Experimental Cell Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Novel proteins that regulate cell extension formation in fibroblasts

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ARTICLE INFO

Keywords:
Actin
Collagen
Matrix remodeling
Cell signaling

ABSTRACT

Cell extensions are critical structures that enable matrix remodeling in wound healing and cancer invasion but the regulation of their formation is not well-defined. We searched for new proteins that mediated cell extension formation over collagen by tandem mass tagged mass spectrometry analysis of purified extensions in 3T3 fibroblasts. Unexpectedly, importin-5, ENH isoform 1b (PDLIM5) and 26 S protease regulatory subunit 6B (PSMC4) were more abundant (> 10-fold) in membrane-penetrating cell extensions than cell bodies, which was confirmed by immunostaining and immunoblotting and also observed in human gingival fibroblasts. After siRNA knockdown of these proteins and plating cells on grid-supported floating collagen gels for 6 h, formation of cell extensions and collagen remodeling were examined. Knockdown of importin-5 reduced collagen compaction (1.9-fold), pericellular collagen degradation (~ 1.8-fold) and number of cell extensions (~ 69%). Knockdown of PSMC4 reduced collagen compaction (~ 1.5-fold), pericellular collagen degradation (~ 1.7-fold) and number of cell extensions (~ 42%). Knockdown of PDLIM5 reduced collagen compaction (~ 1.6-fold) and number of cell extensions (~ 21%). Inhibition of the TGF-β RI kinase, Smad3 or ROCK-II signaling pathways reduced the abundance of PDLIM5 in cell extensions but PSMC4 and importin-5 were reduced only by Smad3 or ROCK-II inhibitors.

We conclude that these novel proteins are required for cell extension formation and their recruitment into extensions involves the Smad3 and ROCK signaling pathways.

1. Introduction

Cell extensions play a critical role in several fundamental processes including physiological matrix remodeling [1], wound healing [2] and extracellular matrix invasion by cancer cells [1,3]. The formation of cell extensions relies in part on actin filament assembly, which contributes to the formation of spike-like protrusions (filopodia) and broader, fan-shaped protrusions (lamellipodia) [4,5]. Both of these structures are enriched with arrays of actin filaments that are contiguous with the adjacent sub-membrane actin cortex [6]. Filopodia play critical roles in binding and remodeling extracellular matrix (ECM) proteins, processes that are dependent on actin assembly and the formation of actin-rich adhesion complexes [7]. The formation of lamellipodia is also dependent on tightly regulated actin filament assembly at the leading edge of the cell [5], which is regulated in part by actin filament bundling and cross-linking proteins that help to regulate locally the mechanical properties of the actin polymer network. These mechanical properties are important for resisting deformation and preservation of cell shape as filopodia and lamellipodia expand and spread into the adjacent extracellular matrix [4]. The generation of filopodia and lamellipodia is associated with a large number of actin regulatory proteins including formins, VASP, actin capping proteins, Arp2/3 and WAVE, however how extension formation is regulated when these organelles extend into extracellular matrices like collagen is not well understood [5,8,9], particularly in collagen networks with well-defined mechanical properties.

Our understanding of cell-matrix interactions and the migration of cells into the ECM has been advanced by the use of progressively more sophisticated 3D hydrogel models [7,10,11]. Cognizant of the limitations imposed by even the most innovative 3D hydrogel models, we developed a floating collagen gel system supported by nylon grids of defined dimensions that provided insight into the influence of rigid physical boundaries on cell extension formation into collagen gels of known physiological stiffness [12]. As the signaling systems that control cell extension formation in the new gel system are not defined, we used mass-tagged tandem mass spectrometry to screen for proteins that are involved in the generation of cell extensions. With a previously described method [13] to separate cell extensions from cell bodies using micropore membranes, we found that in particular, ENH isoform 1b (PDLIM5) and 26 S protease regulatory subunit 6B (PSMC4) were novel, unexpected proteins that participated in the generation of cell extensions in the floating collagen gel model. The recruitment of these

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https://doi.org/10.1016/j.yexcr.2018.02.024
Received 19 October 2017; Received in revised form 19 February 2018; Accepted 20 February 2018

Table 1
Mass spectrometry analysis of differentially enriched proteins in cell body and cell protrusions.

Identified proteins	Accession number	4 h Upper chamber	4 h Bottom chamber	8 h Upper chamber	8 h Bottom chamber	24 h Upper chamber	24 h Bottom chamber
Tubulin beta-5 chain	TBB5_MOUSE	0	1.6	1	1.3	0.1	0.3
Importin-5	IPO5_MOUSE	0	1	0.5	0.8	− 0.1	0.1
Cytoplasmic Dynein 1 heavy chain 1	DYHC1_MOUSE	0	1	0.3	0.7	−0.3	0.5
ENH Isoform 1b	D9J2Z9_MOUSE (+5)	0	1.8	1.3	1.4	0.7	1.4
26S protease regulatory subunit 6B	PRS6B_MOUSE (+4)	0	1.7	1.3	1.6	1.0	0.6

Data are $\log_{10}$ fold difference of the abundance of predicted proteins in the indicated fractions compared with their abundance in whole cell lysates. The Upper chamber fractions represent the cell bodies and the Bottom chamber fractions represent the cell extensions. These data are derived from one experiment. For each experiment, the type 1 error associated with the protein assignment based on peptide analyses was $p < 0.01$. Repeats of this experiment ($n = 3$) showed very similar results with < 10% deviation of the fold-differences of enrichment that are shown here for each protein.

proteins into nascent cell extensions was in turn mediated by the Smad and ROCK signaling pathways.

2. Materials and methods

2.1. Reagents

Bovine dermal type I collagen was purchased from Advanced BioMatrix (6.2 mg/ml: ~ 97% type I collagen; San Diego, CA). TGX221, SB431542 and SIS3 were purchased from Calbiochem (San Diego, CA). Fasudil HCL was purchased from Selleck Chem (Houston, TX). Rabbit monoclonal antibody against β-tubulin was purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal antibody against DYNC1H1 was purchased from Protein Tech. (Rosemont, IL). Rabbit polyclonal antibodies to importin- 5 and PDLIM5 were purchased from Gene Tex (Irvine, CA). Rabbit polyclonal antibody to PSMC4 was purchased from US Biological (Salem, MA). Affinity purified, rabbit antibody to a neo-epitope of a ¾ collagen fragment (collagen cleavage site) was purchased from Immunoglobe (Himmelstadt, Germany). Alexa Fluor 568-conjugated goat anti-rabbit and anti-mouse antibodies and Alex Fluor 488-conjugated phalloidin were purchased from Invitrogen (Waltham, MA). IRDye 800CW anti-rabbit goat IgG (H + L) and IRDye 680RD anti-mouse goat antibody were obtained from Mandel (Guelph, ON). Oligonucleotides for siRNA knockdown were obtained from Dharmacon, GE Healthcare, Mississauga, ON.

2.2. Cell culture

NIH 3T3 mouse fibroblasts and human gingival fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (10% FBS) and a 10% antibiotic solution (146 units/ml penicillin G, 50 µg/ml gentamycin and 0.25 µg/ml amphotericin). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell protrusion assay and tandem mass spectrometry

Purification of cell extensions was conducted as described [13]. Briefly, NIH 3T3 fibroblasts were seeded into T-75 flasks (Becton Dickinson, Oakville, ON) in 10% FBS in DMEM and grown to subconfluence. Prior to the conduct of migration and pseudopodia preparation assays, cells were starved (0% FBS in DMEM) for 3 h. Cell protrusions were separated with a pseudopodia purification kit (ECM 660; Millipore, Billerica, MA) that used six-well polycarbonate membrane Transwell® inserts (3.0 µm pore size; Corning, Tewksbury, MA). The polycarbonate porous membranes used in the pseudopodia purification inserts were coated with a sufficient volume of collagen (bovine dermal type I collagen; 1 mg/ml) to provide a 100 µm thickness of protein on the membranes. Collagen was polymerized at 37 °C for 2 h and cells (in a total volume of 1.5 ml medium comprised of 0% FBS in DMEM)) were seeded on to the upper chamber inserts, placed into the wells of six-well plates that contained 2.5 ml of 20% FBS in DMEM and incubated at 37 °C.

We harvested cell extensions and cell bodies using previously described methods [13]. For purification of cell extensions (located on the bottom of the inserts), after 4, 8 and 24 h, cotton swabs were used to remove the cell body from the upper insert surface and the remaining pseudopodia were collected in lysis buffer. For harvesting of cell bodies (on the top of the membranes), cotton swabs were used to remove the cell extensions from the bottom of the membrane and the cell bodies were collected in lysis buffer. Samples were frozen at − 20 °C. Subsequently, acetone was added to samples followed by drying with an evaporator.

Samples were reduced, alkylated, digested, and TMT-labelled according to the manufacturer's directions (Thermo Fisher TMT 10 Plex). Labelled peptides from the top and bottom samples were combined and lyophilized. Peptides were re-suspended in 2.5% TFA and were then bound to a homemade Strong Cation Exchange column (SCX). Peptides were eluted off in fractions (5% Ammonium Hydroxide, 80% ACN), and then lyophilized.

Samples were analyzed on an Orbitrap analyzer (Q-Exactive, ThermoFisher, San Jose, CA) outfitted with a nanospray source and EASY-nLC nano-LC system (ThermoFisher, San Jose, CA). Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75 µm x 50 cm PepMax RSLC EASY-Spray column filled with 2 µM C18 beads (ThermoFisher San, Jose CA). Peptides were eluted using formic acid acetonitrile. Peptides were introduced by nano-electrospray into the Q-Exactive mass spectrometer.

Tandem mass spectra were extracted and all MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.1.14) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Sequest was set up to search Uniprot_Mouse_Nov_18_2015.fasta (Downloaded Nov 18 2015, 74993 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search the Uniprot_Mouse_Nov_18_2015 database. Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability. Peptide probabilities from X! Tandem were assigned by the Peptide Prophet algorithm [14] with Scaffold delta-mass correction. Peptide Probabilities from Sequest were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.