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Tensin 2 Modulates Cell Contractility in 3D Collagen Gels Through the RhoGAP DLC1

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ABSTRACT

Cytoskeletal proteins of the tensin family couple integrins to the actin cytoskeleton. They are found in both focal adhesions and the fibrillar adhesions formed between cells and the fibronectin matrix. There are four tensin genes which encode three large (~200 kDa) tensin isoforms (tensin 1, 2, 3) and one short isoform (cten). However, the subcellular localization and function of the individual isoforms is poorly understood. Using human foreskin fibroblasts (HFFs), and imaging on both fixed and live cells, we show that GFP-tensin 2 is enriched in dynamic focal adhesions at the leading edge of the cell, whereas GFP-tensin 3 translocates rearward, and is enriched in fibrillar adhesions. To investigate the possible role of tensins in cell-matrix remodeling, we used siRNAs to knockdown each tensin isoform. We discovered that tensin 2 knockdown significantly reduced the ability of HFFs to contract 3D collagen gels, whilst no effect on fibronectin fibrillogenesis was observed. This inhibition of collagen gel contraction was associated with a substantial reduction in Rho activity, and it was reversed by depletion of DLC1, a RhoGAP that binds to tensin in focal adhesions. These findings suggest that focal adhesion-localized tensin 2 negatively regulates DLC1 to permit Rho-mediated actomyosin contraction and remodeling of collagen fibers. J. Cell. Biochem. 109: 808–817, 2010.

KEY WORDS: TENSIN; FIBRONECTIN MATRIX ASSEMBLY; FIBRILLAR ADHESION; COLLAGEN CONTRACTION; DLC1; RhoGAP

Many interactions between animal cells and the extracellular matrix (ECM) are mediated by members of the integrin family of cell adhesion molecules. Integrins are α/β heterodimers that bind ECM proteins through their large extracellular domain whilst their cytoplasmic tails, particularly those of the integrin β subunit, are frequently coupled to the actin cytoskeleton via a series of linker proteins that include tensin, talin, filamin, α-actinin, melusin, integrin-linked kinase, and skelemin [Otey et al., 1993; Reddy et al., 1998; Brancaccio et al., 1999; Legate and Fassler, 2009]. Although linkage of integrins to the cytoskeleton is essential to integrin function, it is less apparent why so many different proteins are involved. Clearly, each linker protein has a different structure, shape, mode of regulation and binding partners. Therefore, each linker may support a different type of actin cytoarchitecture at distinct locations within the cell. For example, focal adhesions are enriched in integrin αVβ3, tensin, talin, vinculin and phosphotyrosine-containing proteins such as paxillin, whilst fibrillar adhesions, formed between cells and fibronectin, are enriched in the integrin α5β1 and tensin [Zamir et al., 1999; Pankov et al., 2000].

In vertebrates, the term tensin describes a family of large (~200 kDa) cytoskeletal proteins encoded by three genes that are also subject to alternative splicing. A fourth gene cten encodes a much smaller protein that is homologous to the C-terminal part of the larger isoforms [Lo, 2004]. The large tensin isoforms share a common domain structure with an N-terminal region that has homology with the lipid phosphatase and C2 domains of the tumor suppressor PTEN, and a C-terminal region that contains both an SH2 and a PTB domain. The intervening region is not conserved and is predicted to be unstructured. Tensin has both actin cross-linking and capping activity [Lo et al., 1994; Chuang et al., 1995], and the SH2 domain has been implicated in binding to a number of signaling proteins (e.g., PI3K, FAK, p130Cas,

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Pyk2, Dok2 and PDK1) that regulate cell migration [Salgia et al., 1995; Auger et al., 1996; Benzing et al., 2001; Szabo et al., 2002; Calderwood et al., 2003; Wavreille and Pei, 2007]. Indeed, overexpression of GFP-tensin 1 or 2 in HEK293 cells increases cell migration in a manner that is dependent on a functional SH2 domain [Chen et al., 2002; Chen and Lo, 2003]. Intriguingly, cten is overexpressed in mammary tumors that contain high levels of EGFR and Her2, and the EGF-mediated upregulation of cten and down-regulation of tensin 3 has been shown to enhance cell migration [Katz et al., 2007]. All tensins interact with DLC1 [Yam et al., 2006; Liao et al., 2007; Qian et al., 2007], a RhoGAP that is down-regulated in a variety of human cancers either through gene deletion or DNA methylation [Liao and Lo, 2008]. Re-expression of DLC1 inhibits cell growth in several cancer cell lines, and the interaction with tensin is required for this tumor suppressor-like activity [Liao et al., 2007; Qian et al., 2007].

Tensin interacts with integrins via its PTB domain, which binds to the membrane proximal NPxY motif in β-integrin tails, the same motif recognized by the talin PTB domain [McCleverty et al., 2007]. However, while phosphorylation of the NPxY motif inhibits talin binding [Oxley et al., 2008], it has no effect on binding of the tensin PTB domain, suggesting that phosphorylation of integrin tails might act as a switch, driving the disassembly of integrin/talin complexes and favoring the formation of integrin/tensin complexes [McCleverty et al., 2007]. Analysis of the dynamics of fibrillar adhesion formation in human foreskin fibroblasts (HFFs) shows that although both α5β1 and αVβ3 integrins are initially localized in focal adhesions, α5β1 translocates out of these structures into tensin-containing fibrillar adhesions in a Src-dependent manner, and tensin remains localized in large focal adhesions in cells lacking Src [Volberg et al., 2001]. Engagement of the αVβ3 integrin, an intact actin cytoskeleton and dephosphorylation of paxillin on tyrosines 31 and 118 are also required for this process [Wierzbicka-Patynowski et al., 2007], although it is independent of cell migration [Pankov et al., 2000]. Cytoskeletal linker proteins such as talin, ILK and PINCH have also been shown to be important in fibronectin matrix assembly [Wu et al., 1998; Guo and Wu, 2002; Feral et al., 2007; Shi et al., 2008]. However, the role of the various tensin isoforms in these integrin-mediated events remains unclear.

In the present study, we have characterized the sub-cellular localization and dynamics of each of the three large tensin isoforms in HFFs, and their contribution to matrix remodeling. We find substantial differences in localization and dynamics of these isoforms. Tensin 2 is highly dynamic and enriched in focal adhesions at the leading edge of cells, whereas tensin 3 is enriched in fibrillar adhesions. In contrast tensin 1 appears to be equally distributed between focal and fibrillar adhesions. To determine the role of each of the tensin isoforms in remodeling the ECM, we analyzed the effects of siRNA-mediated tensin knockdown on the ability of HFFs to contract a collagen gel and to make a fibronectin matrix. Consistent with its dynamics and characteristic localization in focal adhesions, tensin 2 knockdown was particularly effective in inhibiting gel contraction, and this was accompanied by a substantial reduction in Rho activity. The inhibition was reversed by depleting cells of the tensin-binding RhoGAP DLC1 that is also present in focal adhesions, suggesting a role for focal adhesion-localized tensin 2 in negatively regulating DLC1 and promoting Rho-dependent remodeling of the ECM.

MATERIALS AND METHODS

The antibodies used in this study were: rabbit anti-GFP (A6455, Invitrogen); mouse anti-tensins (610064, BD Transduction Laboratories), (MAB1649, Chemicon International), (05–183, Upstate Biotechnology). Rabbit anti-tensin 3 (Rb33 raised against tensin 3 residues 439–457 (Biosource)); mouse anti-α5 integrin (SNAKA51), rabbit anti-fibronectin, mouse anti-vimentin (V5255, Sigma); mouse anti-GAPDH (MAB374, Chemicon International).

pEGFP TENSIN cDNAs

RNA isolated from HFFs using RNeasy kit (Qiagen) was DNaseI treated using DNA Free (Ambion), and 1 μg of RNA was then reverse transcribed using a poly-T primer and SuperScript II (Invitrogen). Primers specific to the 5′ and 3′ ends and internal primers spanning unique restriction sites of the tensin cDNAs were designed (Table S1). These primers were used to amplify cDNA by PCR, using Platinum Pfx DNA polymerase (Invitrogen), either from the reverse transcribed RNA or from the tensin 2 cDNA clone IHS1380 (Open Biosystems). The amplified material was digested with the appropriate restriction enzymes and cloned into the pEGFP-C1 (Clontech) or pmCherry vectors using standard molecular biology techniques.

TRANSFECTION OF HUMAN EMBRYONIC KIDNEY (HEK293) CELLS

HEK293 were cultured in DMEM supplemented with 10% FCS and 100 μM MEM non-essential amino acids (Invitrogen). Confluent cells were split 1:5 the day before transfection. Plasmid DNA and Fugene 6 (Roche Applied Science) were mixed in a 1:3 ratio in DMEM and incubated for 40 min. Transfection complexes were added to the cells dropwise. Transfected cells were harvested in RIPA buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, complete protease inhibitor (Roche Applied Science) 24 h post-transfection, mixed with SDS sample buffer and boiled for 5 min.

WESTERN BLOTTING

Samples were run on a 7.5% SDS–polyacrylamide gel and transferred by wet transfer to Hybond-C nitrocellulose (Amersham Biosciences). The nitrocellulose was blocked with 5% non-fat milk in 10 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST) and then incubated with primary antibody followed by appropriate HRP conjugated secondary antibody (anti-mouse IgG HRP NA931, Amersham. Anti-rabbit IgG HRP A6154, Sigma). Blots were visualized using the ECL system (Amersham Biosciences) and Super RX Fujifilm.

TRANSFECTION OF HUMAN FORESKIN FIBROBLASTS (HFFs)

Primary HFFs were a gift from Susan Yamada (NIDCR, NIH) and were transfected at passages 7–18 using lipofection or electroporation.

Cells were seeded onto 19 mm coverslips (Menzel Glaser) in 12-well plates (6×10^4 cells/well) and cultured overnight in DMEM and 10% FCS without antibiotics. Plasmid DNA (1 μg) was diluted in 100 μl Opti-MEM medium before adding 0.5 μl Plus reagent (Invitrogen), and incubated for 15 min. Then 1.75 μl of Lipofectamine LTX (Invitrogen) was added, mixed and incubated 25 min. Transfection complexes were added dropwise to the cells and cultured overnight at 37°C, 10% CO_2. For electroporation of HFFs, cells were incubated overnight with 0.2 M thymidine. Cells were washed with Hanks balanced salts solution (Invitrogen) and harvested by trypsinization. 1.5×10^6 cells were resuspended in 550 μl electroporation buffer (20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and transferred into a 0.4 cm gap cuvette (Bio-Rad). Cells were electroporated at 170 V and 960 μFd using a Gene Pulser (Bio-Rad). Cells were cultured in media containing 5 mM sodium butyrate.

INDIRECT IMMUNOFLUORESCENCE

Lipofected cells were fixed for 20 min with 4% (w/v) paraformaldehyde, 5% (w/v) sucrose in PBS. Cells were stained with rabbit anti-fibronectin and mouse anti-α5 integrin antibody (SNAKA51) followed by, fluorescent secondary antibodies with minimal cross-reactivity to other species, Cy5-conjugated $F(ab')_2$ fragment donkey anti-rabbit IgG (H + L) and Rhodamine Red-X-conjugated $F(ab')_2$ fragment donkey anti-mouse IgG (H + L) (711-176-152, 715-296-151 Jackson ImmunoResearch Laboratories respectively). Stained samples were mounted in Prolong Gold Antifade Reagent (Invitrogen) and immunofluorescence images were obtained with a Leica TCS SP5 confocal microscope; the collected data were deconvolved using Huygens Essential 3.3.0p3 (Specific Volume Imaging B.V.).

TIRF MICROSCOPY

HFFs were transiently co-transfected with either GFP-tensin 2 and mCherry-tensin 3 or GFP-tensin 1 and mCherry-tensin 3 by electroporation. At 24–48 h post-transfection, the cells were passaged and allowed to adhere overnight in complete medium to culture dishes (MatTek) pre-coated with 1 μg/ml human plasma fibronectin. The cells were visualized using two-channel total internal reflection fluorescence (TIRF) microscopy. The Olympus IX71 TIRF microscope was equipped with a TIRF illuminator, back-thinned 512 × 512 EM-CCD camera (Hamamatsu Photonics K.K., Japan), 60×/1.45 NA TIRF objective, Argon 457, 488, 514 nm laser lines, and krypton–argon 568, 647 nm laser lines. Images of eGFP and mCherry fluorescence were collected every 30 s. Acquired fluorescence images were processed by applying the following MetaMorph (MDS Analytical Technologies, Inc.) filters: (i) low pass 3 × 3 pixels filter; (ii) sharpen high filter; (iii) median 3 × 3 pixels filter; (iv) flat background filter. Pictures of fluorescence images were assembled with Adobe Photoshop CS2.

HFF TRANSFECTION WITH siRNA

HFFs were transfected with siRNA as described by Endo et al. [2006]. 1.5×10^5 HFF were plated in culture media in the absence of antibiotics into a 6 well plate and incubated at 37°C, 10% CO_2 overnight. Five microliters of Lipofectamine 2000 (Invitrogen) was diluted in 250 μl of Opti-MEM and incubated for 5 min before adding to siRNA diluted in 250 μl of Opti-MEM. The mixture was incubated for 30 min and added to cells with an additional 0.5 ml culture media. The transfected cells were left for 4 h before addition of another 1 ml culture medium. siRNA was obtained from Dharmacon, siRNA pools were used unless stated otherwise (Table S2).

QUANTITATIVE REAL-TIME PCR

RNA was isolated and reverse transcribed as for the construction of the cDNAs. PCR reactions were set up in triplicate using 10 ng reverse transcription mixture, 0.8 μM of each forward and reverse primers (Table S1) and 1× iQ SYBR Green supermix (Bio-Rad) per sample. PCR reactions were run in a MiniOpticon real-time PCR system (Bio-Rad).

PREPARATION OF DEOXYCHOLATE INSOLUBLE MATRIX FROM HFFs

Cells were harvested in 20 mM Tris HCl pH 8.5, 1% (w/v) deoxycholic acid, 2 mM *N*-ethylmaleimide, 2 mM iodoacetic acid, 2 mM EDTA, 2 mM PMSF (DOC buffer), extruded through a 23-gauge needle five times and centrifuged at 20,000*g* for 20 min at 4°C. The pellet was washed once with DOC buffer and prepared for SDS–polyacrylamide electrophoresis.

COLLAGEN GEL CONTRACTION ASSAY

Collagen gels were formed by mixing 2 ml collagen, (3 mg/ml PureCol from Nutacon), 1 ml 3× DMEM, 0.5 ml FCS, and 1×10^5 siRNA transfected HFFs in 1.5 ml MEM 25 mM HEPES on ice. The cell/collagen mixture was plated into a 60 mm uncoated Petri dish in the presence or absence of 10 μg/ml of the inhibitory β1 integrin antibody mAb13 or 0.2 μg/ml of cell permeable C3 exotransferase Rho inhibitor (CT04, Cytoskeleton Inc.). Triplicate dishes were set up for each siRNA transfected HFF condition. The plates were incubated at 37°C and set within 1 h. Images were taken of each gel at 24 h time points using the Gel Doc system (Bio-Rad laboratories) for up to 96 h, and the gel area was quantified using image J.

RHO ACTIVITY ASSAY

Tissue culture 15 cm diameter plates (Nunc) were coated with a 0.3 mg/ml solution of collagen (PureCol) for at least 3 h. 2×10^6 HFFs were plated onto the collagen-coated plates and incubated for 1 h (unless stated otherwise) at 37°C, 10% CO_2. The Rho activity assay was performed as described in the manufacturers protocol (Millipore). Cells were washed twice with ice cold TBS and harvested in 500 μl MBS buffer. Cell lysate was centrifuged for 5 min at 14,000*g* at 4°C. A 40 μl sample was taken for measuring total Rho, and the remaining supernatant was mixed with 40 μl Rhotekin RBD agarose and mixed for 45 min at 4°C. The agarose was pelleted by centrifugation, washed 3 times with MBS, and mixed with 25 μl SDS sample buffer, 2 μl of 1 M DTT and samples boiled for 5 min. All samples were analyzed on 12% polyacrylamide gels and Western blotted with anti-Rho antibody (clone 55, Millipore) at 3 μg/ml. Westerns were visualized using the SuperSignal West Dura substrate (Thermo Scientific) and Hyperfilm X-ray film (Amersham Pharmacia Biotech).

RESULTS

TENSIN ISOFORMS LOCALIZE TO DIFFERENT ADHESION STRUCTURES

Localization studies of endogenous tensin isoforms have been hampered by the limited number of antibodies available and lack of isoform specificity. Previous studies have shown that in NIH 3T3 cells, GFP-tensin 2 localizes to focal adhesions and does not target fibrillar adhesions as efficiently as tensin 1 [Chen et al., 2002]. Tensin 3 was found in focal adhesions of A549 lung epithelial cells [Cui et al., 2004]. Rat tensins are localized in both focal and fibrillar adhesions in REF52 cells [Zamir et al., 1999]. However, it was unclear whether the anti-tensin antibody from BD Transduction Laboratories used in the latter study recognized all tensin isoforms or whether it was isoform-specific. To address this issue of specificity, all three human large tensin isoforms were expressed as GFP-tagged proteins in HEK293 cells, and cell extracts were probed with the same antibody as well as other commercially available anti-tensin antibodies from Chemicon International and Upstate Biotechnology. The results showed that all three antibodies are specific for tensin 1 while no commercially available antibodies exist that are specific for tensin 2 or 3 (Fig. S1). To determine the distribution of the tensin isoforms in primary human cells, we expressed each of the pEGFP-tensin cDNAs in human foreskin fibroblasts (HFFs), and compared their localization to fibronectin and α5 integrin, a subunit of the fibronectin receptor α5β1. All three tensins were found to be present in both focal and fibrillar adhesions but to markedly differing degrees. Thus, tensin 2 was localized mainly in focal adhesions, tensin 3 was mostly found in fibrillar adhesions and tensin 1 was found in both types of adhesion (Fig. 1).

The localization and dynamics of the three tensin isoforms were explored further using TIRF microscopy and live-cell imaging

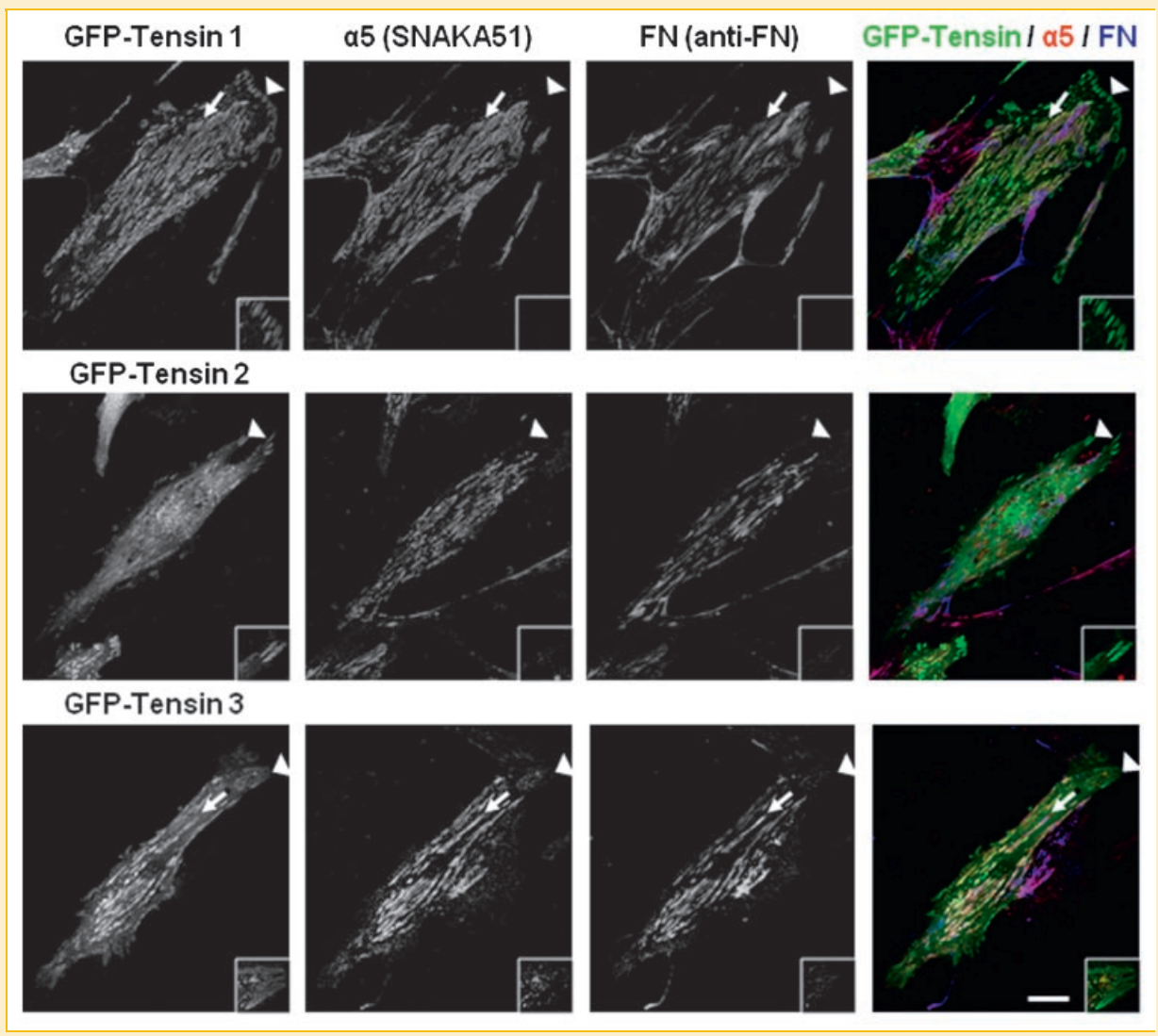

Fig. 1. Representative images of the localization of GFP-tensin isoforms expressed in human foreskin fibroblasts (HFFs). HFFs transfected with the pEGFP-tensin cDNAs were fixed and stained with anti-α5 integrin (SNAKA51), which detects the ligated α5β1 receptor, and anti-fibronectin (anti-FN) to identify fibrillar adhesions (arrows). Arrowheads indicate focal adhesions. Scale bar, 20 μm. Insets are an enlargement of a representative region of focal adhesions.

following co-transfection of HFFs with mCherry-tensin 3 and either GFP-tensin 2 or GFP-tensin 1 (Fig. 2). Comparisons of the TIRF images showed that tensin 2 had the most unique distribution—it was restricted to newly formed adhesions at the leading edge and periphery of the cell and was found only rarely in fibrillar adhesions on the ventral (bottom) surface of cells. The distribution of tensin 1 and tensin 3 were similar to each other, but tensin 1 localized toward the leading edge to a greater degree. Adhesions in the cell body behind the leading edge showed prominent localization of both tensin 1 and tensin 3, but not tensin 2.

Examination of tensin dynamics by time-lapse TIRF microscopy revealed that at the leading edge of the cell, tensin 2 (green) remains localized at the front of growing adhesions (white asterisk, Fig. 2A), whereas tensin 3 (red) translocates posteriorly (Fig. 2A, ROI 1). Behind the leading edge, tensin 2 leaves the adhesions as the cell migrates forward, while tensin 3 remains in adhesions under the cell body (Fig. 2A, ROI 2, supplementary movies 1–3). Like tensin 3 (red), tensin 1 (green) also remains localized to the adhesions under the cell body (Fig. 2B, ROI 1). However, the amounts of tensin 3 in the adhesions under the cell body behind the leading edge increase as the cell migrates forward, while the amount of tensin 1 does not increase. The net effect is that the intensity of tensin 3 becomes greatest in adhesions at the cell body while the intensity of tensin 1 is greatest in adhesions toward the front of the cell (Fig. 2B, ROI 1

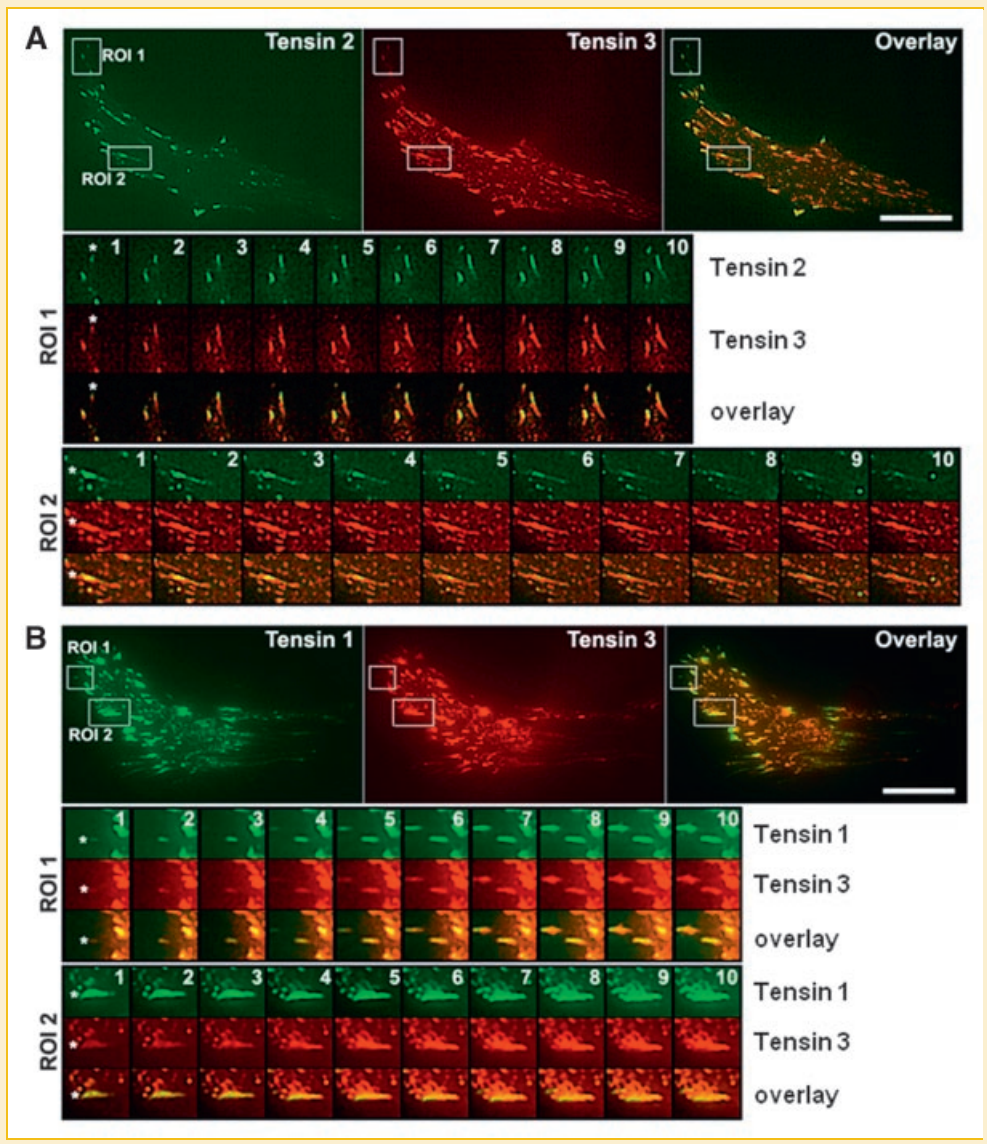

Fig. 2. Analysis of tensin dynamics using live-cell imaging. HFFs transfected with GFP- or mCherry-tensin cDNAs were plated onto fibronectin-coated (1 μg/ml) glass-bottom culture dishes and visualized using total internal reflection fluorescence (TIRF) microscopy. A: Image of whole cell co-expressing pEGFP-tensin 2 and pmCherry-tensin 3. Region of interest 1 (ROI 1) indicates the dynamics of tensin 2 and tensin 3 in an adhesion (asterisk) (6.5 min frame intervals). ROI 2 shows tensin 2 and tensin 3 dynamics in a preformed adhesion under the cell body (9 min frame intervals). B: Image of whole cell co-expressing pEGFP-tensin 1 and pmCherry-tensin 3. ROI 1 indicates the accumulation of tensin 1 and tensin 3 in an adhesion (asterisk) (3.5 min frame intervals). ROI 2 shows tensin 1 and tensin 3 in a preformed adhesion under the cell body (12.5 min frame intervals). Asterisk indicates front of adhesion. Scale bar, 30 μm.

and ROI 2; Supplementary movies 4–6). Taken together, these findings indicate that because of its distinctive localization and dynamics, tensin 2 is the most unique of the isoforms, and its localization in focal adhesions may indicate a function in remodeling of the ECM.

TENSIN ISOFORM-SPECIFIC KNOCKDOWN BY RNA INTERFERENCE

We examined the role of each tensin isoform in remodeling the ECM using Dharmacon siRNA SMARTpools to knockdown the expression of the individual tensin isoforms. The specificity and efficiency of siRNA knockdown of tensin was analyzed in HFFs at both the mRNA and protein level. Quantitative real-time PCR showed substantial mRNA knockdown at 10 nM siRNA for each of the tensin isoforms (approximately fivefold reduction, Fig. S2). The same siRNA concentrations produced maximal and specific knockdown of tensin 1 and 3 proteins as detected using isoform specific antibodies (Supplementary Fig. 2). However, we have been unable to generate an antibody specific for tensin 2 to confirm siRNA-mediated knockdown at the protein level.

KNOCKDOWN OF ANY OR ALL TENSIN ISOFORMS HAS NO EFFECT ON FIBRONECTIN MATRIX ASSEMBLY

To explore the role of the tensin family in fibronectin matrix assembly, HFFs were transfected with all three tensin siRNA SMARTpools individually or combined. After 24 h the cells were trypsinized to destroy the pre-existing fibronectin matrix and replated. After a further 24 h, the cells were fixed and stained for fibronectin. Tensin knockdown had no observable effect on the fibronectin matrix compared to control cells (Fig. S3A). To investigate this further, cells were treated in an identical manner and harvested in deoxycholate buffer (DOC). The amounts of fibronectin incorporated into the DOC-insoluble matrix were quantified by Western blotting with anti-fibronectin (Fig. S3B). Although there were fluctuations in the amount of fibronectin matrix assembled, there was no statistically significant difference between cells transfected with the individual or combined tensin siRNAs or control siRNAs. We therefore conclude that knockdown of the large tensin isoforms has no effect on the organization of the fibronectin matrix of HFFs.

TENSIN 2 IS INVOLVED IN CONTRACTION OF THE EXTRACELLULAR MATRIX

Collagen gel assays provide a convenient platform to investigate cell behavior and matrix remodeling in 3D more closely representing cell behavior in vivo [Grinnell, 2003]. To establish whether tensin isoforms are required for the cell to exert the mechanical force required to remodel the surrounding matrix, we tested the ability of tensin-depleted cells to contract collagen gels. HFFs were incorporated into collagen gels 24 h after they were transfected with tensin or control siRNAs. The percentage of collagen used, cell number and culture dish size were such that cell contraction exerted sufficient force to separate the collagen gel from the sides of the dish, thereby allowing the gel to relax and float. A series of images of the collagen gels were taken at 24 h time points (Fig. 3A). The area of the gels was calculated for each time point (Fig. 3B), cell morphology and viability was noted. HFFs transfected with the tensin 2 siRNA SMARTpool showed a greatly reduced ability to contract the collagen gel, and a significant difference in collagen gel area was observed. In contrast, knockdown of tensin 1 or 3 had no significant effect compared to that of the control siRNA. Individual siRNAs against tensin 2 gave the same phenotype as that observed with the SMARTpool of 4 siRNA oligos (Fig. S4). All transfected cells remained morphologically similar to controls, with retention of membrane processes and no difference in cell viability observed. The fact that tensin 2 is the dominant isoform involved in HFF mediated collagen gel contraction correlates with the fact that it is predominantly localized to focal adhesions at the cells leading edge where force is exerted by actomyosin contraction on the extracellular matrix, to initiate remodeling.

TENSIN 2 EXPRESSION INDIRECTLY MODULATES Rho ACTIVITY

To explore the mechanisms by which tensin 2 might contribute to HFF-induced collagen gel contraction, we first sought to confirm that the process was dependent on the β1-integrin family of collagen receptors. The collagen gel contraction assay was therefore performed in the presence of an inhibitory β1 integrin antibody (mAb13), which proved to be a potent inhibitor of collagen gel contraction. Similarly, treatment of the cells with the cell-permeable C3 exotransferase Rho inhibitor confirmed that active Rho is essential for gel contraction (Figs. 3A and S5). Subsequently, we investigated whether depletion of tensin 2 in some way compromised the activity of Rho. Unfortunately, it was not possible to measure Rho activity in cells within collagen gels due to interference by the large amounts of collagen, and we therefore assayed Rho in HFFs plated on collagen-coated plastic. Maximal activation of Rho was achieved 1 h after plating, but it then rapidly became inactive (Fig. 4A). Knockdown of any of the three tensin isoforms significantly reduced Rho activity, but the tensin 2 siRNA produced the greatest effect with only 20% Rho activity remaining compared to the control (Fig. 4B).

The activity of Rho is controlled by a variety of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), and it has recently been shown that tensin 2 interacts with the RhoGAP Deleted in Liver Cancer1 (DLC1) in focal adhesions [Yam et al., 2006]. To investigate whether DLC1 contributes to the reduced ability of tensin 2-depleted HFFs to contract collagen gels, cells were co-transfected with both tensin 2 and DLC1 siRNAs. Transfection of the DLC1 siRNAs alone gave similar results to the control and did not alter the cells ability to contract the gel (data not shown). Strikingly, two different individual DLC1 siRNAs rescued the tensin 2 contraction defect, such that cells transfected with both the tensin 2 and DLC1 siRNAs were able to contract 3D gels to a similar extent to the controls (Fig. 4C).

DISCUSSION

The major findings of this study comparing the location and function of the three large tensin isoforms are as follows: (1) Each tensin isoform has a distinctive subcellular location. Tensin 2 is present at the front edge of growing focal adhesions at the leading

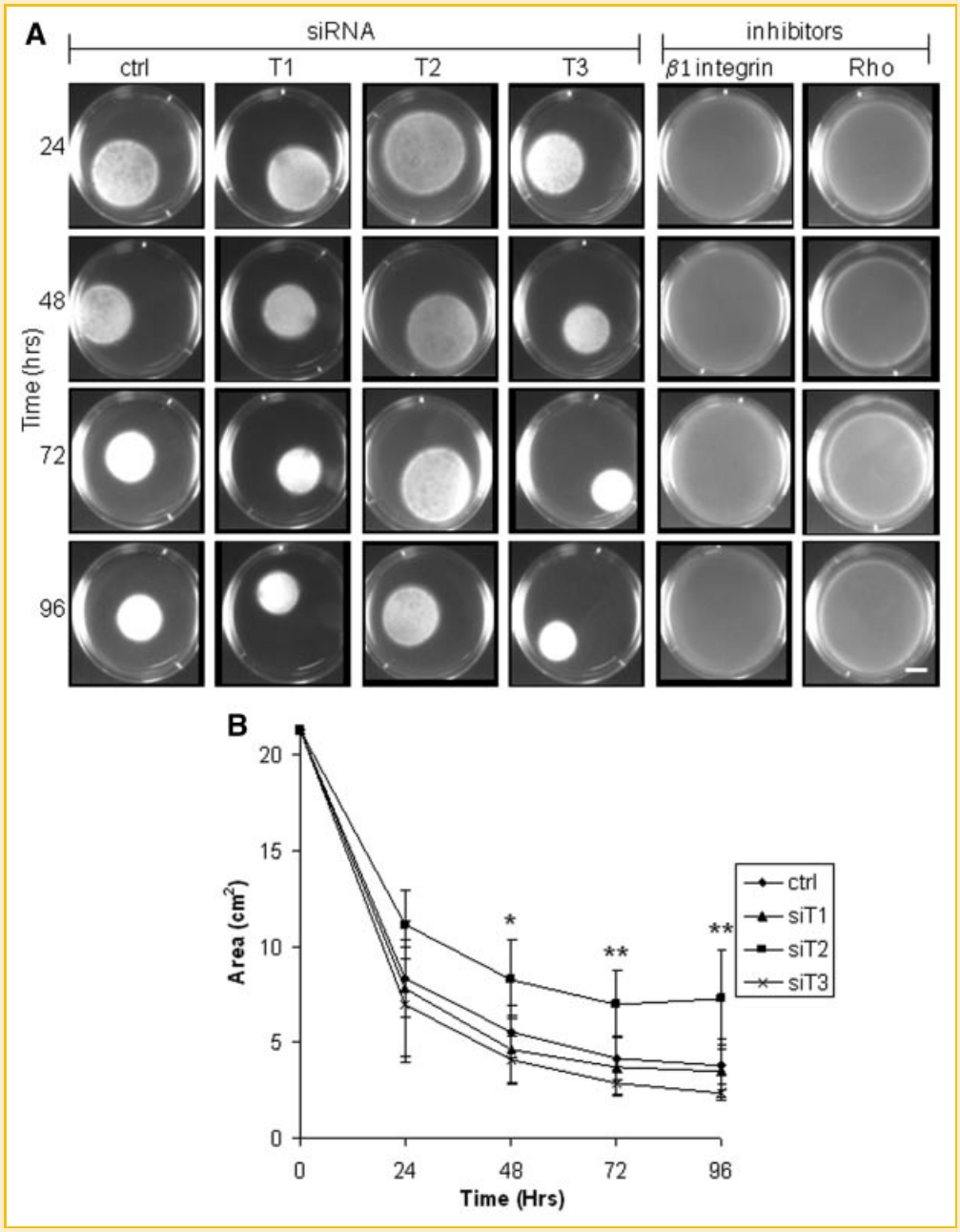

Fig. 3. Collagen gel contraction by HFFs is dependent on tensin 2. A: HFFs were transfected with siRNA directed against each of the three tensin isoforms (T1, T2, T3) or treated with 10 μg/ml an inhibitory β1 integrin antibody (mAb13) or 0.2 μg/ml cell permeable C3 exotransferase Rho inhibitor (Rho), and incubated within 3D collagen gels. The extent of collagen gel contraction was recorded at 24 h time points (scale bar, 1 cm). B: Quantification of collagen gel contraction using measurement of gel area (n = 6, error bars represent SD). Tensin 2 siRNA transfection was compared to control using a one way ANOVA with Dunnett post-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

edge of primary human fibroblasts; its levels are dynamic but it does not translocate along adhesions; tensin 1 and tensin 3 are present in fibrillar adhesions further back from the leading edge. However, tensin 1 remains stable and higher in anterior adhesions, while tensin 3 translocates back along adhesions and is enriched in fibrillar adhesions under the cell body. (2) Contraction of 3D collagen gels by human fibroblasts is dependent on tensin 2 through modulation of Rho activity, and it does not depend on tensins 1 or 3. (3) Depletion of the RhoGAP DLC1, which binds to tensin in focal adhesions, reverses the inhibition of collagen gel contraction after tensin 2 knockdown.

Although some previous work has attempted to localize the tensin isoforms in different types of cell adhesions, no direct comparisons of localization of the various isoforms has been performed in the same cell type. In NIH 3T3 cells, GFP-tensin 2 localized to focal adhesions and did not target fibrillar adhesions as efficiently as tensin 1 [Chen et al., 2002]. Tensin 3 was found in focal adhesions of A549 lung epithelial cells, but these cells are unlikely to make

fibrillar adhesions and were used because of antibody cross-reactivity problems with tensin 1 [Cui et al., 2004]. We clarified by determining the antibody specificity that the rat tensin localized in both focal and fibrillar adhesions in REF52 cells [Zamir et al., 1999] is tensin1. Extending these results, we find that there is a hierarchy of tensin localization to cell adhesions, with tensin 2 being closest to the leading edge followed by tensin 1, and then tensin 3. The anterior location and dynamics of tensin 2 at sites ideally suited for early interactions with extracellular matrix suggest a role in matrix remodeling. In contrast, tensin 3 which has the ability to fully translocate centripetally as the adhesion grows is predominantly localized in more posterior fibrillar adhesions. Previous live cell imaging using GFP-chicken tensin also indicated translocation of tensin into longer fibrillar adhesion-like structures [Zamir et al., 2000]. The highly conserved nature of the N- and C-terminal regions of these proteins suggests that it is the central divergent and apparently unstructured regions of the tensin isoforms that are likely involved in their differing cellular localizations and functions.

Although tensins are a major cytoskeletal component of fibrillar adhesions [Zamir et al., 1999], and expression of a chicken tensin fragment, the AH2 region, blocked the formation of fibrillar adhesions and the process of fibronectin fibrillogenesis [Pankov et al., 2000], knockdown of all three tensin isoforms had no apparent effect on the assembly of fibronectin matrix under the conditions we used. It is not understood how the AH2 region mediates its dominant-negative effect on fibrillar adhesion formation. The sequence of this region of chicken tensin is not conserved within the human tensin isoforms, but it falls within the unstructured central region and may function by coupled folding and binding to its functional partners [Wright and Dyson, 2009]. The tensin family of proteins may not be essential for fibronectin fibrillogenesis, or compensation by other linker proteins such as talin, ILK or PINCH may occur [Wu et al., 1998; Guo and Wu, 2002; Feral et al., 2007; Shi et al., 2008; Green et al., 2009]. Indeed, a recent study has shown that ILK and PINCH1 are essential for focal adhesion maturation into fibrillar adhesions, and tensin availability did not alter the fibrillar adhesion defect in the ILK or PINCH knock-out cells. However, ILK and α-parvin are required for tensin recruitment to fibrillar adhesions [Stanchi et al., 2009]. Since tensin 2 is a dynamic component of focal adhesions, we therefore investigated its role in remodeling of a 3D ECM.

Collagen gel contraction has been used as a model for pro-contractile remodeling of the ECM by fibroblasts in processes such as wound healing [Grinnell, 2003]. The collagen gel system we used

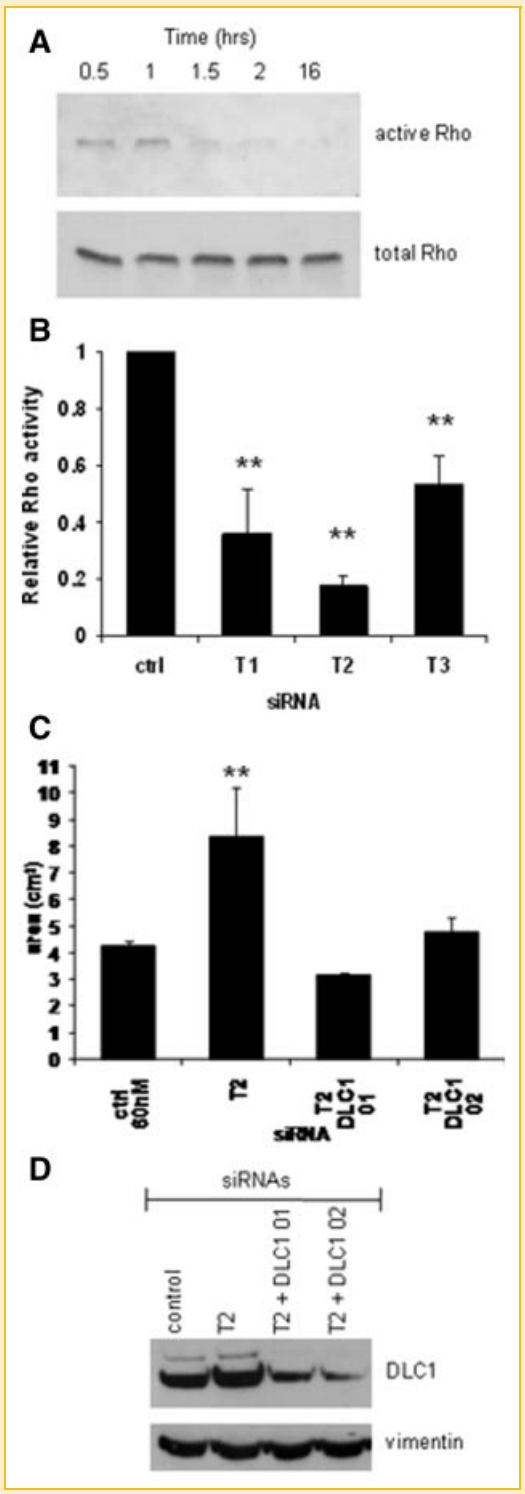

Fig. 4. Tensin expression levels modulate Rho activity through the RhoGAP deleted in liver cancer 1 (DLC1). A: HFFs were plated onto collagen-coated plastic plates, cell lysates were harvested at set time points, and the levels of active Rho were determined using a rhotekin pull-down assay. Active and total Rho were assessed by Western blotting. B: HFFs transfected with siRNAs directed against either tensin 1 (T1), tensin 2 (T2) or tensin 3 (T3), or control (ctrl) siRNA were plated for 1 h onto collagen coated plastic, and Rho activity was measured as above. The activity was quantified by densitometry; active Rho was normalized to the amount of total Rho protein, and results are presented relative to cells transfected with control siRNA ($n = 3$, error bars represent SD). Data were compared to controls using a one-way ANOVA with Dunnett post-test ($^{*}P < 0.05$ $^{**}P < 0.01$). C: Measurement of collagen gel area comparing HFFs transfected with control (ctrl), tensin 2 (T2) or a combination of tensin 2 and DLC1 siRNAs (DLC1 01, DLC1 02) ($n = 3$, error bars represent SD). Tensin 2 siRNA transfection was compared to cell transfected with the control siRNA or tensin 2 alone or tensin 2 plus DLC1 siRNAs using a one-way ANOVA with Tukey Kramer post-test ($^{**}P < 0.01$). D: HFFs transfected as for the collagen contraction assay and cell lysates were harvested for Western blotting with anti-DLC1 and anti-vimentin as a loading control.

allows cells to develop endogenous tension strong enough to separate the gel from the dish edge. This method depends on signaling mechanisms important for cell contractility [Grinnell, 2000]. Using this system, we showed that HFF gel contraction is dependent on β1 integrin, most probably the two major collagen receptors α2β1 and α11β1 integrin [Carver et al., 1995; Cooke et al., 2000] and is inhibited by very low doses of the Rho inhibitor C3 exotransferase, indicating that it is highly sensitive to the global level of Rho activity. Therefore, it was particularly significant to find that knockdown of tensin 2 expression substantially inhibited the activity of Rho and impaired the ability of the cells to contract the collagen gel. Depletion of tensin 1 or 3 had no effect on collagen gel contraction. In contrast, fibroblasts taken from plastic surgery and transfected with high concentration of tensin 1 siRNA demonstrated a reduced collagen gel contraction, this correlated with aged fibroblasts having reduced tensin 1 expression and capability to contract collagen gels [Saintigny et al., 2008]. The higher concentration of siRNA used in this study may cause off target effects, and the source and passage number of the fibroblasts may also influence the experimental outcomes.

Although depletion of all tensin isoforms affected Rho activation levels, tensin 2 knockdown had the greatest effect, reducing Rho activity by 80%. The RhoGAP DLC1 has been shown to localize to focal adhesions [Kawai et al., 2004] and was subsequently identified as a tensin-binding protein [Yam et al., 2006]. DLC1 interacts with the SH2 domain of all tensins in a tyrosine phosphorylation-independent manner, via Tyr442, which is essential for DLC1 localization to the focal adhesion [Liao et al., 2007; Qian et al., 2007]. DLC1 has been found subsequently to have an essential binding site for the tensin 2 PTB domain, which may be involved in tensin 2-specific recruitment of DLC1 to the focal adhesion [Chan et al., 2009]. Interestingly transfection of MDA-MB 231 breast cancer cells with tensin 1 mutants F302A or R1488A, which bind reduced levels of DLC1, have twofold lower levels of active Rho compared to wild-type tensin 1 [Hall et al., 2009]. DLC1 has been identified as a tumor suppressor and is an inhibitor of cell growth. However, because the collagen gel contraction assays were performed in relaxed gels where the cells are quiescent, the ability of DLC1 to suppress cell growth is unlikely to have affected the outcome of the results reported here. In addition, no difference in cell viability was observed between the different siRNA-transfected cells. We therefore speculated that experimental depletion of tensin 2 might result in unregulated DLC1 activity at the focal adhesion, and its RhoGAP function would reduce Rho activity in a specific localized manner and limit gel contraction. Consistent with this hypothesis, knockdown of DLC1 dramatically rescued the ability of fibroblasts depleted of tensin 2 to contract collagen gels. The DLC1 knockdown alone had no effect on gel contraction, indicating a tensin2 regulatory role. DLC1 RhoGAP activity can be inhibited by intramolecular interaction through its SAM domain [Kim et al., 2008] or by the more recently discovered interaction with p120Ras-GAP, a DLC1 inhibitor [Yang et al., 2009]. p120Ras-GAP co-localizes with DLC1 at the focal adhesion, where tensin 2 is also present. The recently identified binding site for the tensin 2 PTB domain within DLC1 [Chan et al., 2009] indicates a different relationship between these proteins to that between DLC1 and the other tensin isoforms. This leads us to suggest that tensin 2 inhibits DLC1 either by stabilizing the intramolecular interaction with the SAM domain or promoting the interaction between DLC1 and p120Ras-GAP. Loss of tensin 2 could therefore reduce Rho activity and contractility by permitting activation of the DLC1 RhoGAP.

In conclusion, we have demonstrated distinct localization and dynamics of all three large tensin isoforms. Tensin 2 is highly dynamic and is enriched in focal adhesions, whereas tensin 3 mainly locates to fibrillar adhesions, and tensin 1 appears to be equally distributed between both adhesion types. Tensin 2 plays a critical role in regulating Rho activity and collagen gel contraction by a novel mechanism in which it locally modulates DLC1 RhoGAP function in focal adhesions at the leading edge, permitting Rho activation and subsequent matrix remodeling.

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REFERENCES

Auger KR, Songyang Z, Lo SH, Roberts TM, Chen LB. 1996. Platelet-derived growth factor-induced formation of tensin and phosphoinositide 3-kinase complexes. J Biol Chem 271:23452–23457.

Benzing T, Gerke P, Hopker K, Hildebrandt F, Kim E, Walz G. 2001. Nephrocystin interacts with Pyk2, p130(Cas), and tensin and triggers phosphorylation of Pyk2. Proc Natl Acad Sci USA 98:9784–9789.

Brancaccio M, Guazzone S, Menini N, Sibona E, Hirsch E, De Andrea M, Rocchi M, Altruda F, Tarone G, Silengo L. 1999. Melusin is a new muscle-specific interactor for beta(1) integrin cytoplasmic domain. J Biol Chem 274: 29282–29288.

Calderwood DA, Fujioka Y, de Pereda JM, Garcia-Alvarez B, Nakamoto T, Margolis B, McGlade CJ, Liddington RC, Ginsberg MH. 2003. Integrin beta cytoplasmic domain interactions with phosphotyrosine-binding domains: A structural prototype for diversity in integrin signaling. Proc Natl Acad Sci USA 100:2272–2277.

Carver W, Molano I, Reaves TA, Borg TK, Terracio L. 1995. Role of the alpha 1 beta 1 integrin complex in collagen gel contraction in vitro by fibroblasts. J Cell Physiol 165:425–437.

Chan LK, Ko FC, Ng IO, Yam JW. 2009. Deleted in liver cancer 1 (DLC1) utilizes a novel binding site for Tensin2 PTB domain interaction and is required for tumor-suppressive function. PLoS ONE 4:e5572.

Chen H, Lo SH. 2003. Regulation of tensin-promoted cell migration by its focal adhesion binding and Src homology domain 2. Biochem J 370:1039–1045.

Chen H, Duncan IC, Bozorgchami H, Lo SH. 2002. Tensin1 and a previously undocumented family member, tensin2, positively regulate cell migration. Proc Natl Acad Sci USA 99:733–7338.

Chuang JZ, Lin DC, Lin S. 1995. Molecular cloning, expression, and mapping of the high affinity actin-capping domain of chicken cardiac tensin. J Cell Biol 128:1095–1109.

Cooke ME, Sakai T, Mosher DF. 2000. Contraction of collagen matrices mediated by alpha2beta1A and alpha(v)beta3 integrins. J Cell Sci 113(Pt 13): 2375–2383.

Cui Y, Liao YC, Lo SH. 2004. Epidermal growth factor modulates tyrosine phosphorylation of a novel tensin family member, tensin3. Mol Cancer Res 2:225–232.

Endo Y, Even-Ram S, Pankov R, Matsumoto K, Yamada KM. 2006. Inhibition of rho GTPases by RNA interference. Methods Enzymol 406: 345–361.

Feral CC, Zijlstra A, Tkachenko E, Prager G, Gardel ML, Slepak M, Ginsberg MH. 2007. CD98hc (SLC3A2) participates in fibronectin matrix assembly by mediating integrin signaling. J Cell Biol 178:701–711.

Green JA, Berrier AL, Pankov R, Yamada KM. 2009. beta 1 integrin cytoplasmic domain residues selectively modulate fibronectin matrix assembly and cell spreading through talin and AKT-1. J Biol Chem

Grinnell F. 2000. Fibroblast-collagen-matrix contraction: Growth-factor signalling and mechanical loading. Trends Cell Biol 10:362–365.

Grinnell F. 2003. Fibroblast biology in three-dimensional collagen matrices. Trends Cell Biol 13:264–269.

Guo L, Wu C. 2002. Regulation of fibronectin matrix deposition and cell proliferation by the PINCH-ILK-CH-ILKBP complex. FASEB J 16:1298–1300.

Hall EH, Daugherty AE, Choi CK, Horwitz AF, Brautigan DL. 2009. Tensin1 requires protein phosphatase 1a in addition to RHO(GAP) DLC-1 to control cell polarization, migration and invasion. J Biol Chem 284:34713–34722.

Katz M, Amit I, Citri A, Shay T, Carvalho S, Lavi S, Milanezi F, Lyass L, Amariglio N, Jacob-Hirsch J, Ben-Chetrit N, Tarcic G, Lindzen M, Avraham R, Liao YC, Trusk P, Lyass A, Rechavi G, Spector NL, Lo SH, Schmitt F, Bacus SS, Yarden Y. 2007. A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. Nat Cell Biol 9:961–969.

Kawai K, Yamaga M, Iwamae Y, Kiyota M, Kamata H, Hirata H, Homma Y, Yagisawa H. 2004. A PLCdelta1-binding protein, p122RhoGAP, is localized in focal adhesions. Biochem Soc Trans 32:1107–1109.

Kim TY, Healy KD, Der CJ, Sciaky N, Bang YJ, Juliano RL. 2008. Effects of structure of Rho GTPase-activating protein DLC-1 on cell morphology and migration. J Biol Chem 283:32762–32770.

Legate KR, Fassler R. 2009. Mechanisms that regulate adaptor binding to {beta}-integrin cytoplasmic tails. J Cell Sci 122:187–198.

Liao YC, Lo SH. 2008. Deleted in liver cancer-1 (DLC-1): A tumor suppressor not just for liver. Int J Biochem Cell Biol 40:843–847.

Liao YC, Si L, deVere White RW, Lo SH. 2007. The phosphotyrosine-independent interaction of DLC-1 and the SH2 domain of cten regulates focal adhesion localization and growth suppression activity of DLC-1. J Cell Biol 176:43–49.

Lo SH. 2004. Tensin. Int J Biochem Cell Biol 36:31–34.

Lo SH, Janmey PA, Hartwig JH, Chen LB. 1994. Interactions of tensin with actin and identification of its three distinct actin-binding domains. J Cell Biol 125:1067–1075.

McCleverty CJ, Lin DC, Liddington RC. 2007. Structure of the PTB domain of tensin1 and a model for its recruitment to fibrillar adhesions. Protein Sci 16:1223–1229.

Otey CA, Vasquez GB, Burridge K, Erickson BW. 1993. Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. J Biol Chem 268:21193–21197.

Oxley CL, Anthis NJ, Lowe ED, Vakonakis I, Campbell ID, Wegener KL. 2008. An integrin phosphorylation switch: The effect of beta3 integrin tail phosphorylation on Dok1 and talin binding. J Biol Chem 283:5420–5426.

Pankov R, Cukierman E, Katz BZ, Matsumoto K, Lin DC, Lin S, Hahn C, Yamada KM. 2000. Integrin dynamics and matrix assembly: Tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J Cell Biol 148:1075–1090.

Qian X, Li G, Asmussen HK, Asnaghi L, Vass WC, Braverman R, Yamada KM, Popescu NC, Papageorge AG, Lowy DR. 2007. Oncogenic inhibition by a deleted in liver cancer gene requires cooperation between tensin binding and Rho-specific GTPase-activating protein activities. Proc Natl Acad Sci USA 104:9012–9017.

Reddy KB, Gascard P, Price MG, Negrescu EV, Fox JE. 1998. Identification of an interaction between the m-band protein skelemin and beta-integrin subunits. Colocalization of a skelemin-like protein with beta1- and beta3-integrins in non-muscle cells. J Biol Chem 273:35039–35047.

Saintigny G, Bernard FX, Juchaux F, Pedretti N, Mahe C. 2008. Reduced expression of the adhesion protein tensin1 in cultured human dermal fibroblasts affects collagen gel contraction. Exp Dermatol 17:788–789.

Salgia R, Brunkhorst B, Pisick E, Li JL, Lo SH, Chen LB, Griffin JD. 1995. Increased tyrosine phosphorylation of focal adhesion proteins in myeloid cell lines expressing p210BCR/ABL. Oncogene 11:1149–1155.

Shi X, Qu H, Kretzler M, Wu C. 2008. Roles of PINCH-2 in regulation of glomerular cell shape change and fibronectin matrix deposition. Am J Physiol Renal Physiol 295:F253–F263.

Stanchi F, Grashoff C, Nguemeni Yonga CF, Grall D, Fassler R, Van Obberghen-Schilling E. 2009. Molecular dissection of the ILK-PINCH-parvin triad reveals a fundamental role for the ILK kinase domain in the late stages of focal-adhesion maturation. J Cell Sci 122:1800–1811.

Szabo IL, Pai R, Jones MK, Ehring GR, Kawanaka H, Tarnawski AS. 2002. Indomethacin delays gastric restitution: Association with the inhibition of focal adhesion kinase and tensin phosphorylation and reduced actin stress fibers. Exp Biol Med (Maywood) 227:412–424.

Volberg T, Romer L, Zamir E, Geiger B. 2001. pp60(c-src) and related tyrosine kinases: A role in the assembly and reorganization of matrix adhesions. J Cell Sci 114:2279–2289.

Wavreille AS, Pei D. 2007. A chemical approach to the identification of tensin-binding proteins. ACS Chem Biol 2:109–118.

Wierzbicka-Patynowski I, Mao Y, Schwarzbauer JE. 2007. Continuous requirement for pp60-Src and phospho-paxillin during fibronectin matrix assembly by transformed cells. J Cell Physiol 210:750–756.

Wright PE, Dyson HJ. 2009. Linking folding and binding. Curr Opin Struct Biol 19:31–38.

Wu C, Keightley SY, Leung-Hagesteijn C, Radeva G, Coppolino M, Goicoechea S, McDonald JA, Dedhar S. 1998. Integrin-linked protein kinase regulates fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. J Biol Chem 273:528–536.

Yam JW, Ko FC, Chan CY, Jin DY, Ng IO. 2006. Interaction of deleted in liver cancer 1 with tensin2 in caveolae and implications in tumor suppression. Cancer Res 66:8367–8372.

Yang XY, Guan M, Vigil D, Der CJ, Lowy DR, Popescu NC. 2009. p120Ras-GAP binds the DLC1 Rho-GAP tumor suppressor protein and inhibits its RhoA GTPase and growth-suppressing activities. Oncogene

Zamir E, Katz BZ, Aota S, Yamada KM, Geiger B, Kam Z. 1999. Molecular diversity of cell-matrix adhesions. J Cell Sci 112(Pt 11):1655–1669.

Zamir E, Katz M, Posen Y, Erez N, Yamada KM, Katz BZ, Lin S, Lin DC, Bershadsky A, Kam Z, Geiger B. 2000. Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. Nat Cell Biol 2:191–196.