Developmental Biology 327 (2009) 386–398

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The physical state of fibronectin matrix differentially regulates morphogenetic movements *in vivo*

Tania Rozario [a], Bette Dzamba [a], Gregory F. Weber [a], Lance A. Davidson [b], Douglas W. DeSimone [a,*]

[a] Department of Cell Biology and the Morphogenesis and Regenerative Medicine Institute, University of Virginia, PO Box 800732, School of Medicine, Charlottesville, VA 22908, USA
[b] Department of Bioengineering, University of Pittsburgh, Bioscience Tower 3-5059, 3501 Fifth Avenue, Pittsburgh, PA 15260, USA

ARTICLE INFO

Article history:
Received for publication 8 September 2008
Revised 1 December 2008
Accepted 19 December 2008
Available online 30 December 2008

Keywords:
Fibronectin
Gastrulation
Xenopus
Morphogenesis
Integrin
Matrix assembly
Epiboly
Polarity
Migration
Convergent extension

ABSTRACT

This study demonstrates that proper spatiotemporal expression and the physical assembly state of fibronectin (FN) matrix play key roles in the regulation of morphogenetic cell movements *in vivo*. We examine the progressive assembly and 3D fibrillar organization of FN and its role in regulating cell and tissue movements in *Xenopus* embryos. Expression of the 70 kD N-terminal fragment of FN blocks FN fibril assembly at gastrulation but not initial FN binding to integrins at the cell surface. We find that fibrillar FN is necessary to maintain cell polarity through oriented cell division and to promote epiboly, possibly through maintenance of tissue-surface tension. In contrast, FN fibrils are dispensable for convergence and extension movements required for axis elongation. Closure of the migratory mesendodermal mantle was accelerated in the absence of a fibrillar matrix. Thus, the macromolecular assembly of FN matrices may constitute a general regulatory mechanism for coordination of distinct morphogenetic movements.

Introduction

A fundamental question in development is whether regulated changes in the physical organization of the ECM can inform both cell fate and morphogenetic decisions *in vivo*. Many multifunctional ECM proteins bind to receptors at cell surfaces and through intermolecular associations, assemble into higher-order structured states such as fibrils, cables and other macromolecular networks. In recent years, it has become increasingly apparent that the architectural state of assembled ECMs may have important functional consequences for the regulation of cell differentiation, cell motility and tissue organization.

The relationship between ECM and intracellular processes is one of dynamic reciprocity (Nelson and Bissell, 2006), in which constant feedback reinforces and maintains a cellular microenvironment that is critical to cell, tissue and organ level physiology. ECM confers spatial, physical and biochemical information to cells about their microenvironments (Green and Yamada, 2007). Spatial cues are transduced through the 3D organization of ECM. For example, cell behaviors such as fibroblast migration speed and directionality are enhanced on 3D vs. 2D ECM substrates (Green and Yamada, 2007). Cell fates and morphogenesis are also dependent on the 3D organization of ECM. On 2D substrates, mammary epithelial cells lose their identity, flatten and fail to respond to lactogenic cues. In 3D matrices, they assemble into polarized acinar structures similar to alveoli *in vivo* and secrete milk proteins (Barcellos-Hoff et al., 1989; Lee et al., 1985). Cell fate decisions are also dependent on mechanical properties of the ECM. Human mesenchymal stem cells, grown in the presence of appropriate inductive signals, will differentiate specifically into neurons, myoblasts or osteoblasts when placed on collagen substrates that have been "tuned" to approximate the elastic modulus of brain, muscle and bone tissues, respectively (Engler et al., 2006). In other studies, the differentiation of human mesenchymal stem cells to adipocyte or osteoblast lineages was affected by constraining cell shape using micropatterned ECM substrates (McBeath et al., 2004). These and other *in vitro* approaches highlight the importance of physiomechanical stimuli from ECM in the control of cell behavior and fate. A significant challenge, however, has been to elucidate whether and how changes in ECM architecture may regulate cell and tissue responses *in vivo*.

Fibronectin (FN) is a multifunctional adhesive glycoprotein of vertebrate ECMs. FN loss-of-function leads to defects in axial extension (Davidson et al., 2006; Marsden and DeSimone, 2003; Yang et al., 1999), polarized cell division (Marsden and DeSimone, 2001), mesoderm specification (George et al., 1993; Georges-Labouesse et al., 1996), and heart development (Trinh and Stainier, 2004). FN is present in at least three distinct physical states in the extracellular compartment: soluble FN dimers, cell-surface bound FN,

* Corresponding author. Fax: +1 434 982 3912.
E-mail address: desimone@virginia.edu (D.W. DeSimone).

0012-1606/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.12.025

and FN fibrils. Secreted FN dimers bind initially at cell surfaces to form a pericellular matrix that subsequently can be assembled and remodeled into 3D fibrillar structures (Mosher et al., 1992; Schwarzbauer and Sechler, 1999). In amphibian embryos, FN fibril assembly is spatio-temporally regulated; FN fibrils are assembled on the blastocoel roof (BCR) but not on the blastocoel floor (Boucaut and Darribère, 1983; Davidson et al., 2004; Lee et al., 1984). This spatial localization occurs even though all cells that line the blastocoel cavity are in contact with soluble FN and express integrin α5β1, a FN receptor that has been shown to initiate FN fibril assembly in these and other cells (Fogerty et al., 1990; McDonald et al., 1987). Fibrillogenesis begins at the onset of gastrulation in amphibians and is coincident with a variety of cell and tissue movements at this stage that are known to require FN (Davidson et al., 2006; Marsden and DeSimone, 2001, 2003).

A variety of approaches have been used to perturb FN-dependent cell adhesion and signaling at gastrulation in *Xenopus*. Most recently, mAbs directed against FN cell-binding domains (Marsden and DeSimone, 2001, 2003) or the integrin responsible for binding FN (Davidson et al., 2002) were employed along with integrin dominant negative constructs (Marsden and DeSimone, 2003) and morpholino knockdowns (Davidson et al., 2006) to identify the movements and cell fate decisions that involve cell–FN interactions at gastrulation. Each of these studies utilized either acute or chronic functional perturbations to demonstrate that FN–integrin interactions are important for epiboly, convergent extension and mesendoderm migration. However, once FN is expressed *in vivo* it undergoes a progressive process of assembly from surface-bound dimeric to fibrillar, coincident in space and time with the dramatic morphogenetic and signaling events of gastrulation. Whether or not these different organizational states of FN assembly are functionally equivalent has not been addressed by loss-of-function experiments in *Xenopus* or any other developmental system.

Does the 3D architecture of fibrillar FN matrix influence polarized cell movements that are crucial to gastrulation? To investigate functional differences between fibrillar and non-fibrillar states of FN in this system, we expressed the 70 kD N-terminal fragment of FN in embryos in order to interfere with FN–FN interactions important for fibril assembly (McDonald et al., 1987; McKeown-Longo and Mosher, 1985) but not FN-dimer binding to integrins. This method permits integrin binding to the Arg–Gly–Asp (RGD) containing central-cell-binding domain of FN, thus maintaining endogenous FN in a surface-bound pericellular state.

Materials and methods

Embryos and antibody staining

Embryos were obtained and cultured using standard methods and staged according to Nieuwkoop and Faber (1994). Keller sandwiches were made from stage 10 embryos as described by Keller and Danilchik (1988) and cultured for 6 h in DFA (Davidson et al., 2002) before fixation. Immunostaining was carried out as described previously (Marsden and DeSimone, 2001, 2003) using the following antibodies: mouse anti-FN mAb directed against Type III_{10} repeat of FN (4H2) 1:300; rabbit anti-FN polyclonal Ab (32FJ) 1:2000; rabbit anti-C-Cadherin (Xcad) 1:2000; and mouse anti-α tubulin 1:1000, followed by goat anti-mouse and rabbit IgG conjugated to Alexa-488, -555 or -647 fluorophores. BCRs were mounted in 50% glycerol/PBS on glass slides. Bisected embryos and Keller sandwiches were dehydrated in methanol and cleared in BB:BA (benzyl alcohol, benzyl benzoate) for microscopy.

RNA constructs and microinjection schemes

X. laevis 70 kD FN was PCR amplified from cDNA pD8 (DeSimone et al., 1992) and cloned into the pCS2+MT vector. For the control construct, the first 1.2 kbp was excised by restriction digest with SacI and religated. RNAs were transcribed *in vitro* using SP6 RNA polymerase. Control and 70 kD FN transcripts were injected in 5 nl containing ~1–3 ng of RNA.

Western blot

Xenopus embryos were solubilized in 200 μl lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 8.0, 1% Triton X-100, 2 mM PMSF [phenylmethylsulphonylfluoride], 2 μl protease inhibitor cocktail [Sigma P2714]). Protein extracts were diluted in 2× reduced Laemmli buffer (2% β-mercaptoethanol), separated by SDS-PAGE (6 or 7%) and blotted onto nitrocellulose for probing with antibodies.

DOC insolubility assay

10 embryos were solubilized in DOC buffer (2% DOC, 1 mM EDTA, 20 mM Tris–HCl pH 8.8, 2 mM Iodoacetic acid and Sigma protease inhibitor cocktail). DOC soluble extract was obtained after centrifugation at 14 K *g* for 30 min. The DOC insoluble pellet was dissolved in SDS buffer (substitute 2% DOC with 1% SDS). 5% of the DOC soluble extract and 100% of the DOC insoluble extract were analyzed by Western blot and probed with anti-FN mAb 4H2.

Mesendodermal mantle closure

Embryos were pre-fixed in MEMFA (3.7% formaldehyde, 0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$), dehydrated in methanol and rehydrated in PBST (PBS/0.1% Tween). The BCRs were peeled back using forceps and the mesendodermal mantle was imaged using a Zeiss LumarV12 stereomicroscope and Zeiss AxioCam MRm camera. Morphometric measurements were made using AxioVision software.

Mesendoderm migration assay

Glass coverslips were alkaline–ethanol washed and flamed prior to coating with FN. Coverslips were coated with 200 μl of 1–15 μg/ml bovine plasma FN (Calbiochem) overnight at 4 °C to yield a maximum coating density of 0.15–2.26 μg/cm^2. Mesendoderm tissue was excised from stage 10 *Xenopus* embryos and placed in Ca^{2+}/Mg^{2+}-free 1× MBS (1× MBS: 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES pH 7.8, 2.5 mM $NaHCO_3$) to dissociate the tissue to single cells. Cells were then transferred into 1× MBS containing Ca^{2+}/Mg^{2+} on FN-coated coverslips. Cells were allowed to attach for 1 h, then time-lapse phase-contrast images were acquired for approximately 1 h using a Hamamatsu Orca camera mounted on a Zeiss Axiovert 35 microscope equipped with a motorized stage driven by OpenLab software (Improvision). OpenLab image files were imported into ImageJ software (NIH) for analysis. Sobel edge detection kernels were used to create an outline defining the perimeter of each cell, and thresholds were set to select individual cells. The centroid of each cell area was tracked over time to determine total cell path length, which was then divided by the total time to calculate average cell speed. Only mesendoderm cells that were attached and spread at the beginning of image acquisition were analyzed for migration speed.

Quantification of FN by Ab binding to live BCRs

Live BCRs were dissected, cultured in 1/4 × MBS and incubated in anti-FN mAb 4H2 or anti-Fak mAb 2A7 for 30 min. Caps were then washed, solubilized in lysis buffer (non-reduced) and analyzed by Western blot. Blots were probed for IgG using anti-mouse secondary antibody.

Whole-mount in situ hybridization

In situ hybridizations were performed with slight modifications to the standard protocol (Harland, 1991). Protease K digestion was