulation of Fn conformation. We achieved specificity in quantifying conformational changes in this region of Fn by measuring the ratio of two fluorescent monoclonal antibodies, one that is insensitive to Fn conformational changes and a second whose binding is reduced or enhanced by non-equilibrium conformational changes. Importantly, this technique is shown to work on Fn adsorbed on surfaces, single Fn fibers, and Fn matrix fibers in cell culture. Using our dual antibody approach, we show that heparin and mechanical strain co-regulate Fn conformation in matrix fibrils, which is the first demonstration of heparin-dependent regulation of Fn in its physiologically-relevant fibrillar state. Furthermore, the dual antibody approach utilizes commercially available antibodies and simple immunohistochemistry, thus making it accessible to a wide range of scientists interested in Fn mechanobiology.

1. Introduction

Cell function within multicellular organisms must be tightly coordinated to maintain homeostasis and to respond to changing demands placed on the organism. Consequently, cells constantly communicate with one another by releasing and receiving chemical, mechanical and electrical signals, and the ECM is one such medium used for transfer of information between cells (Vogel and Sheetz, 2006). This information is encoded in the chemical composition, molecular conformation, and supermolecular structure of the ECM. Whereas the chemical composition of the ECM in various tissues and organs has been defined through traditional biochemical methods, few tools are available to evaluate the conformational state of the ECM (Smith et al., 2007; Cao et al., 2012; Hertig et al., 2012). Furthermore, current approaches are insufficient to effectively evaluate the functional activity of the ECM as it relates to the conformational state of its components. These limitations are highlighted in studies that aim to understand the rapid responses of cells and tissues during development, wound repair and disease.

The ECM is principally comprised of proteins and polysaccharides, with the glycoprotein Fn being a prevalent component of the ECM during times of dynamic ECM remodeling such as wound healing, development, and the progression of diseases such as cancer and atherosclerosis (Hynes, 2009). The expression of Fn at these times and the large number of binding partners for Fn, including integrins and growth factors, make it a prime candidate for regulation of cell fate and signaling (Pankov and Yamada, 2002). Protein structure determines function, and both molecular Fn and Fn assembled into supermolecular fibers were demonstrated to have altered binding properties for ligands, and even altered bioactivity due to changes in their conformation (Zhong et al., 1998; Mitsi et al., 2006; Little et al., 2008; Little et al., 2009). A number of factors can influence Fn conformation, including denaturants, pH, mechanical forces, and allosteric binding partners (Alexander et al., 1979; Khan et al., 1990; Mitsi et al., 2006; Bradshaw and Smith, 2011). Multiple factors are presented simultaneously in vivo, although the combined influence of structure-altering factors is rarely considered in concert.

Heparan sulfate represents a family of structurally related linear polysaccharides that are found on cell surfaces and in the ECM

* Correspondence to: M.A. Nugent, University of Massachusetts Lowell, 198 Riverside Street, Olsen 414A, Lowell, MA 01854, United States. Tel.: +1 978 934 2888.
** Correspondence to: M.L. Smith, Boston University, 44 Cummington Mall ERB 502, Boston, MA 02215, United States. Tel.: +1 617 358 5489.
E-mail addresses: matthew_nugent@uml.edu (M.A. Nugent), msmith@bu.edu (M.L. Smith).

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throughout all animal tissues (Sarrazin et al., 2011). Heparin is a highly sulfated member of the heparan sulfate family that is found mainly in the storage granules of connective tissue mast cells (Sarrazin et al., 2011) and is released at cites of injury and inflammation where it has been shown to help the growth of embryonic stem cells (Furue et al., 2008). Heparan sulfates bind reversibly to Fn type III modules 12 to 14, thereby inducing a conformational change in Fn that is retained even after heparin unbinding (Mitsi et al., 2006, 2008). We have previously shown through ^{3}H-heparin binding assays that heparin is not retained by Fn after sample washing (Mitsi et al., 2006), which is consistent with the finding that heparin binding to Fn is relatively weak and destabilized under physiological ionic strength (Yamada et al., 1980; Gold et al., 1983; Sekiguchi et al., 1983). After heparin-dependent alteration of Fn conformation, the apparent affinity of Fn for growth factors, including vascular endothelial growth factor-A (VEGF), is dramatically increased as a consequence of increased availability of binding sites on Fn (Mitsi et al., 2006, 2008; Smith et al., 2009; Martino and Hubbell, 2010). This interaction is specific for heparan sulfate, as chondroitin sulfate and desulfated derivatives of heparin do not increase VEGF binding (Mitsi et al., 2006).

Cell derived forces can mechanically strain Fn fibers (Smith et al., 2007), and the application of mechanical stress to Fn fibers leads to strain-induced alterations in the binding of numerous Fn ligands (Little et al., 2008, 2009; Cao et al., 2012). These interactions can also alter cell attachment, as recent work has suggested that Fn binding sites for bacterial adhesins are disrupted with high levels of Fn fiber strain (Chabria et al., 2010), and alterations in the conformation of the 9th and 10th type III repeats can reduce cell attachment (Grant et al., 1997; Wan et al., 2013). The Fn molecule contains a large repertoire of binding sites for cell adhesion molecules, other ECM components, and cell signaling molecules (Pankov and Yamada, 2002; Hynes, 2009), and thus the role of mechanical forces in regulation of Fn competence for attachment of Fn binding partners has been of interest for some time.

In vivo, the ECM is exposed to both mechanical regulation and chemical regulation of its conformation, and the combined effects are hypothesized to influence cell-signaling events. There is great interest in monitoring conformation changes of Fn, although currently available methods focus on mechanical strain-based conformation changes (Cao et al., 2012; Hertig et al., 2012). Antibodies (Abs) have been used for monitoring conformational changes of Fn for some time (Underwood et al., 1992; Ugarova et al., 1995; Zhong et al., 1998; Klein et al., 2003), however binding of an Ab cannot account for changes in Fn quantity. Here, we report on a dual Ab approach for monitoring heparin-mediated conformational changes in Fn within cell-generated Fn fibers in the ECM. A control Fn Ab with consistent binding affinity regardless of mechanical strain or heparin binding is used in conjunction with a conformation specific Ab. The ratiometric approach accounts for differences in Ab binding due to Fn quantity, thus overcoming limitations in previous approaches. Furthermore, this approach was used to determine the relative contribution of mechanical strain and heparin binding on the regulation of the activity of the growth factor-binding region of Fn in the 12th to 14th type III repeats of Fn. The Abs were initially screened using ELISAs, identifying heparin-sensitive Abs as well as a control Fn Ab that is conformation insensitive. The dual Ab technique was tested at the single fiber level and used to evaluate the mechanical impact on binding. Finally, the conformation of native cell made matrix was examined using the dual Ab screening system, demonstrating that this approach is competent for the detection of heparin-dependent regulation of Fn conformation even in cell-derived ECM.

2. Results

Heparan sulfates are expressed by nearly every animal cell type and, as a pervasive component of the ECM, are regularly in contact with Fn, where they can induce conformational changes of Fn to promote the binding of growth factors such as VEGF (Mitsi et al., 2006; Mitsi et al., 2008; Martino and Hubbell, 2010). Detection of altered conformational states is a major technical challenge, especially in vivo, and thus we sought to identify Abs that are sensitive to heparin-induced conformational changes in Fn. We chose to probe Abs that bind the Hep2, growth factor-binding domain of Fn, due to the importance of growth factor binding and presentation in regulation of cell behavior (Symes et al., 2010; Hudalla et al., 2011). Such Abs could then be used to detect heparin-mediated conformational changes in Fn matrix that render it competent for growth factor binding, even in complex cell culture and tissue environments, using widely accessible immunohistochemical approaches.

Quartz crystal microbalance with dissipation (QCMD) was chosen as a platform for examining the conformational regulation of heparin on surface absorbed Fn in real-time in aqueous conditions. For these experiments, Fn or bovine serum albumin (BSA) was adsorbed onto the chip surface causing a sharp reduction in frequency and increase in dissipation (Fig. 1). When the Fn-coated chip was exposed to phosphate buffered saline (PBS) alone or when the BSA coated chip was exposed to heparin for the remainder of the experiment, minimal changes in frequency or dissipation were observed. However when Fn-coated chips were exposed to heparin, a rapid increase in frequency and a decrease in dissipation were observed (Fig. 1C, D). Both concentrations of heparin tested (10 μg/ml and 100 μg/ml) caused a similar maximal change in frequency and dissipation after prolonged exposure (Fig. 1C, D). However, the initial rates of change were greater for the higher heparin concentration. The differences in the rates of change are consistent with our previous work showing that heparin catalytically converts Fn from a globular to a stable elongated structure (Mitsi et al., 2008). The heparin-mediated change in Fn structure is also consistent with an overall reduction in the roughness of a fibronectin layer on a polystyrene surface (Mitsi et al., 2006), which would predict a loss of associated water (increased frequency) and a stiffer and more ordered surface (reduced dissipation). Moreover, the fact that heparin did not induce these changes on the BSA coated surface suggests that they are not an artifact of the addition of the highly charged heparin. Thus, QCMD provides additional evidence that heparin catalytically modifies Fn structure and offers a means to quantitatively monitor the kinetics of this process in real-time (Mitsi et al., 2006; Molino et al., 2012).

To determine if the heparin-induced conformational alteration in Fn might lead to altered Ab binding to the Hep2 region, we conducted a series of ELISAs on Fn treated with and without heparin using anti-Fn Abs specific for the Hep2 region and a control Ab raised to full-length Fn. Fn was adsorbed onto polystyrene plates and treated with heparin over a range of 0 to 100 μg/ml. After washing the plates to remove heparin (demonstrated in (Mitsi et al., 2006)), primary Abs were incubated with samples, followed by HRP-conjugated secondary Abs for analysis of binding with a spectrophotometer. Heparin treatment at the range of concentrations did not affect the binding of the control Fn Ab to the Fn-coated surfaces, as confirmed by ANOVA (Fig. 2A). However, the binding of two Abs raised against the Hep2 domain was dependent upon whether Fn was pre-treated with heparin. A32 showed increased binding to heparin-pretreated Fn (Fig. 2B). Alternatively, MAB1935 showed decreased binding to Fn as the heparin concentration was increased (Fig. 2C). Thus, the heparin-induced conformational change in Fn appears to have altered the availability of the epitopes for these two Abs, with increased availability for A32 and reduced availability for MAB1935.

Cell contractile forces mechanically stretch Fn matrix fibers, and mechanical stress alters the molecular conformation of Fn within fibers (Smith et al., 2007; Bradshaw and Smith, 2011). Thus, we sought to determine whether mechanical tension applied to single fibers of Fn also altered the binding of monoclonal Ab A32. A32 was used since it demonstrated the largest relative change in binding to Fn in response