Tensin1 Requires Protein Phosphatase-1α in Addition to Rhogap DLC-1 to Control Cell Polarization, Migration, and Invasion*

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Tensin is a family of multidomain scaffold proteins that bind the cytoplasmic tail of β-integrins and localize to adhesions that anchor stress fibers in cells. Tensin expression is suppressed in cancer, especially metastatic cancer. The N-terminal domain of tensin1 associates with protein phosphatase-1α (PP1α) and mediates PP1α localization to adhesions. Here, we show F302A mutation in a KVXT motif of tensin1 abrogates binding to PP1α. The SH2 domain in tensin family member c-ten requires R474 to bind a Rhogap called DLC-1 (deleted in liver cancer). We mutated the corresponding residue in tensin1, R1488A, and showed this reduces association with DLC-1. Unexpectedly, tensin1 F302A also had reduced association with DLC-1. Expression of tensin1 F302A or R1488A showed similar dominant phenotypes, with reduced cell polarization, lowered MLC20 phosphorylation and reduced levels of RhoA(GTP) compared with cells expressing tensin1 WT. However, migration and invasion of metastatic MDA MB 231 breast cancer cells were differentially affected by tensin1 mutated at F302A or R1488A. Cancer cells stably expressing F302A tensin1 showed increased migration and invasion compared with cells stably expressing either R1488A tensin1 or WT tensin1. This suggests that PP1α bound to tensin1 has additional effects in reducing migration and invasion that are not mediated through DLC-1. Our results show the importance of PP1α binding to tensin1 for the regulation of cell polarization, migration, and invasion.

Focal adhesions are not simply structural elements of the cytoskeleton but are also sites for integrin-based signaling that connect the actin cytoskeleton of tissue culture cells to the extracellular matrix. Focal adhesions generate signals that allow cells to protrude their leading edge during cell spreading and migration (1, 2). Different types of focal adhesions have been described such as initial adhesions that contain focal adhesion kinase, paxillin, and vinculin and fibrillar adhesions that contain tensin and are relatively low in paxillin. These fibrillar adhesions bind to actomyosin filaments (stress fibers) in the cytoskeleton and are responsible for generating contractile force in cells spread on extracellular matrix (3–7).

The tensin family of proteins include tensin1, tensin2, tensin3, and c-ten, which lacks the N-terminal PTP-related region in the other tensin proteins (5). Common to all tensin family members are tandem SH2 and PTB domains at the C terminus. EGF addition to epithelial cells triggers epithelial-mesenchymal transition, which is accompanied by reduced expression of tensin3 and increased expression of c-ten, suggesting different tensin family proteins can affect differentiation and cell migration (8–10).

Tensin1 is expressed in normal tissues (11); however, analysis of human clinical specimens reveals that human breast carcinoma, prostate carcinoma, head and neck squamous cell carcinoma, and melanoma have greatly reduced expression of tensin1 (12). It has also been shown that some cancer cell lines do not express detectable levels of tensin1 protein relative to normal fibroblasts that have abundant expression (11). Deletion of the single tensin gene in Drosophila gives a phenotype of blistered wings that was attributed to destabilizing integrin links to the cytoskeleton (13, 14). Tensin knock-out mice were viable, and the phenotype noted was kidney defects (15). Only years later was it appreciated that there are multiple tensin genes.

An important function of tensins is association with a Rhogap protein called deleted in liver cancer-1 (DLC-1), which itself is a negative regulator of tumor formation and plays a role in cell migration (16–18). There is some controversy about how and where DLC-1 binds to tensin family members. Tensin 2 was found in a yeast 2-hybrid screen using DLC-1 as bait (19). GST-DLC1(375–509) pulled down the Myc-tagged PTB domain of tensin2, indicating the PTB domain alone was sufficient for binding DLC-1. On the other hand, binding of DLC-1 with c-ten was mapped by yeast two-hybrid with the SH2 domain, but not the PTB domain (17). Either the SH2 or PTB domain of chicken tensin1 fused to GST exhibited DLC-1 binding (18). Endogenous tensin1 and DLC-1 were co-immunoprecipitated from human Mel1011 melanoma cells (18). Mutation R474A in the c-ten SH2 domain eliminated co-precipitation of DLC-1 (17). This R residue aligns with other tensin family proteins in the P-Tyr-binding pocket of the SH2 domain. However, binding of DLC-1 to the c-ten SH2 domain does not require DLC-1 tyrosine phosphorylation of reside Tyr-442, which was determined to be the critical tyrosine residue in DLC-1 for binding to c-ten (17). The SH2 domains from tensin

2 The abbreviations used are: SH2, Src homology 2 domain; PP1α, protein phosphatase-1α isoform; DLC-1, deleted in liver cancer 1; MLC20, myosin light chain; FBS, fetal bovine serum; MOPS, 4-morpholinopropanesulfonic acid; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; PTB, phosphotyrosine binding domain; WT, wild type.
family members also interact in a P-Tyr-dependent manner with PI 3-kinase, p130Cas, and FAK, suggesting that tensins have multiple links to signal transduction pathways with a potential role in cell motility, tumor suppression, and cell proliferation (5). We previously found that the N-terminal region of tensin1, related in sequence to the tumor suppressor protein PTEN (phosphatase and tensin homologue), is sufficient for isoform-specific association with PP1α, a protein phosphatase involved in different signaling pathways (20). In the current study we individually mutated KVXF motifs in tensin1 and identified residue Phe-302 as required for binding to PP1α. In addition, we mutated Arg-1488 in tensin1, and showed this mutation reduces co-precipitation of DLC-1. Unexpectedly, the F302A mutant also showed reduced binding of DLC-1 compared with WT. On the other hand, the R1488A mutant bound PP1α the same as WT tensin1. Expression of either F302A or R1488A tensin1 resulted in loss of cell polarization, lower phospho-MLC (myosin light chain), and lower RhoA activation in cells compared with cells expressing tensin1 WT. In contrast to the same effects on MLC and RhoA, the F302A and R1488A mutations had opposite effects on MDA MB 231 human breast cancer cell migration and invasion, suggesting that PP1α regulates both DLC-1 dependent and independent functions of tensin1, and is required as a critical partner for tensin1.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK-293 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen), rat embryo fibroblasts (REF52), and NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Cells were transfected according to the manufacturer’s instructions with Arrest-In™ Transfection Reagent (Open Biosystems). MDA MB 231 were cultured in L-15 medium supplemented with 10% FBS. CHO.K1 cells were cultured in F-12K medium supplemented with 10% FBS and transfected using Lipofectamine (Invitrogen). All cell cultures except MDA MB 231 cells (grown without CO2), were maintained at 37 °C in a humidified atmosphere of 5% CO2. Cells obtained from the University of Virginia Tissue Core Facility originated from ATCC. S-protein-agarose and S-Tag™ monoclonal antibody were purchased from Novagen. HA-probe (F-7) antibody, EGFP (B-2)-agarose, and EGFP (FL) rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology. DLC-1 mouse monoclonal antibody was purchased from BD Transduction Laboratories. Secondary antibodies Alexa Fluor® 680 goat anti-rabbit IgG and anti-mouse IgG were purchased from Invitrogen, and IR Dye 800 conjugated affinity-purified anti-rabbit IgG and anti-mouse IgG were purchased from Rockland.

Tensin Point Mutations—Full-length and 1–740 pTriEX (S-tag)-tensin1 and EGFP-tensin1 DNA were utilized for the construction of tensin1 point mutations. The tensin F302A point mutation was produced by PCR amplification with primers; sense: 5’-CAG AGT ATG GCA AAG TGG AGG CTG TAT TTT CTT ATG GCC CAG-3’ and antisense: 5’-CGC CAT AAG AAA ATA CAG CCT CCA TCT TGC CAT ACT CTG-3’. The tensin R1488A point mutation was produced by PCR amplification with primers; sense: 5’-GGG GCC TTC ATC ATC GCC GAC AGT CAC TCC TT-3’ and antisense: 5’-AAG GAG TGA CTG TCG GCC ATG ATG AAG GCC CC-3’. PCR products were digested with DpnI for 1 h at 37 °C and transformed into DH5α chemically competent cells. Colonies were selected, and DNA sequence confirmed point mutations.

PP1α Binding—HEK293 cells were co-transfected with HA3-PP1α and either pTriEX-tensin-(1–740) or pTriEX-tensin-(1–740) F302A or with either pTriEx-tensin or pTriEx-tensin F302A, and after 48 h, cells were lysed on ice for 15 min and sonicated briefly in 700 μl of 50 mM MOPS, pH 7.0, 0.1 M NaCl, 1 mM EGTA, 5% glycerol, 0.1% Tween 20, 0.1% 2-mercaptoethanol, 0.4 mM Pefabloc. The lysate was centrifuged at 13,000 rpm for 15 min, and the supernatant was incubated with S-protein agarose for 3 h at 4 °C. The agarose was washed three times in lysis buffer, and samples were boiled in 1X SDS for 5 min. Samples were resolved on 12% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were incubated with anti-HA and anti-S-tag antibodies, washed, and incubated with secondary antibodies. Immunoblots were scanned by the Odyssey Infrared Imaging System (LiCor®) and quantitated with the Odyssey Application Software version 2.0.41 (LiCor®).

Cell Spreading Assay—CHO.K1 cells transfected with EGFP-tensin WT or EGFP-tensin F302A were trypsinized for 3 min at 37 °C. Trypsin was neutralized with culture medium following by centrifugation. Next, cells were resuspended in CC1M medium and plated on glass-bottomed dish coated with 2 μg/ml fibronectin at 37 °C, pH 7.4. Bright field images of cells spreading were captured by an Olympus IX70 inverted microscope (UPlanFL 40x/0.75 objective) every 10 s and analyzed using ImageJ (NIH). Beginning of spreading is determined by a cell touching down to the surface and expanding uniformly in all directions (i.e. isotropic spreading); the end point is determined when a cell stops the expansion and starts to generate protrusions in one or two dominant directions.

Quantification of Adhesion Turnover—CHO.K1 cells expressing Paxillin-mOrange (CoralHue monomeric Kusabira Orange (mOrange) from MBL) and either EGFP-tensin WT or EGFP-tensin F302A were plated on 2 μg/ml fibronectin-coated glass-bottomed dishes in CC1M for 1 h and maintained at 37 °C, pH 7.4. TIRF images were acquired using an Olympus IX70 inverted microscope (1.45 NA (oil) PlanApo 60x TIRFM objective), fitted with a Ludl modular automation controller (Ludl Electronic Products) and a charge-coupled device camera Retiga Exi (Qimaging). EGFP and mOrange were excited using the 488 nm laser line of an Ar ion laser and the 543 nm laser line of a He-Ne laser (Melles Griot), respectively. For dual EGFP/mOrange acquisition, a polychromatic mirror (Z488/543rpc), a dual emission filter (Z488/543), and HQ525/50 and HQ620/60 emission filters were utilized (Chroma Technology). All images were acquired with Metamorph software (Molecular Devices) and analyzed using ImageJ. Changes in fluorescent intensity of individual adhesions near the leading edge were measured over time with background and photobleaching corrections. Assembly and disassembly rates were plotted and calculated via Microsoft Excel (Microsoft Corp.) (21).
**DLC-1 Binding**—HEK293 cells were co-transfected with pcDNA3-DLC-1 (a generous gift from Dr. Lowy) alone or with either pTriEx-tensin1, pTriEx-tensin F302A, or pTriEx-tensin R1488A. After 48 h, cells were lysed on ice for 15 min in 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.9, 0.137 M NaCl, 1 mM EGTA, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1× protease inhibitor mixture set V). The lysate was centrifuged at 13,000 rpm for 15 min, and the supernatant was incubated with S-protein-agarose for 3 h at 4 °C. The agarose was washed three times in lysis buffer, and samples were boiled in 1× SDS for 5 min. Samples were resolved on 4–15% criterion pre-cast gradient gel (Bio-Rad) and transferred to nitrocellulose membrane. Membranes were incubated with anti-DLC-1, washed, and incubated with secondary antibodies. Immunoblots were visualized by enhanced chemiluminescence or scanned by the Odyssey Infrared Imaging System (LiCor®) and quantitated with the Odyssey Application Software version 2.0.41 (LiCor®).

**Focal Adhesion and Shape Factor Assays**—REF52 and NIH3T3 cells were plated in BiOptechs Delta T Culture Dishes (Fisher Scientific) and 1 μg of EGFP-tensin WT, F302A, or R1488A plasmid were transiently transfected with Arrest-In. Cells were imaged with a Nikon TE2000 with TIRF adaptor and a Spectra Physics 488/514 air-cooled laser for tensin localization in focal adhesions. Images were captured with a QCapture Pro software equipped with a Hamamatsu model C4742-95 CCD camera, and images were analyzed and quantitated with Improvision OpenLab 5.0.1 Software.

**Stable Cell Lines**—EGFP empty vector, EGFP-tensin, EGFP-tensin WT F302A, and EGFP-tensin R1488A constructs were linearized with HindIII (New England BioLabs) for three hours at 37 °C and purified by phenol chloroform extraction. One 25-mm plate of cells (70% confluent) (HEK293 and MDA MB 231) was transfected with 50 μg of plasmid by Fugene according to the manufacturer’s directions. Three days post-transfection, selection began with the addition of 700 μg/ml geneticin to the growth medium (MEM supplemented with 10% FBS), and colonies expressing EGFP were selected after 2 weeks. To produce S-tag stable cells, pTriEx empty vector, pTriEx-tensin WT, pTriEx-tensin F302A, and pTriEx-tensin R1488A constructs were linearized with MluI (New England BioLabs) for 3 h at 37 °C and purified by phenol/chloroform extraction. One 25-mm plate of MDA MB 231 cells (70% confluent) was transfected as stated above, and 3 days later selection began with the addition of 500 μg/ml geneticin. Colonies were selected and tested for expression after 3 weeks.

**Phospho-MLC Immunofluorescence**—MDA MB 231 EGFP-tensin WT, EGFP-tensin F302A, and EGFP-tensin R1488A stable cells were plated on glass bottom slides coated with 2 μg/ml fibronectin. Twenty-four hours later, cells were fixed in 3.7% formaldehyde/PBS, permeabilized with 0.1% Triton, stained with anti-pS19/20 myosin light chain antibody (Rockland) and Hoechst, washed, and incubated with secondary antibodies. Cells were viewed with a Nikon Eclipse E800 microscope equipped with a Hamamatsu model C4742-95 CCD camera, and images were analyzed and quantitated with Improvision OpenLab 5.0.1 Software.

**GST-Rhotekin-RBD Pulldown**—MDA MB 231 EGFP-tensin WT, EGFP-tensin F302A, and EGFP-tensin R1488A stable cells were plated in 10-cm dishes and grown to 80% confluency. Cells were lysed and incubated with GST-Rhotekin-RBD according to the manufacturer’s instructions (Cytoskeleton Inc.). The recommended lysis buffer was modified to include β-mercaptoethanol and dithiothreitol. Samples were run on a 4–15% gradient gel (Bio-Rad) and immunoblotted with anti-RhoA (Upstate) and anti-GST antibodies, scanned by the Odyssey Infrared Imaging System (LiCor®) and quantitated with the Odyssey Application Software version 2.0.41 (LiCor®).

**Cell Migration Assay**—MDA MB 231 EGFP-tensin WT, EGFP-tensin F302A, and EGFP-tensin R1488A stable cell lines were grown to a confluent monolayer on glass bottom chamber slides, scrape wounds were created, and time lapse images were acquired for 24 h every 15 min. Multiple individual cell migration patterns were tracked; cell migration distance and displacement were quantified with Improved OpenLab 5.0.1 Software.

**Cell Invasion Assay**—MDA MB 231 pTriEx4, pTriEx4-tensin WT, pTriEx4-tensin F302A, and pTriEx4-tensin R1488A stable cells were plated in triplicate in the upper chamber of a BD BioCoat Growth Factor Reduced BD Matrigel Invasion Chamber (BD Bioscience) in serum-free medium. Cells were allowed to invade into the bottom chamber containing 10% FBS for 22 h. Upper chambers were rinsed with phosphate-buffered saline and wiped with cotton swabs to remove non-invaded cells. Lower chambers were washed with phosphate-buffered saline, and the matrigel inserts were fixed in methanol for 15 min and stained with 0.005% crystal violet/methanol to visualize, image, and count invaded cells.

**Sequence Alignments**—The EMBL-EBI ClustalW2 alignment program (22) was used to align tensin1 (NP_072174), tensin2 (NP_736610), tensin3 (NP_073585), and tensin4 (NP_116254).

**RESULTS**

The Phe-302 Residue in Tensin1 Is Required for PP1α Binding—Human tensin1 co-localizes by immunofluorescence and co-immunoprecipitates with protein phosphatase-1α (PP1α), showing association but not direct interaction (16). PP1α has a surface site that binds directly to (R/K)VF motifs in regulatory subunits (23), and in tensin1 there are six such motifs, at residues 27, 268, and 302 (position of F) in the N-terminal domain and at residues 1455, 1639, and 1723 in the C-terminal domain (Fig. 1A). Because our previous results showed that PP1 co-precipitates with the N-terminal region of tensin1, rather than the C-terminal region, we initially introduced individual Phe to Ala point mutations in S-tag-tensin1 (1–740) and used co-precipitation and immunoblotting to assay for binding to HA3-PP1α. Neither F27A nor F268A reduced co-precipitation relative to WT (not shown). In contrast, a F302A mutation significantly reduced HA3-PP1α co-precipitation, down to background levels of binding to the blank beads in this assay (Fig. 1B). After identifying Phe-302 as the primary site for PP1α interaction, we introduced this mutation into both full-length EGFP-tensin1 and S-tag-tensin1. Binding with co-expressed HA3-PP1α was essentially eliminated (Fig. 1C). The equal recovery of tensin1 in the pull downs was confirmed by immu-
noblotting, and the expression levels of the co-expressed proteins in the extracts was demonstrated by immunoblotting for HA and S-tag (Fig. 1C). This KVXF motif in tensin1 is not preserved in other tensin family members (Fig. 1D). These data demonstrate that the F302A mutation prevents PP1 binding to tensin1 in cells.

Tensin1 F302A Significantly Reduces the Rate of Cell Spreading but Not Adhesion Turnover—We examined cell spreading as an initial test of the cellular effects of the F302A mutation in tensin1. CHO.K1 cells were transiently transfected to express either EGFP-tensin1 WT or F302A, which allowed identification of the transfected cells by EGFP fluorescence. Cells were replated on glass-bottomed dishes coated with fibronectin and images collected every 10 s. Isotropic cell spreading was analyzed from touch down on the surface until the appearance of one or two dominant protrusions (defined as start of anisotropic action). Cells expressing either EGFP-tensin1 WT or F302A are shown at the start and finish points of isotropic spreading (Fig. 2A). The average time of isotropic spreading for cells expressing EGFP-tensin1 F302A versus WT is significantly higher duration of spreading for cells expressing EGFP-tensin1 F302A versus WT. C, average area of twenty cells per group, demonstrating no significant difference between cells expressing EGFP-tensin1 F302A versus WT after isotropic spreading.

**FIGURE 1.** Phe-302 in tensin1 is required for PP1 binding. A, schematic showing the location of the six VXF motifs in human tensin1 (F positions). The PTP (dots), SH2 (diagonal lines), and PTB (wedges) domains are indicated as well as the region found previously to be sufficient for isoform-specific PP1 binding (16). B, HEK293 cells were transfected to express HA3-PP1 alone (C, control) or to co-express either S-tag-tensin1-(1–740) WT or S-tag-tensin1-(1–740) F302A. Immunoblotting of the S-peptide with anti-HA shows PP1 binding with the tensin1-(1–740) WT protein compared with nonspecific background binding of PP1 to tensin1-(1–740) F302A or control beads without tensin1. Three independent experiments showed significant (*, p < 0.03) reduction in PP1 binding to the tensin1 F302A mutant. The bands shown are from the same gel of one experiment. C, HEK293 cells were co-transfected to express HA3-PP1 and full length S-tag-tensin1 WT or F302A. The S-peptide pull downs were immunoblotted with anti-HA to show the relative binding of PP1 to the WT versus F302A TNS1 protein. Immunoblotting with anti-S-tag demonstrated equal recovery of TNS1 in the pull down. Whole cell extracts were immunoblotted to show relative expression levels. D, sequence alignment of the region surrounding the PP1 binding site in TNS1 (shaded) compared with tensin2, tensin3, and c-ten.

**FIGURE 2.** Tensin1 F302A reduces cell spreading but not cell area. A, images of CHO.K1 cells expressing either EGFP-tensin1 WT (top panels) or EGFP-tensin1 F302A (bottom panels). Images were acquired at the start of cell spreading (left panels) and when cells were finished isotropically spreading (right panels). The final cell outline was traced and shown with a white line. B, average time of isotropic spreading for twenty cells per group, showing a significantly (*, p < 0.001) higher duration of spreading for cells expressing EGFP-tensin1 F302A versus WT. C, average area of twenty cells per group, demonstrating no significant difference between cells expressing EGFP-tensin1 F302A versus WT after isotropic spreading.
Tensin1 Requires PP1 and DLC-1

Significantly different (Fig. 2, A and C). Thus, cell spreading but not cell size was affected by the F302A mutation in tensin1.

Following isotropic spreading cells extend and retract lamellipodia and near this leading edge nascent adhesions undergo rapid, continuous formation and disassembly (turnover) (3). Because there was a reduced rate of cell spreading, we examined whether the F302A mutation in tensin1 affected adhesion turnover. High-resolution TIRF microscopy was utilized to examine assembly and kinetics of these adhesions. Dual-color, time-lapse images of CHO.K1 cells co-expressing paxillin-mOrange with either EGFP-tensin1 WT or EGFP-tensin1 F302A showed coincident localization in large stable adhesions around the perimeter of the cells. There was no obvious difference in the distribution of EGFP-tensin1 WT (Fig. 3A, top panel) compared with EGFP-tensin1 F302A (Fig. 3A, bottom panel) in these adhesions. Thus, the F302A mutation did not compromise localization of tensin1 to adhesions. Paxillin was localized in nascent adhesions of the protruding lamellipodia along with low levels of tensin1 (Fig. 3A). This contrasts with previous immunofluorescence results that have indicated tensin was not present in these adhesions. Time-lapse recording of the paxillin was used to analyze the kinetics of formation and dissolution of these nascent adhesions (Fig. 3B) (21). No significant difference in rate of turnover was detected between cells expressing EGFP-tensin1 WT and EGFP-tensin1 F302A (Fig. 3C). Taken together, these data indicate tensin1 participates in initial adhesions in protrusions; however, because cells expressing WT and F302A tensin1 behaved the same, it is unlikely that tensin-bound PP1 affects adhesion formation and turnover.

Morphology and Polarization of Cells Expressing Tensin1 WT and Tensin1 Mutants—Fibroblasts express endogenous tensins and exhibit prominent adhesions. Using TIRF microscopy we observed elongated adhesions in fibroblasts overexpressing EGFP-tensin1 F302A (Fig. 4). In these experiments, the EGFP-tensin1 was expressed at relatively low levels to act as a tracer for visualization of adhesions by EGFP fluorescence. Adhesions containing EGFP-tensin1 were primarily distributed along the perimeter of the cell and adhesion length was measured in more than 100 adhesions and sorted into quartiles. This analysis showed the average length of adhesions containing EGFP-tensin1 F302A was longer relative to adhesions containing EGFP-tensin WT (Fig. 4C). Thus, the F302A mutant tensin1 deficient in binding PP1α was dominant in altering focal adhesion morphology.

Alterations in adhesion length and size might reflect changes in the contractility of the cytoskeleton, related to the activity of RhoA. Therefore, we produced the R1488A mutant form of tensin1, which corresponds to the residue in the SH2 domain of c-ten that is required for the phosphotyrosine-independent association with DCL-1 (Fig. 5A). Binding of DCL-1 to S-tag-tensin1 WT, F302A, and R1488A was compared by S-peptide pull down and immunoblotting (Fig. 5B). There was effective co-precipitation of DCL-1 with WT tensin1. In comparison, the amount of DCL-1 recovered with tensin1 R1488A was reduced by more than half (Fig. 5B), as expected based on the corresponding mutation in c-ten. Not expected was that binding of DCL-1 to tensin1 F302A was reduced to about the same extent as to R1488A, relative to WT. On the other hand, there was no difference in co-precipitation of HA3-PP1α with R1488A relative to WT tensin1 (Fig. 5C). Therefore, mutation R1488A reduced DCL-1 but not PP1 association with tensin1, whereas F302A reduced binding of both DCL-1 and PP1. We concluded that if cells expressing F302A or R1488A showed common phenotypes it was probably due to the reduction in DCL-1 binding to tensin1.

Fibroblasts expressing EGFP-tensin1 WT assumed the classic elongated fan shape of a polarized cell, with a broad leading edge opposite a narrow extended tail (Fig. 5D). In contrast, cells expressing EGFP-tensin1 F302A (Fig. 5E), or R1488A (Fig. 5F) appeared rounder in shape, without a distinctive leading edge.
or extended tail. We noted that in cells expressing either the F302A or R1488A mutated form of tensin1, the adhesions around the perimeter of the cell showed exaggerated length and density. To quantitate the change in morphology, we determined a shape factor. Cell shape was significantly altered for cells expressing either the F302A or R1488A mutated forms of tensin1 compared with WT tensin1 (Fig. 5G). Mutations at either Phe-302 or Arg-1488 dominantly interfered with tensin1 function in fibroblasts, disturbing cell polarization.

Myosin Light Chain Phosphorylation and RhoA-GTP Require Tensin1 Binding to PP1 and DLC-1—Cell polarization and adhesions depend on phosphorylation of the myosin IIB light chain, under control of the opposing light chain kinases and myosin phosphatase (24). Human breast cancer MDA MB 231 cells express low levels of tensin1 we discovered by RT-PCR, even though the protein was not detected by immunoblotting, as previously reported (11). At equivalent cycles of PCR only tensin1, not tensin2, tensin3, or c-ten were detected in MDA

![Tensin1 Requires PP1 and DLC-1](image)

**FIGURE 4.** Tensin1 F302A produces elongated adhesions. TIRF images of adhesions in REF52 fibroblast expressing EGFP-tensin1 WT (A) or EGFP-tensin1 F302A (B). C, average focal adhesion lengths show that EGFP-tensin1 F302A (red) produces longer adhesions than EGFP-tensin1 WT (blue).

**FIGURE 5.** Association of DLC-1 and PP1α with tensin1 WT, F302A, and R1488A related to cell polarization. A, sequence alignment of the DLC-1 binding site in tensin family proteins with the critical arginine shown in bold. Tensin 4 is the same as c-ten. B, HEK293 cells were transfected to express DLC-1 alone or co-express DLC-1 with S-tag-tensin1 WT, F302A, or R1488A. The S-peptide complexes were recovered and immunoblotted to show the amounts of DLC-1, compared with control background binding to the S-protein beads in the absence of tensin1 (control). The image is representative of three independent experiments. The expression levels of DLC-1 in the extracts are shown by immunoblotting in the lower panel. C, S-tag-tensin1 was co-expressed with HA3-PP1α and complexes recovered by S-protein beads and immunoblotted for HA to show recovery of associated PP1α. The image is representative of three independent experiments, showing equivalent binding of PP1α to WT and R1488A tensin1. D–F, images from TIRF microscopy showing NIH3T3 cells expressing EGFP-tensin1 WT (D), EGFP-tensin1 F302A (E), or EGFP-tensin1 R1488A (F). G, cells expressing tensin1 F302A and R1488A mutants have a significantly (*, p ≤ 0.001) lower shape factor scores (average of 30 cells per group) compared with cells expressing EGFP-tensin1 WT.

![Tensin1 R1488A](image)
Using transfection and selection we isolated MDA MB 231 cells stably expressing EGFP, EGFP-tensin1 WT, F302A, or R1488A and analyzed the phosphorylation of endogenous myosin light chains by immunofluorescent staining using a phospho site-specific antibody (Fig. 6A). The phospho-myosin appeared concentrated at the perimeter of the cells, and quantification of this immunofluorescent staining by digital imaging with a CCD camera showed that cells expressing EGFP-tensin F302A or R1488A had about 2-fold lower levels of MLC20 phosphorylation compared with cells expressing EGFP alone or EGFP-tensin WT (Fig. 6A). There was not a significant difference in MLC20 phosphorylation between cells expressing either F302A or R1488A. Immunoblotting extracts of these cell lines using the same phosphosite-specific antibody showed a less dramatic difference in MLC20 phosphorylation that nonetheless was significantly different in cells expressing the tensin1 mutants compared with WT (not shown).

Lower levels of phosphorylated MLC20 in cells expressing F302A- or R1488A-mutated tensin1 could result from either reduced myosin light chain kinase activity or elevated myosin LC phosphatase activity. The myosin phosphatase itself is negatively regulated by phosphorylation of its MYPT1 subunit by RhoA-activated kinase. RhoA(GTP) can be assayed by pull down with the GST-Rhotekin-RBD, which selectively binds the active, not the inactive form of RhoA. MDA MB 231 cells stably expressing EGFP-tensin1 F302A or R1488A had on average about 2-fold lower levels of active RhoA, compared with cells expressing EGFP-tensin1 WT (Fig. 6B). The difference of RhoA activation in this assay comparing cells expressing WT tensin1 versus mutants did not achieve statistical significance. However, the F302A and R1488A mutants had identical effects in suppressing activation of RhoA and MLC20 phosphorylation in human breast cancer cells.

Migration and Invasion of Human Breast Cancer Cells Expressing Ectopic Tensin—In contrast to the identical responses to the F302A and R1488A mutations in tensin1, migration and invasion of metastatic MDA MB 231 breast cancer cells were differentially affected by these mutations. Migration of individual cells were analyzed by time-lapse microscopy of scrape wounds made in confluent monolayers of MDA MB 231 cells stably expressing WT or mutated EGFP-tensin1. The differences in cell migration are displayed by mapping the movement of three individual cells of each type with the direction shown by arrows (Fig. 7A). Movies revealed significantly more rapid multidirectional movement of the EGFP-tensin1 F302A cells compared with less rapid, directional movement of EGFP-tensin1 WT (Fig. 7, A and B). In contrast, cells expressing EGFP-tensin1 R1488A migrated significantly slower with persistent directionality compared with cells expressing EGFP alone. B, GST-Rhotekin-RBD binding of RhoA(GTP) from MDA MB 231 cells expressing EGFP-tensin1 WT (lane 1), EGFP-tensin1 F302A (lane 2), or EGFP-tensin1 R1488A (lane 3). Complexes were pulled down and immunoblotted with anti-RhoA to visualize the amount of active RhoA (upper panel) and with anti-GST as loading control (lower panel). RhoA was quantified by scanning fluorescence to compare average staining intensity (n = 3).
expressing tensin1 WT (Fig. 7, A and B). The distance between the initial (open circle) and final positions (closed circle) is referred to as displacement. Displacement was slightly increased for cells expressing the F302A mutant, but was significantly reduced relative to WT for R1488A tensin1 (Fig. 7C). These two mutations in tensin1 produced near opposite effects on migration.

In an in vitro cell invasion assay MDA MB 231 cell lines expressing tensin1 WT or R1488A were indistinguishable, and both showed significantly reduced invasion compared with control cells (Fig. 8, A and B). In contrast, cells expressing tensin1 F302A were significantly more invasive than either control cells or cells stably expressing tensin1 WT or R1488A (Fig. 8, A and B). These results showed that PP1 binding is critical for tensin1 to reduce invasion of human cancer cells.

**DISCUSSION**

The results of this study show that association with PP1 is critical for cellular functions of tensin1. We found that mutation of F302A in one of six KVXF motifs in tensin1 eliminated binding of PP1, and used this mutation to assess the contributions of PP1α to tensin1 actions in cells. We used a different mutation in tensin1 to compare how binding to the RhoGAP DLC-1 contributes to tensin1 function. Unexpectedly, the F302A mutation that prevented PP1α binding also reduced binding of DLC-1 to tensin1. Thus, mutation of either one of these two side chains >1100 residues apart in tensin1 reduced DLC-1 binding. We imagine that the absence of phosphatase activity due to reduced PP1α binding allows an increase in the Ser/Thr phosphorylation of tensin1 and/or DLC-1, which
could interfere with their association. It is important to point out that whereas mutation of either Phe-302 or Arg-1488 in tensin1 reduced DLC-1 binding, the R1488A mutation did not affect binding of PP1α. Therefore, we propose that phenotypes common to cells expressing either F302A or R1488A mutated tensin1 are probably because of loss of DLC-1 binding, whereas distinctive phenotypes of cells expressing F302A tensin1 can be attributed to the loss of PP1α binding.

Expression of WT tensin1 did not alter several phenotypes of different parental cell lines, and our interpretation was that overexpression reinforced the actions of endogenous tensin1, with no changes in behavior. In contrast, expression of F302A or R1488A mutant tensin1 enhanced adhesion size and disoriented cell polarization, evidence for dominant effects of these mutated proteins over the endogenous tensin1. Our hypothesis is that alterations in adhesions and the cytoskeleton arise from reduced MLC20 phosphorylation and lower levels of activated RhoA(GTP). RhoA is known to activate the kinase ROCK to phosphorylate the MYPT1 subunit of myosin phosphatase as well as MLC. Phosphorylation of MYPT1 reduces phosphatase activity, thereby increasing MLC20 phosphorylation and contractility of actomyosin. Thus, RhoA activity and MLC20 phosphorylation are predicted to change in parallel, which is what we observed. But, what might be the basis for dominant effects from expression of F302A or R1488A mutants? The EGFP-tensin1 fusion proteins were localized to adhesions, regardless of the mutations, so intracellular protein targeting and localization were preserved, and therefore not dependent on tensin1 association with either PP1α or DLC-1. We speculate that changes in the RhoA-ROCK-MYPT1 pathway were dependent on the activity of DLC-1 bound to tensin1 in adhesions, and in turn DLC-1 activity depends on PP1α binding to tensin1. According to this view both the mutated forms of tensin1 displaced endogenous tensin1 from adhesions, thereby reducing the adhesion-localized RhoGAP activity, to produce the same changes in RhoA(GTP), P-MLC and actomyosin cytoskeleton. There are reports in the literature that DLC-1 is phosphorylated at multiple sites (25, 26) but it is not known whether this regulates RhoGAP activity. Based on our results we would imagine that PP1 dephosphorylation activates DLC-1 when both are bound to tensin1. The regulation of DLC-1 by phosphorylation is an important question for future study.

During anisotropic lamellapodia formation, we observed tensin1 localized to adhesions at the leading edge, challenging the concept that tensin1 is concentrated exclusively in large contractile or fibrillar adhesions. This localization and the rates of adhesion assembly and disassembly were not significantly different in cells expressing tensin1 WT or tensin1 F302A. Either tensin1 does not regulate adhesion turnover during anisotropic spreading, or, if it is involved this function, it is not dependent on binding to PP1α or DLC-1. We also used fibroblasts with mature contractile adhesions and endogenous tensin1 (20) and observed ectopic mutated tensin1 (F302A or R1488A) exerted a dominant effect, elongating fibroblast adhesions and reorganizing the cytoskeleton, with a loss of polarization. Because both mutants produced the same effect, we propose that these functions depend on tensin1 binding to DLC-1.

We studied the function of tensin1 in human breast cancer MDA MB 231 cells that express very low levels of tensin1, that are not detectable by immunoblotting. Stable expression of tensin1 WT, F302A, and R1488A mutants produced distinctly different migration patterns. Tensin1 F302A expressing cells had higher motility, but their movement was multidirectional instead of directional. Tensin1 R1488A cells had significantly reduced cell migration, but preserved directional movement. Thus, directional movement depended on which tensin1 mutant was expressed and did not simply reflect the activation state of RhoA, which was the same in cells expressing either tensin1 mutant.

Expression of either WT or R1488A tensin1 significantly suppressed MDA MB 231 breast cancer cell invasion, so this response did not require binding of DLC-1 to tensin1. In contrast, cells expressing tensin1 F302A showed increased invasion in Matrigel. We concluded that PP1α binding to tensin1 is critical for tensin1 to limit cancer cell invasion, and the response is separate from effects on DLC-1, and probably not dependent on changes in RhoA activity. Thus, we propose PP1α is required for tensin1 function, especially in restricting cell invasion, and in effect, tensin1 performs as a PP1α subunit in adhesions for effects on migration and invasion. Based on what is known of other PP1 regulatory subunits, the catalytic activity of PP1 is allosterically controlled, and PP1 substrates are selectively recruited through their association with the regulatory subunit. We speculate that tensin1 itself, as well as other proteins bound to tensin1, are dephosphorylated by tensin1-bound PP1. Metastatic tumor cells down-regulate expression of tensin1 and/or DLC-1 (12), and our results help to provide some insight of the consequences of these genetic changes. Overall our studies demonstrate that tensin1 requires PP1α as a partner for its role in the maintenance of cell polarization, and especially the regulation of cell migration and suppression of invasion that are involved in metastasis.

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