Tropomyosin isoform modulation of focal adhesion structure and cell migration

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Orderly cell migration is essential for embryonic development, efficient wound healing and a functioning immune system and the dysregulation of this process leads to a number of pathologies. The speed and direction of cell migration is critically dependent on the structural organization of focal adhesions in the cell. While it is well established that contractile forces derived from the acto-myosin filaments control the structure and growth of focal adhesions, how this may be modulated to give different outcomes for speed and persistence is not well understood. The tropomyosin family of actin-associating proteins are emerging as important modulators of the contractile nature of associated actin filaments. The multiple non-muscle tropomyosin isoforms are differentially expressed between tissues and across development and are thought to be major regulators of actin filament functional specialization. In the present study we have investigated the effects of two splice variant isoforms from the same α-tropomyosin gene, TmBr1 and TmBr3, on focal adhesion structure and parameters of cell migration. These isoforms are normally switched on in neuronal cells during differentiation and we find that exogenous expression of the two isoforms in undifferentiated neuronal cells has discrete effects on cell migration parameters. While both isoforms cause reduced focal adhesion size and cell migration speed, they differentially effect actin filament phenotypes and migration persistence. Our data suggests that differential expression of tropomyosin isoforms may coordinate acto-myosin contractility and focal adhesion structure to modulate cell speed and persistence.

Introduction

Both the speed and direction of mesenchymal cell migration is influenced by the structural organization of focal adhesions.1-4 From their earliest description it was appreciated that focal adhesions are linked to bundles of polymerized actin known as stress fibers5 and it was subsequently established that focal adhesions grow and elongate in response to mechanical tension derived through the acto-myosin stress fibers.6,7 Given the relationship between the contractile stress fibers and focal adhesion growth, molecules that determine the contractile properties of the actin stress fibers may also determine the structure of the associated focal adhesion.8 The non-muscle tropomyosins are emerging as important contributors to the myosin-mediated contractile properties of actin filaments.3,9-11 Therefore, investigating the relationship between the tropomyosins, which determine the contractile properties of the actin cytoskeleton, and focal adhesion structure is a key step toward understanding how speed and persistence are controlled during cell migration.

Focal adhesions are elongated (3–10 μm long), dash-shaped structures that form at the boundary between the fast and slow actin flow zones at the cell’s leading edge.12 Following Rho-GTPase dependent transition from a pre-cursor/focal complex into a focal adhesion, there is a linear relationship between the area occupied by the focal adhesion and tension derived through the associated acto-myosin stress fibers.7 The physical association between the adhesion and the bundled actin filaments allows the transmission of the tensile force to the adhesion.13 While the strength, size and number of these macromolecular structures is a key determinant of cell migration rates, there is not a simple, direct relationship between the extent of cell adhesion and rates of cell migration.1,2,4,14 Rather, maximal migration rates are determined by combined spatial and temporal organization of the focal adhesions and stress fibers and their associated regulatory proteins. Moreover, adhesion structure plays a decisive role in regulating intrinsic persistence (directional migration)—specifically the focal adhesions influence the stability of lamellipodial protrusions and thus migration persistence.15 The tropomyosins form head to tail dimers that lie along the major groove of the actin filament and an important consequence of tropomyosin association is to regulate myosin motor activity on the associated actin filament.3,5-11 Isoform-specific structural associations between tropomyosins and actin filaments have been proposed to result in differential access to binding sites for actin-associating regulatory molecules such as myosin16 and thus altered contractile properties of the actin filament. Expression of tropomyosins

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that promote extensive myosin recruitment to actin stress fibers leads to increased focal adhesions and formation of adhesions closer to the membrane edge. An important question is whether, conversely, tropomyosin isoforms that are associated with reduced myosin activity, such as the brain specific isoform TmBr3, may cause reduced focal adhesion area and thereby alter migration parameters of speed and persistence.

In the present study we characterize focal adhesion structure in neuronal B35 cells expressing exogenous TmBr3 and compare the effects with a second brain-specific isoform, TmBr1. Both isoforms derive from the same α-tropomyosin gene and differ only by unique N-termini generated through alternative exon splicing. TmBr1 and TmBr3 are not normally expressed in undifferentiated neuronal cells but are specifically induced during neuronal morphogenesis and maturation. Our results show that alternative tropomyosin expression can lead to reduced focal adhesion area and altered parameters of cell migration in an isoform-specific manner.

Results

Levels of TmBr1 and TmBr3 expression were compared between control B35 cells and the cell lines created to express either exogenous TmBr1 or TmBr3, using antibody WS19c that specifically recognizes these two isoforms. Agreeing with previous reports that TmBr1 and TmBr3 are not expressed in undifferentiated neuronal cells, no TmBr1 or TmBr3 is detected in undifferentiated B35 cells by either western blot or immunofluorescence analysis (Fig. 1A and B). Exogenous TmBr1 has a punctate appearance throughout the cytoplasm while exogenous TmBr3 shows punctate staining in the cytoplasm and enrichment at the cell periphery (Fig. 1B). As reported previously, cells expressing exogenous TmBr3 display little evidence of stress fibers that traverse the cell length (Fig. 1C). Instead, these cells have shorter, more disorganized filaments and an enriched level of F-actin at the periphery. Interestingly, the arrangement of the actin filaments in the cells expressing exogenous TmBr1 is noticeably different to this pattern. The actin organization in the TmBr1 expressing cells is similar to the arrangement seen in the control cells with clear actin stress fibers traversing the cytoplasm in both cultures (Fig. 1C).

Analysis of focal adhesion phenotypes in cells expressing exogenous TmBr3 revealed small paxillin positive adhesions at the cell edge (Fig. 2A). As predicted based on the appearance of the actin filaments, quantification revealed that individual focal adhesion area is significantly decreased in the TmBr3 cell line (Fig. 2B). Surprisingly, despite the presence of substantial actin stress fibers, focal adhesion area was also significantly reduced in the cells expressing exogenous TmBr1 (Fig. 2B) and this was equivalent to the average focal adhesion area in cells expressing exogenous TmBr3. Moreover, the total ventral cell area occupied by focal adhesions proved to be significantly less in both exogenous tropomyosin expressing cell lines (Fig. 2C). Thus, these two tropomyosin isoforms led to reduced focal adhesion area, however this was associated with distinct actin filament arrangements. We have previously shown a strikingly altered activation of adhesion signaling molecules, dependent on the tropomyosin expression profile. Thus we next questioned whether TmBr1 and TmBr3 might differentially effect these same signaling pathways. Firstly, we examined the hyper-phosphorylation status of p130Cas, a mechanosensory focal adhesion protein, and find that p130Cas is significantly more hyper-phosphorylated (p-p130Cas) in the TmBr1 cells than in the control cells (Fig. 3A). Next, we determined the expression of Src kinase, a major regulator of focal adhesion dynamics and p130Cas phosphorylation. Corresponding with the increased levels of hyper-phosphorylated p130Cas, we observe significantly increased levels of total Src protein in the TmBr1 cells (Fig. 3B). Together these data suggest increased activation of adhesion signaling pathways in the TmBr1 cells.

We next measured the effect of reduced focal adhesion area observed in the TmBr1 and TmBr3 on the migration parameters of speed and persistence, by performing time-lapse imaging of sparsely plated cells. Graphic representations of the cell tracks (Fig. 4A) show that the paths transcribed by the TmBr1 cells appear shorter than the control cells. Confirming this, these cells have significantly reduced migration speeds when compared with control cells (Fig. 4B). In contrast, while the cell tracks of the TmBr3 cells are not obviously different to the control cells (Fig. 4A) the speed of these cells is reduced to the same level as the TmBr1 cells (Fig. 4B). Thus, we questioned whether the TmBr3 cells might be moving in a more persistent manner than the TmBr1 cells. When compared with the distribution of persistence ratios for the control cells (white bars, Fig. 4C), the TmBr1 cells exhibit a shift to decreased persistence while in contrast the TmBr3 cells exhibit a shift towards increased persistence. Correlated with this, analysis of the time-lapse movies indicates that both the control and TmBr3 cells exhibit membrane protrusion restricted to the direction of migration, consistent with a more directional pattern of migration (Fig. 4D). Thus the TmBr3 cells maintain a polarized shape in the direction of motility, however in contrast to the controls these cells show reduced translocation of the cell body over the same time course. In contrast and in agreement with the more random migration pattern dynamic membrane protrusions occur randomly from the TmBr1 cells. In the example shown (Fig. 4D), membrane protrudes first from one end of the cell and 10 min later from the opposite end of the cell and correspondingly there is no evidence of translocation of the cell body during this time-frame.

As a second measure of cell migration, we performed scratch wound healing assays. At 12 h and 24 h after wound healing both the TmBr1 and TmBr3 cells exhibited a reduced extent of wound closure when compared with the control cells (Fig. 5). Notably, at 24 h the TmBr1 cells have still failed to close the wound area, while the TmBr3 cells have almost completely covered the wound area. By 48 h after wounding, all cell lines had efficiently covered the wound. Despite the equally reduced rate of migration seen for both the TmBr3 and TmBr1 cells (Fig. 4B), the enhanced directional migration of the TmBr3 cells (Fig. 4C) likely explains why these cells close the wound more rapidly than the TmBr1 cells (Fig. 5).
In the scratch wound healing assay the organization of the focal adhesions and the actin filaments in cells at the wound edge is critical to the migration of cells into the wound area. Therefore, we examined the distribution of actin and focal adhesions in cells closest to the scratched region. Control cells immediately proximal to the wound (Fig. 6A, arrow heads) have arrays of actin filaments aligned parallel to the wound edge. Notably, the parallel actin filaments are predominantly arrayed on the wound side of the nucleus. Actin-rich filopodia are also seen extending towards the wound from the cell (see inset). Paxillin positive focal adhesions are seen associated with the ends of the parallel actin stress fibers (Fig. 6, top row, arrows).

Actin stress fibers are also clearly visible in TmBr1 cells at the wound edge (Fig. 6A, middle row). Some of these cells appeared to have their stress fibers aligned perpendicularly to the edge of the wound, in contrast to the arrangement seen in the control cells. Consequently, this raised the possibility that the focal adhesions may be differently oriented with respect to the wound in the TmBr1 cells (Fig. 6A, middle row, arrows). Following measurement of focal adhesion orientation, we noted a small increase in TmBr1-cell focal adhesions at ~45 degree angle to...
Figure 2. Focal adhesion area is decreased in cells expressing either exogenous TmBr1 or TmBr3. (A) Cells immunostained with antibodies to paxillin to detect focal adhesions. Insets = 4.2x magnifications of a region from the lower magnification image. (B) Histogram showing the average area and SEM of focal adhesions in arbitrary units (a.u.) (n > 190 focal adhesions per cell line). (C) Histogram showing the average total focal adhesion area per cell as a percentage of the total cell area and the SEM *p < 0.001.

Figure 3. Activation of adhesion signaling pathways in TmBr1 cells. (A) Western blot analysis of p130Cas. The histogram shows hyper-phosphorylated p130Cas (p-p130Cas, upper form) levels expressed as a percentage of the total p130Cas protein detected in each cell line. (B) Western blot analysis of Src protein expression. The histogram shows the level of Src protein expressed relative the levels detected in control cells. Protein levels were determined by densitometry of triplicate repeats. *p < 0.05, NS, not significant.
the wound and conversely a small increase in control cell focal adhesions oriented at $\sim$75 degrees (i.e., more parallel to the wound edge) (Fig. 6B). However, the overall distribution of focal adhesion orientations was very similar between the two cell lines. In contrast, and correlating with the data seen earlier (Fig. 2B), the focal adhesions at the leading edges of the TmBr1 cells have a significantly reduced area when compared with control cells (Fig. 6C). Finally, the TmBr3 cells show a distinct phenotype, displaying fine, disorganized fibers throughout the entire cytoplasm (Fig. 6A, bottom row). Corresponding to the fine actin filaments, the adhesions in these cells are notably smaller and rounded in shape.

**Discussion**

In the present study we have directly compared the phenotypic effects caused by splice variants of the same $\alpha$-tropomyosin gene. We find that cells expressing exogenous TmBr1 maintain well-defined actin stress fibers, yet display significantly reduced focal...
adhesion area, cell motility and directional persistence accompanied by increased phosphorylation of p130Cas and expression of Src. In contrast, cells expressing exogenous TmBr3 have lost the well-defined actin stress fibers and instead display fine, disorganized filaments. This is also accompanied by a reduction in focal adhesion area and a loss of cell migration speed, but contrasting the data for cells expressing exogenous TmBr1, there is no loss of directional persistence.

It has previously been established that acto-myosin contractility leads to the growth of focal adhesions and contractility inhibitors lead to the rapid disassembly of focal adhesions (reviewed in ref. 24). We therefore hypothesized that expression of tropomyosins may display isoform-specific modulation of focal adhesion structure. Indeed in previous work we have shown that exogenous expression of the isoform Tm5NM1 leads to enhanced stress fiber formation and numerous long and stable focal adhesions.4 By contrast, we here show that exogenously expressed TmBr3 causes loss of defined actin stress fibers and a correlated loss of focal adhesion area. These two pieces of data therefore fit well with the model that the presence of the actin stress fibers can play a critical determining role in the structure of the focal adhesions. However, we also found that while TmBr1 expression did not obviously alter the actin filament structure when compared with the control cells, it did lead to significantly reduced focal adhesion area. Recent models suggest that actin filaments are initiated by polymerization at the focal adhesion and that myosin is incorporated once the filament is several micrometers long.25 Thus it appears that initial focal adhesion formation is not contractility-dependent, so presumably a certain size of focal adhesion can be achieved prior to contractility-dependent growth of the adhesion. In this light, it is interesting to note that the focal adhesions of the TmBr1 and TmBr3 cells have identical focal adhesion areas and to speculate that this may represent the minimal contractility-independent size of focal adhesions in the B35 cells. This further suggests that focal adhesion growth may only occur once a certain threshold of contractility has been achieved at the filament.

Aligned with the differential effects of tropomyosins on FA structure we have observed an interesting pattern of regulation of focal adhesion signaling molecules. While Tm5NM1 overexpression is associated with significant downregulation of Src kinase protein and loss of p130Cas phosphorylation,4 in the present study we find that Src protein levels and p130Cas hyperphosphorylation are significantly increased. Thus tropomyosins appear to result in altered adhesion signaling. Whether this is a direct effect or a consequence of altered actin dynamics is not yet established, however given the proposed role for actin in the regulation of Src26 and tension in the regulation of mechanosensory adhesion signaling molecules,20 it is interesting to speculate that the tropomyosins may function to coordinate actin filaments with adhesion dynamics.

In assays of individual cell motility we find that exogenous expression of both TmBr1 and TmBr3 result in reduced cell motility. This contrasts with an earlier report using a micro-scale migration assay, suggesting that TmBr3 increases cell motility.11 We note that the micro-scale assay measurements measure net changes in the geographical population distribution of the cells and does not reflect movement of individual cells.32 Potentially, the enhanced directional persistence of the TmBr3 cells reported in the present study may explain the net increase in population distribution seen in the micro-scale assay. This also aligns with our data indicating that the wound healing response of the TmBr3 cells was only marginally delayed when compared with control cells. Combined with our previous study4 we have now demonstrated that tropomyosin expression leading to either increased focal adhesion area (Tm5NM1) or decreased focal

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**Figure 5.** Both TmBr1 and TmBr3 reduce the rate of wound healing. Shown are the wound healing responses for each cell line 6, 12, 24 and 48 h after wounding. Cells were fixed at each of the indicated time points and nuclei stained with DAPI. Data shown are representative of duplicate repeats.
Each of the tropomyosin isoforms we have investigated previously (Tm3 and Tm5NM1) and in the present study have resulted in reduced rates of cell migration. However, a more complex picture has emerged when we have measured the effect of these tropomyosins on intrinsic cell persistence. The reduction in cell migration stimulated by Tm5NM1 and TmBr1 is accompanied by a loss of persistent migration (increased random motility) and importantly, this is paralleled by increased persistence in fibroblasts derived from a Tm5NM1 knockout mouse model.

In contrast, cells expressing either TmBr3 or Tm3 exhibit the adhesion area (Tm3, TmBr1 and TmBr3) correlates with reduced intrinsic cell migration, consistent with the idea that maximal migration rates depend on specific organization of the focal adhesions and actin filaments. The B35 neuronal cells exhibit fairly rapid intrinsic cell motility with an average speed of ~1 µm per minute under the assay conditions used. This suggests that since conditions that either increase or decrease focal adhesion area in these cells both lead to decreased migration speeds, the parental B35 cells may be moving at maximal speed.

Figure 6. Different arrangement of actin filaments and adhesions in cells at the wound edge. (A) Monolayers of the indicated cell types were scratched to form wounds, then 6 h later cells were fixed and immunostained with antibodies to paxillin (false-coloured red in the overlays) and phalloidin to detect filamentous actin (false-coloured green in the overlays). Inset panels show 5x magnifications of a region from the lower magnification image. Arrow heads point to the cells closest to the wound. Arrows point to focal adhesions. (B) Frequency distribution of focal adhesions oriented with the indicated angle towards the wound, values range from 0 degrees for adhesions in which the longest axis of the adhesion runs perpendicular to the wound, to 90 degrees for adhesions that are parallel to the wound. (C) Histogram showing the average area and SEM of focal adhesions in arbitrary units (a.u.) (n > 300 focal adhesions per cell line).
same directional persistence as the control parental cells. Thus, it appears that Tm5NM1 and TmBr1 may specifically suppress directional persistence. We find that the TmBr1 cells display random membrane protrusions from around the cell body, while cells expressing Tm5NM1 appear to have reduced membrane protrusion (data not shown). It is perhaps significant that both the Tm3 and TmBr3 cells exhibit reduced actin stress fibers, while the TmBr1 and Tm5NM1 cells retain and have elevated stress fibers, respectively. Similarly, skeletal muscle tropomyosin caused a change in the actin filament distribution, with cells exhibiting bundled fibers all the way to the cell periphery and this was accompanied by the loss of lamellipodia. Therefore, the expression of tropomyosins may contribute to directional persistence through the regulation of lamellipodial dynamics.

Collectively, data presented in this study together with previous studies, suggest that the tropomyosins play an important role in regulating adhesion structure and consequently the speed and persistence of cell migration. In the present study we have investigated the effect of two isoforms that are developmentally regulated, being switched on specifically in neuronal cells during differentiation. During the processes of differentiation and tissue specialization cells have different requirements for migration and indeed for directional migration; given the spatial and temporal regulation of tropomyosins they may represent important contributors to the specialization of focal adhesions for precise regulation of migration parameters.

Materials and Methods

Cell lines and antibodies. B35 cell clones expressing exogenous TmBr3 were previously described and the same procedure followed to create TmBr1 clones (Schevzov G, et al. submitted). Reagents for immunofluorescence and immunoblotting were purchased from the following companies: anti-paxillin and anti-p130Cas (BD Transduction Laboratories); anti-Src (Upstate Biotechnology); rhodamine-phalloidin and 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich); horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech); and Alexa 488- and CY3-conjugated anti-p130Cas (BD Transduction Laboratories); and Alexa 488- and CY3-conjugated anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech); and Alexa 488- and CY3-conjugated anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech); and Alexa 488- and CY3-conjugated anti-mouse and anti-rabbit antibodies (Amersham Pharmacia Biotech). The rabbit polyclonal anti-peptide antibody W5et/9c has been previously described. Conditions of protein extraction, quantitation and immunoblotting have previously been described for p130Cas and Src antibodies (Jackson ImmunoResearch). The rabbit polyclonal anti-peptide antibody W5et/9c has been previously described. Conditions of protein extraction, quantitation and immunoblotting have previously been described for p130Cas and Src antibodies (Jackson ImmunoResearch).

Microscopy. Immunofluorescence was carried out as previously described. Immunostained cells were mounted using Vector Shield mounting reagent (Vector Laboratories). Images of fixed cells were captured by using a Spot II-cooled charge-coupled device (CCD) digital camera (Diagnostic Instruments) and an Olympus BX50 microscope with a 60x (numerical aperture [NA], 0.65 to 1.25) oil objective.

Focal adhesion morphometry. Focal adhesion area was measured using Metamorph software. Small rectangles were drawn inside the cell perimeter next to, but excluding, the paxillin positive focal adhesions. Using the region measurement function, the average intensity value of the rectangle was determined and this value then subtracted from every pixel in the image. Following size calibration of the images, paxillin positive focal adhesions were thresholded, and using the single function of the integrated morphometry analysis, focal adhesions were selected by reference to the original unmanipulated image and area calculated. All visible focal adhesions were selected for measurement. At the same time polygons were drawn around the entire perimeter of the cell and the cell area separately determined to allow calculation of the percentage cell area covered by focal adhesions.

Migration analysis. Transmitted light images were captured every 5 min for 3 h (40x objective). Cells undergoing division or apoptosis were excluded from analyses, and random migration analyses were performed on sparsely plated cultures. After image capture, nuclear translocation was tracked in time-lapse stacks using Metamorph V6.3 software. For wounding assays, confluent cultures were scratched with a pipette tip. Cell track (reoriented to zero in migration traces) and velocity and persistence ratio (i.e., the ratio of vectorial distance travelled to the total path length described by the cell) calculations were performed using Microsoft Excel.

Image preparation. Images were pseudocolored and overlaid by using Metamorph V6.3 software (Molecular Devices, Sunnyvale, CA). Final micrograph images and gray level adjustments were prepared in Adobe Photoshop.

Statistical analysis. All error bars show the standard error of the mean (SEM). Statistical comparison of means was performed in Graph Pad Prism using one-way ANOVA with Tukey’s Multiple Comparison Test.

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