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Tropomyosins are present in lamellipodia of motile cells

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Abstract

This paper shows that high-molecular-weight tropomyosins (TMs), as well as shorter isoforms of this protein, are present in significant amounts in lamellipodia and filopodia of spreading normal and transformed cells. The presence of TM in these locales was ascertained by staining of cells with antibodies reacting with endogenous TMs and through the expression of hemaglutinin- and green fluorescent protein-tagged TM isoforms. The observations are contrary to recent reports suggesting the absence of TMs in regions,where polymerization of actin takes place, and indicate that the view of the role of TM in the formation of actin filaments needs to be significantly revised.
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Introduction

The actin microfilament system is involved in a multitude of processes essential for life. It is an energy-transducing, force-generating system, contributing to the shape and integrity of the mammalian cell, in addition to providing cells with a system required for dynamic motile activities. Cells migrating on a solid substratum form lamellipodia and filopodia in the direction of movement. These cell surface protrusions are built of actin filaments with associated proteins (microfilaments). Photobleaching lamellipodia and filopodia of cells microinjected with labeled actin has provided evidence that actin polymerization at the advancing cell edge is responsible for the formation of lamellipodial actin filaments (Wang, 1985), and subsequent investigations have corroborated this view and added detailed information about the dynamic assembly/disassembly of the actin filament system (Danuser and Waterman-Storer, 2003; Ponti et al., 2003, 2004; Theriot and Mitchison, 1992).

Several protein complexes regulating actin polymerization have been identified. One is the actin-nucleating Arp2/3 complex. This consists of seven polypeptides that are regulated by the Wiskott–Aldrich syndrome protein (WASP), neural (N)-WASP and WASP family verprolin-homologous (WAVE) proteins known to relay signals from Cdc42 and Rac to the actin-nucleation machinery – the Arp2/3 complex. In this complex, growth of the nucleated actin filament occurs at the free (+)-end (barbed end) (Pollard and Borisy, 2003; Stradal et al., 2004). A second type of actin polymerizing protein

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is mDia, which belongs to the formin family of proteins (Pring et al., 2003; Zigmond, 2004). Both of these actin filament assembly systems are present in lamellipodia and therefore may contribute in varying degrees to the formation of the 2–4 μm zone of densely packed, highly ordered microfilaments constituting the interior of lamellipodia (Hoglund et al., 1980; Small et al., 1982). A third type of actin assembly factor is Spire (Quinlan et al., 2005). This protein contains four formin homology regions, nucleates actin filament assembly, and remains associated with the (−)-end of the actin filament. It is expressed preferentially in the brain and its function is linked to vesicle transport.

An important step in the control of actin filament formation in cells involves actin filament (+)-end-capping proteins such as CP (Mejillano et al., 2004), and the gelsolin family of proteins (Silacci et al., 2004). Increased actin filament formation in growth factor-stimulated cells requires removal of such capping proteins. In Arp2/3-dependent actin filament formation, branching and de novo nucleation of filaments would also contribute to the appearance of increased numbers of actin filament growth points, as would severing of existing filaments, by proteins like cofilin (Chan et al., 1998). The addition of actin monomers onto free filament (+)-ends created in this fashion is thought to be involved in "pushing" the cell membrane forward during the protrusion of lamellipodia and filopodia. Formins, on the other hand, are thought to remain at the plus end of actin filaments and operate by inserting actin monomers onto the actin filament (+)-end (Higashida et al., 2004; Kappe et al., 2003; Pring et al., 2003). With both mechanisms, unpolymerized actin in the form of profilin-actin appears to serve as the precursor (Hajkova et al., 2000; Grenklo et al., 2003; Romero et al., 2004).

Among the many proteins involved in the regulation of the microfilament system in cells are tropomyosin (TM) and gelsolin. TM is a major cell protein, constituting more that 1% of the total protein of non-muscle cells (Lin et al., 1985). In skeletal muscle, TM and troponin confer calcium sensitivity to the actomyosin-dependent force generation system. However, TMs are also present in non-muscle cells, but their function with respect to the microfilament system remains unknown. In mammalian non-muscle cells, there are numerous isoforms produced from multiple promoters and by alternative splicing of four TM genes. These isoforms display a diversity of sorting within cells (for reviews see Carlier et al., 1997; Gunning et al., 1998; Pittenger et al., 1994). TM is comprised of two α-helical chains, which form an elongated coil-coil dimer molecule with 5–7 actin-binding sites, depending on the length of the particular isoform. TM molecules, one on each side of the actin helix are bound to each other end-to-end providing structural stability to the filament. In vitro, TM inhibits Arp2/3-dependent actin filament branching (Blanchoin et al., 2001) and severing by cofilin (Ono and Ono, 2002). However, the extent to which Arp2/3-dependent branching and severing by cofilin regulates actin filament assembly in vivo remains unclear. The presence of TM in cellular domains containing these regulatory factors, such as the lamellipodium, could also modulate actin filament assembly.

We recently reported that TM can interact with even the smallest gelsolin:actin complexes to cause the dissociation of the gelsolin and facilitating the annealing of the released product into long filaments (Nyakern-Meazza et al., 2002). Longer gelsolin-capped oligomers of actin are also annealed into long filaments (Ishikawa et al., 1989). Thus, TM might be involved in controlling the accessibility of the (+)-end of actin filaments for polymerization (uncapping the barbed end), and it could also act as an assembly factor in the formation of stable actin filaments in the cell. There is evidence for the latter function in budding yeast (Evangelista et al., 2002).

Gelsolin is known to sever actin filaments and to bind with high affinity to the barbed end of actin filaments in vitro. It is distributed throughout the cell, even into the most distal parts of the advancing lamellipodium (Hartwig et al., 1989, 1990), where the addition of actin monomers to growing actin filaments takes place (Wang, 1985). It is still unclear, however, whether gelsolin or other capping proteins (e.g. CP (Bear et al., 2002)) control filament (+)-ends in the cell. In addition, it has been shown that gelsolin at increasing concentrations binds to TM-decorated actin filaments. At saturation a 2:2:14 ratio of gelsolin:TM:actin was reached. The results indicated that gelsolin might also play a role in controlling the activity of microfilaments in non-muscle cells (Nyakern-Meazza et al., 2002).

These findings and the observation that TM plays an important role in the formation of actin cables in budding yeast prompted the investigation to determine whether TM is present in regions with active actin polymerization, such as the lamellipodia and filopodia of mammalian cells.

Materials and methods

Plasmid constructs

Vectors containing HA-tagged rat non-muscle TM cDNA for 7 different isoforms were kindly provided by Dr. M. Gimona (Gimona et al., 1995). This collection included DNA sequences coding for high-molecular-weight TM-1, TM-2, TM-3, and low-molecular-weight TM-4, TM-5(NM1), TM-5a, and TM-5b.

GFP-tagged constructs were made by PCR amplifying TM cDNA from the HA-tagged constructs and cloning into pEGFP-C2 (Clontech). TM cDNA start

codons were removed during the polymerase chain reaction. Each TM fusion protein carried the GFP sequence at its amino terminus. Constructs were verified by DNA sequencing.

The expression of the constructs was tested by SDS-PAGE and Western blot analysis of cell extracts. For this, antibodies to hemagglutinin and GFP were used, respectively. In each case the analysis showed one band of the expected size and no abrogated forms of the tagged TMs.

Cell culture, stimulation and transfection

Rat mammary adenocarcinoma MTLn3 cells (generously provided by Dr. J. Condeelis) were cultured in MEM/EBSS (HyClone) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin at 37 °C and 7% CO_2. Cells were plated and grown on glass coverslips for 18–24 h and serum-starved for 3 h prior to stimulation with a final concentration of 4 or 5 nM EGF (Invitrogen) for 2–3 min (DesMarais et al., 2002). Human foreskin fibroblasts (1523) were cultured in the same medium as above, but with 10% FBS. Rat embryonic fibroblast (REF) cells (kindly provided by Dr. G. Pinaev, St Petersburg) were grown in DME/high glucose (HyClone) containing 10% FBS and antibiotics as above. Mouse embryonic fibroblast (MEF) cells were grown in DMEM (Gibco) supplemented with 10% FBS, essential amino acids and antibiotics. MTLn3 and REF cells were transiently transfected with the different TM constructs, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, while plasmids were introduced into MEF cells by electroporation (Yoon et al., 2001).

Antibodies and reagents

Primary antibodies: rabbit antiserum (T3651) and mouse monoclonal antibodies (TM311) against TM (Sigma), rabbit polyclonal antibodies to VASP (0010-10, Immunoglobe), goat polyclonal antibodies to the p34 subunit of Arp2/3 (ARPC2, ab 11798-100, Abcam Ltd, UK), rabbit polyclonal (Y-11, Santa Cruz Biotechnology) and mouse monoclonal antibodies to hemaglutinin (HA; 4C12, Abcam Ltd, UK). Secondary antibodies: TRITC- or FITC-conjugated donkey anti-mouse IgG, TRITC- or FITC-conjugated donkey anti-rabbit IgG, and FITC-conjugated donkey anti-goat IgG antibodies (Jackson ImmunoResearch). FITC- or TRITC-conjugated phalloidin (Sigma) were used to visualize filamentous actin.

Immunofluorescence

For fluorescence microscopy, cells were rinsed once with PBS at 37 °C, fixed with 4% formaldehyde in PBS at 37 °C for 15 min and further incubated at room temperature with 4% formaldehyde and 0.1% Triton X-100 for 5 min, unless otherwise stated. Slides were rinsed with PBS several times after which they were incubated with primary antibodies for 60 min at room temperature and then rinsed again with PBS. Incubation with secondary antibodies and labeled phalloidin was carried out for 30–45 min. Slides were rinsed 3 times in PBS and mounted in Prolong Antifade (Molecular probes) or in Vectashield (Vector laboratories Inc.). A Leica DMLB epifluorescence microscope equipped with a 63 × objective, a DC350F CCD-camera and Image Manager 500 software (Leica Microsystems) was used to document the cell staining.

Live-cell imaging

For videomicrography, MEF cells were grown in DMEM, transfected with GFP-TM constructs, and plated on coverslips as described above. At least 1 h prior to the experiment, the medium was changed to Leibowitz medium without phenol red (Gibco) containing 10% FBS. Coverslips with cells were mounted on slides (Yoon et al., 1998) leaving a thin film of medium. The 'chamber' with cells was then sealed with a mixture of petroleum jelly, beeswax, and lanolin (1:1:1) to prevent leakage and evaporation.

The micrographs were taken 18–48 h after transfection. The slides were maintained at 37 °C with an air stream incubator (Model ASI 400; NEVTEK, Burnsville, VA, USA) on the stage of a ZeissLSM 510. Time-lapse observations were made by using a 100 ×, 1.4 numerical aperture oil-immersion objective. GFP images were acquired by excitation at 488 nm and emission at 515–545 nm. Phase-contrast images of cells were also taken during time-lapse recording to define the position of the leading edge (Yoon et al., 1998). Images of live cells were acquired from the same focal plane at 5–10-s intervals for 5–10-min periods as described previously (Yoon et al., 1998). Image analysis was performed using the Zeiss LSM 510 software.

Results

Distribution of endogenous tropomyosin

Lamellipodia and filopodia are sites of actin polymerization in migrating mammalian cells. To test for the presence of endogenous TMs in these locales, rat mammary carcinoma cells (MTLn3), human foreskin fibroblasts, and rat embryo fibroblasts, were analyzed by indirect immunofluorescence using the commercially available monoclonal antibody TM311 (detecting TM-1, TM-2, TM-3, and TM-6) and polyclonal antibody

T3651 (see Materials and methods). Fluorescently labeled phalloidin was used to mark the presence of filamentous actin. As shown in Fig. 1a, MTLn3 cells stimulated with EGF have a circumferential lamellipodium recognizable by a zone along the cell edge having increased rhodamine-phalloidin staining. TM (green) under these conditions coincided with the actin staining out to the very edge of the advancing lamellum (Fig. 1b–d). Apart from a general diffuse staining, there was also punctate TM-staining all over the cell, with an intense staining in the perinuclear area. The intensity of the TM-staining decreased markedly towards the outer edge of the lamellipodium, where there appeared to be a 0.1–0.2 μm TM-free zone at the advancing edge. However, close examination of this zone revealed the presence of a TM staining also in this zone, although weaker, (see also Fig. 4).

Figs. 2 and 3 focus on lamellipodia of replated, migrating fibroblasts. Like in the MTLn3 cells, the endogenous high-molecular-weight TMs reached out into the lamellipodia. In the rest of the leading lamella, as well as in the perinuclear area there was an intense staining with the anti-TM antibody. As illustrated in Fig. 6, bundles of actin filaments seen in the zone proximal to the lamellipodium were stained periodically with the TM antibodies, which was also the case with stress fibers and thick bundles of actin filaments demarcating inactive cell edges (the latter not illustrated).

To ascertain the location of TM relative to the edge of the advancing lamellipodium of MTLn3 cells, the cells were starved and stimulated with EGF for 2 min. Then cells were fixed and co-stained for F-actin (phalloidin staining) and VASP (Fig 4a), F-actin and Arp 2/3 (Fig. 4b), Arp2/3 and TM (Fig. 4c; TM311 antibody), and finally TM and VASP (Fig. 4d). The results of this analysis located VASP right outside the actin staining in the lamellipodium, (Fig. 4a). Arp2/3 co-localized with actin out to the edge (Fig. 4b), as it did with TM (Fig. 4c), and the TM staining had VASP staining just outside (Fig. 4d). This again positions TM in the very same region where the actin polymerizing machinery has been found.

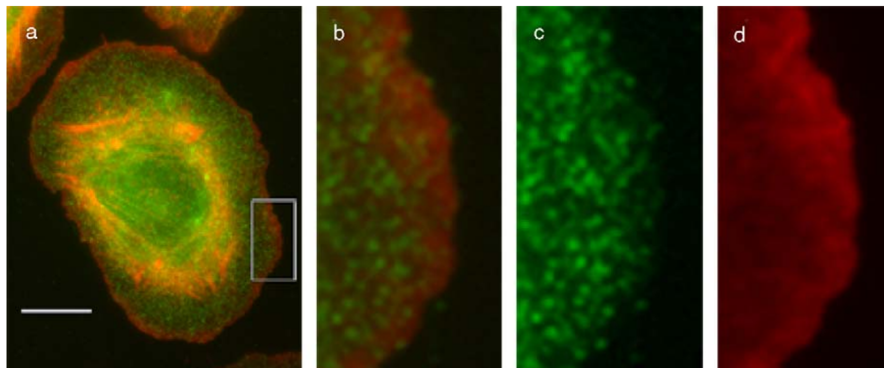

Fig. 1. Distribution of endogenous tropomyosins in EGF-stimulated MTLn3 cells. MTLn3 cells were starved for 3 h followed by stimulation with EGF for 2 min. The cells were then fixed as described in Materials and methods and stained for TM using the TM311 anti-TM antibody (green). Actin was visualized with rhodamine-phalloidin (red). (a, b) Merged images; (b–d) enlargements of the inset (a), with (c) and (d) illustrating TM and actin separately. Bar: 10 μm.

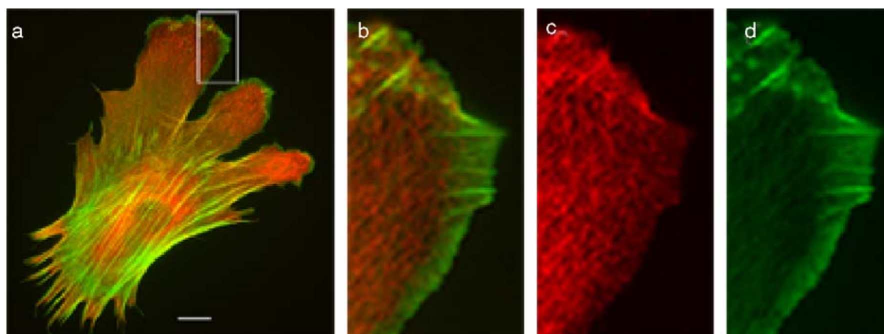

Fig. 2. Distribution of endogenous tropomyosins in lamellipodia of growing human fibroblasts. Human fibroblasts (1523) were replated and left to spread for 3.5 h before fixation with formaldehyde after which the cells were stained for TM using the polyclonal antibody T3651 (red), and for F-actin actin using FITC-phalloidin (green). (a) Overview of a fibroblast with merged images. (b–d) Enlargements of the rectangular area in (a), with (c) and (d) illustrating the distribution of TM and F-actin separately. Bar: 10 μm.

Distribution of HA-tagged tropomyosin isoforms in cells

To investigate the distribution of individual TM isoforms, MTLn3 and rat embryo fibroblasts were transfected with constructs expressing either hemaglutinin-(HA)-TM isoforms or GFP-tagged TM as described in Materials and methods. In both types of constructs the tag was linked to the N-terminus of the TM.

MTLn3 cells transfected with different HA-tagged TM isoforms had been starved for serum and then stimulated with EGF prior to immunofluorescence analysis (Fig. 5a–d). The close up of the area (rectangle) illustrates the relative position of actin (green) and TM (red) for the different isoforms. TM-positive dots were seen throughout the cell, including the lamellipodia along the cell circumference. The long isoforms TM1 (Fig. 5a) and TM2 (Fig. 5b) appeared to be present in lower concentration in the lamellipodia than the short isoforms TM4 (Fig. 5c) and TM5 (Fig. 5d). HA-TM-5 appeared to reach relatively high concentrations all the way out to the edge of the lamellipodium.

A similar situation was observed with HA-TM-5-transfected rat embryo fibroblasts (Fig. 6). The whole cell including the lamellipodia hosted high concentrations of the HA-tagged TM-5. In addition to a more general punctate TM staining, the actin bundles and filopodia in the lamellipodia had TM-positive dots along them. The periodic staining seen along the stress fibers strengthened the conclusion that the HA-tagged TM-5 entered into functional actin organizations (actin staining not shown).

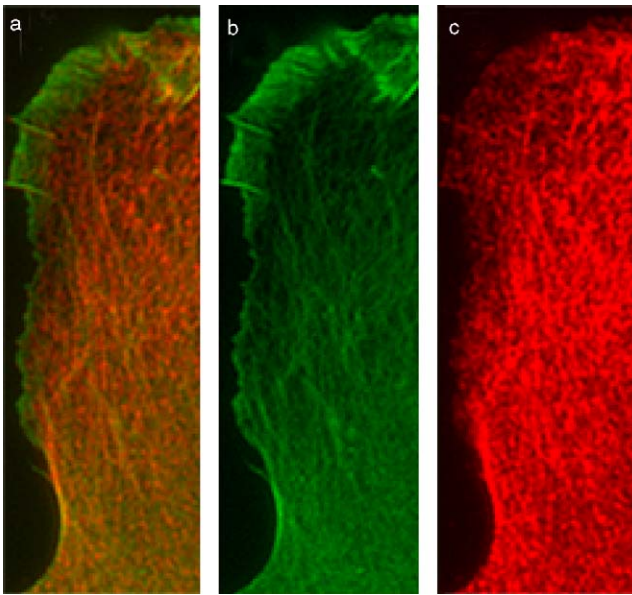

Fig. 3. Distribution of endogensous tropomyosins in the leading lamella of a human skin fibroblast at higher magnification. This figure shows the distribution of anti-TM antibody-reactive material in an extended region of the left portion of the leading lamella of the human fibroblast shown in Fig. 2. It draws the attention to the transition of the less organized TM-containing structures of the lamellipodium (1–4 μm zone), containing densely packed F-actin to distinct strands of F-actin with associated TM.

Distribution of GFP-tagged tropomyosin isoforms in cells

Like in the case of HA-tagged TM isoforms, the GFP-tagged variants were present all over the transfected MTLn3 cells (Fig. 7a–e). In lamellipodia of GFP-TM-5-transfected cells (Fig. 8), there were randomly distributed dots of GFP-labeled TM. Towards the inner part of the lamellipodia, 2–4 μm from the advancing edge, the GFP-TM dots were organized in rows sometimes visibly in close apposition to strands containing filamentous actin. Generally, the GFP fluorescence intensity decreased towards the very edge of the lamellipodia, was weak, but present in the zone 0.1–0.2 μm from the very edge. In the outer parts of the lamellipodium the GFP-tagged TM-5 appeared as punctae. Further in (0.5–1 μm from the edge) the punctae appeared in rows. The filamentous organization

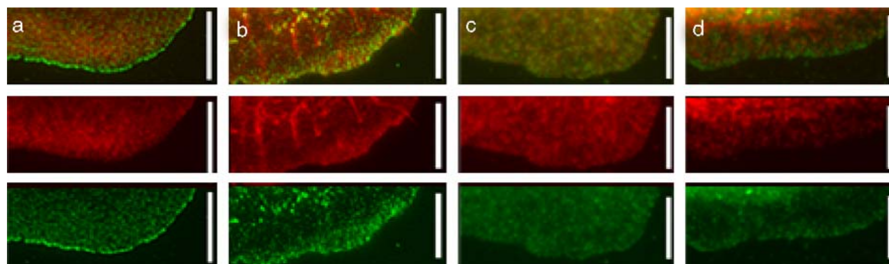

Fig. 4. Distribution of VASP, Arp2/3, and F-actin in relation to tropomyosin in lamellipodia of EGF-stimulated MTLn3 cells. MTLn3 cells were starved for 3 h and then stimulated with EGF for 2 min. The coverslips with cells were then fixed with formaldehyde (see Materials and methods) and co-stained for either F-actin (red) and VASP (green) (a), F-actin (red) and Arp2/3 (green) (b), or TM (red) and Arp2/3 (green) (c), and VASP (green) and TM (red) (d). Upper panels: merged images. Bars: 5 μm.

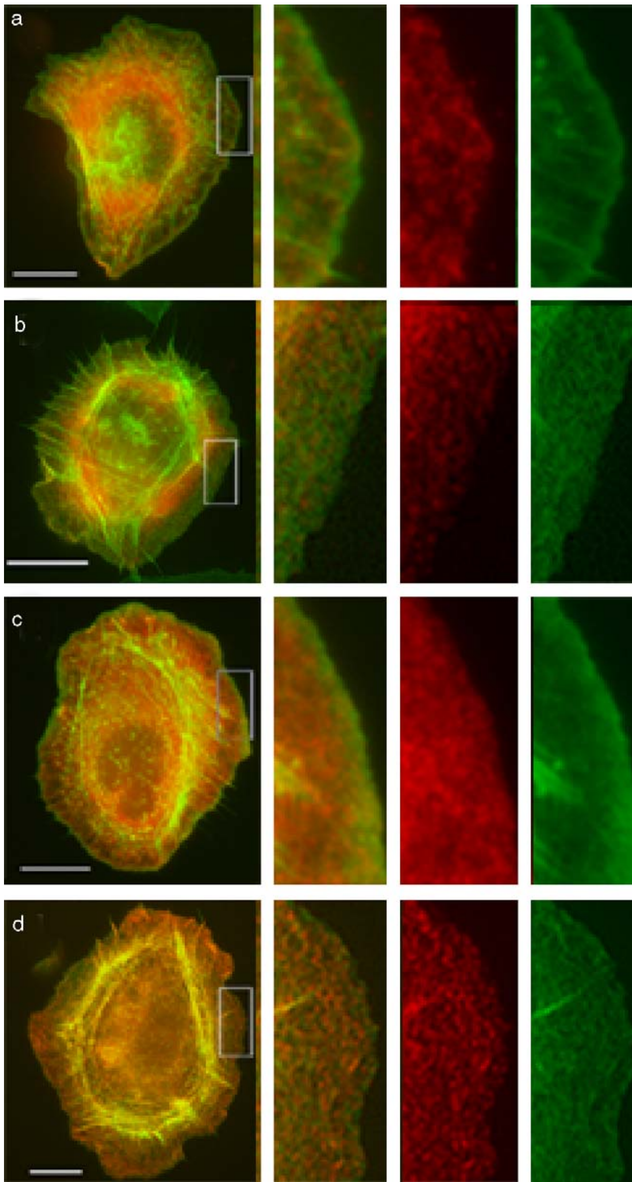

Fig. 5. Localization of HA-tagged TM-1 (a), TM-2 (b), TM-4 (c), and TM-5 (d) in MTLn3 cells. MTLn3 cells were grown and transfected with HA-TM isoform constructs. After 18–24 h the cells were starved for 3 h, stimulated with EGF for 2 min, fixed and stained for TMs using anti-hemaglutinin (red) and for F-actin with FITC-phalloidin (green). The micrographs show cells forming lamellipodia upon EGF stimulation. The parts of lamellipodia marked by the rectangles are shown enlarged to the right of each overview. Bars: 10 μm.

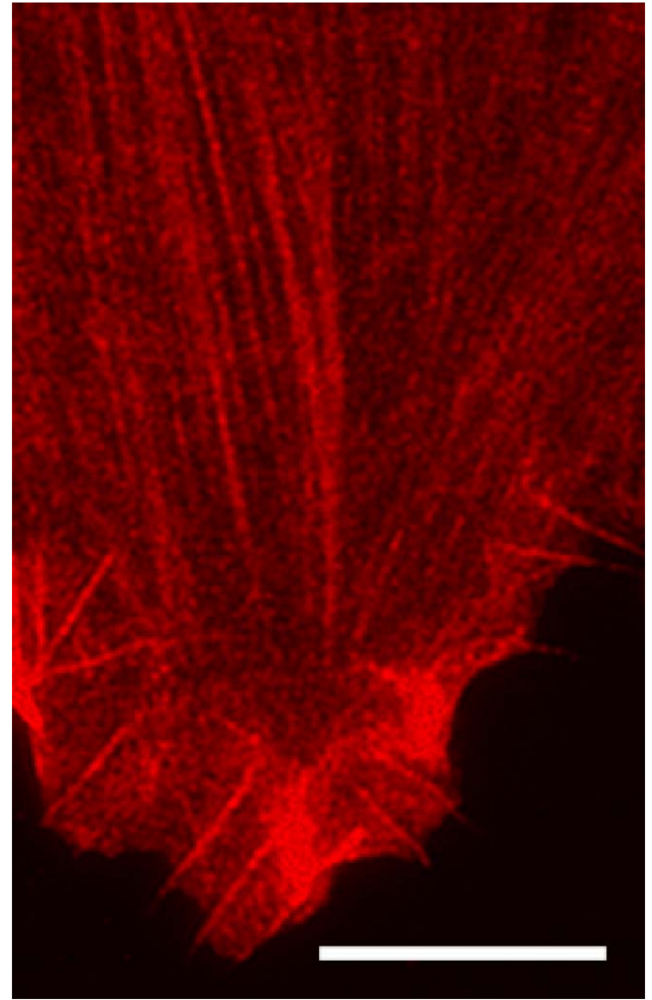

Fig. 6. Organization of HA-TM-5 in the leading lamellum of migrating rat embryo fibroblasts. Rat embryo fibroblasts were transfected with constructs resulting in expression of HA-TM5 for 45 h. The cells were then re-plated and allowed to attach, spread and migrate for a period of 20 h. The cells were then fixed and stained for hemaglutinin to localize the HA-tagged TM isform. Bar: 10 μm.

of the TM became even clearer further into the leading lamellum, where tightly positioned dots of GFP-TM-5, co-aligned with strands of F-actin (F-actin staining not shown). Further into the leading lamella, the GFP-TM-5 gave rise to brightly fluorescing stress fibers, suggesting that even with the rather large GFP tag the TMs retained the capacity to enter into functionally significant F-actin structures.

Live-cell imaging

Time-lapse observations of migrating mouse embryo fibroblasts, expressing GFP-TM-5 (see Fig. 9 and Supplementary video Material) suggested the involvement of this isoform in the dynamic activity, which typifies the leading edge. In the movie, there are GFP-TM-containing elongated structures forming in the lamellipodium. These thicken into strands as they move inwards towards the inner parts of the lamellipodium.

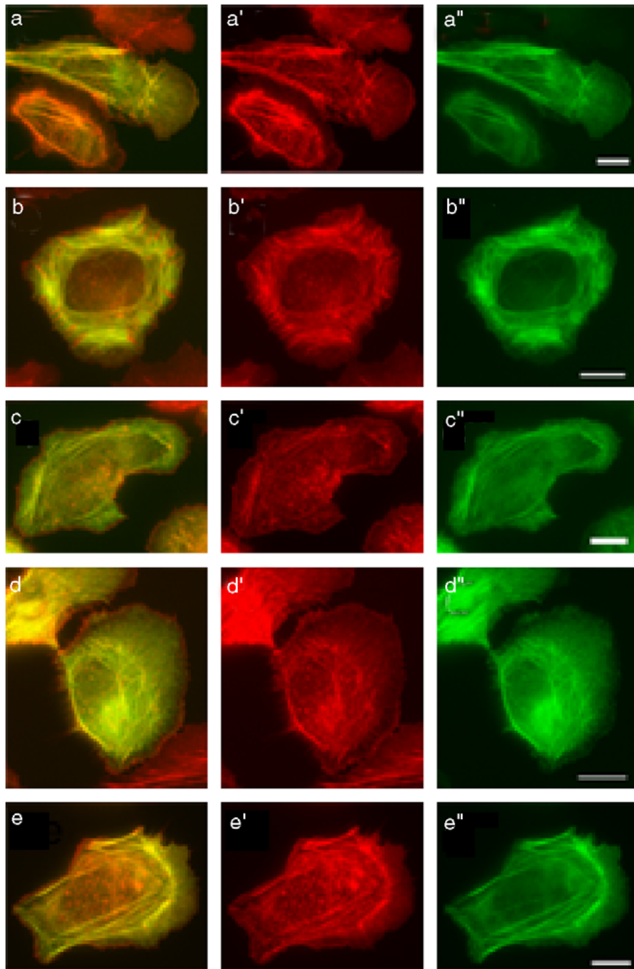

Fig. 7. Localization of GFP-tagged tropomyosins in MTLn3 cells. MTLn3 cells were grown and transfected with GFP-TM-1 (a), GFP-TM-2 (b), GFP-TM-3 (c), GFP-TM-4 (d), and GFP-TM-5 (e). After 18–24 h cells were fixed and stained with TRITC-phalloidin (red). In all cases there was a zone (0.1–0.2 μm) where the GFP fluorescence was relatively weak, indicating relatively low concentrations of the tagged TM isoforms. Bars: 10 μm.

The strands then integrate with stress fibers. These observations are consonant with those made on the fixed cells.

Discussion

Overexpression of the high-molecular-weight TM-1 isoform in transformed cells has been reported to normalize the morphology of the cells (Prasad et al., 1993, 1999), i.e. to result in return to a spread out morphology with adhesion sites and stress fibers. The MTLn3 (adenocarcinoma) cells did not react to transfection by a return to normality in this sense, neither when transfected with HA-tagged TM-1, TM-2,

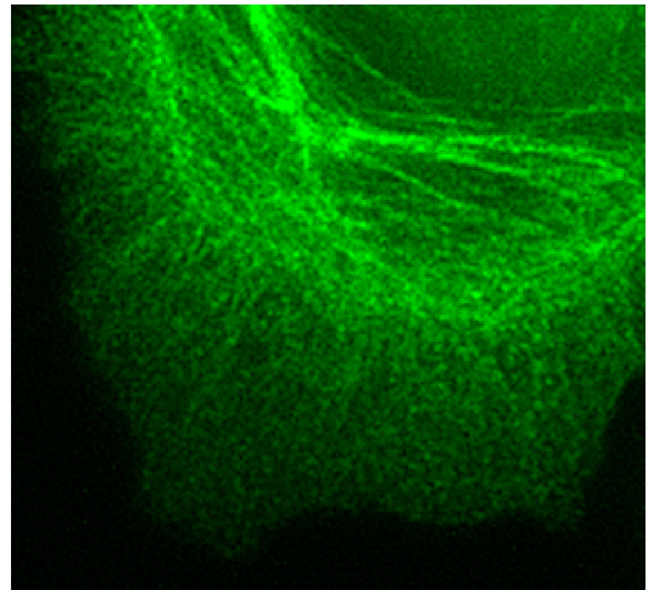

Fig. 8. Visualization of the distribution of GFP-tagged TM-5 in the lamellipodium of an MTLn3 cells. GFP-TM-5 penetrated to within a few tenths of a micrometer from the advancing edge of the lamellipodium, and appeared as a weak punctate staining in its outer parts. Within 0.5–1 μm from the edge the GFP-TM-5 dots appeared to be organized in rows, and further into the lamellipodium there were thicker strands of GFP-TM-5, which appeared to merge with stress fibers in the interior of the cell.

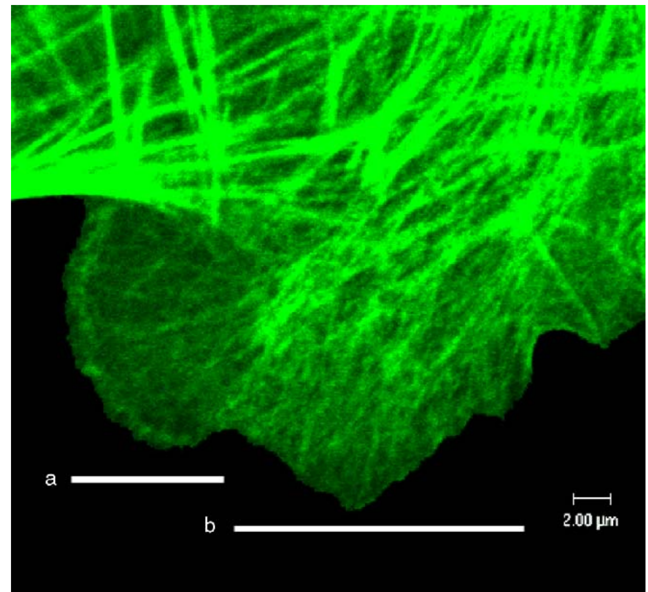

Fig. 9. Live-cell imaging of GFP-TM5-transfected mouse embryo fibroblasts. MEF cells were grown and transfected with GFP-TM-5 construct and observed live during spreading. This micrograph shows one frame from the middle of the Supplementary video. The left part of the lamella, indicated by the a-bar, advanced along the substratum, whereas the right above the b-bar retracted.

nor with TM-3, at least not under the conditions used for the experiments. In fact, it was not possible to distinguish non-transfected from transfected cells by examining their overall morphology as detected by the actin staining.

The interaction between extracellular structures (e.g. growth factors or matrix components) and their cognate cell surface receptors, elicit the assembly of actin filaments, which together with a large number of associated proteins are thought to form the core of lamellipodia and filopodia. Recent analyses have provided detailed information about the assembly and disassembly of actin filaments in lamellipodia. Assembly occurs at the advancing edge with disassembly apparently taking place at sites 1–3 μm inwards from the edge, i.e. in the proximal part of the lamellipodium (Danuser and Waterman-Storer, 2003; Gupton et al., 2005; Ponti et al., 2003, 2004). Adhesion of selected ensembles of filaments via actin filament-associated transmembrane proteins to structures in the extracellular matrix is required for subsequent large-scale translocations, bringing the cell body forward during cell migration. Much information has been gathered concerning molecules and interactions involved in these steps, but there are still many problems to be solved before an understanding of the mechanisms of cell migration is reached.

In vitro, the Arp2/3 complex nucleates actin filament formation giving rise to a collection of branched actin filaments (Mullins et al., 1998), and electron microscopy on detergent-extracted lamellipodia of migrating *Xenopus* fibroblasts and keratocytes have visualized structures suggesting branching of actin filaments in a 1–2 μm zone of up to 4 μm wide lamellipodia (Svitkina and Borisy, 1999). This has led to the suggestion that advancement of the cell edge during motility is based on the formation of arrays of branched actin filaments thought to 'push' the cell membrane forward (Pollard and Borisy, 2003). Furthermore, it has been suggested that the actin-interacting protein cofilin, through a filament severing activity, acts in synergy with Arp2/3 to increase the formation of actin filament growth points in lamellipodia (DesMarais et al., 2004). Interestingly, TM has been shown to inhibit Arp2/3-dependent actin filament branching (Blanchoin et al., 2001), and filament severing by cofilin in vitro (Ono and Ono, 2002). However, recent reports seem to suggest that lamellipodia and filopodia of rat mammary carcinoma cells (MTLn3) as well as PtK_1 epithelial cells are devoid of TM (Danuser and Waterman-Storer, 2003; DesMarais et al., 2002; Gupton et al., 2005; Ponti et al., 2003, 2004). Indeed the apparent absence of TM in the lamellipodia would appear to strengthen the branched-filament-formation model of lamellipodial protrusion (DesMarais et al., 2002).

In contrast, the experimental protocols used here reveal that endogenous TMs are present in lamellipodia and filopodia. The antibodies gave rise to a punctated staining all over the cell, including lamellipodia. Although, these dots of TM staining are sometimes less frequent in the zone 0.1–0.3 μm from the edge (defined by the actin staining) of lamellipodia, the major portion of the 1–4-μm-wide zone within these cytoplasmic domains had a significant anti-TM reactivity (Figs. 1 and 2). Bundled actin filaments in the form of filopodia protruding from the cell surface, or bundles of actin filaments inside lamellipodia were hardly discernable when stained under the conditions used here.

The position of endogenous high-molecular-weight TM isoforms recognized by the monoclonal antibody TM311 relative to the very edge of the lamellipodia was illustrated using anti-VASP antibodies. As shown earlier, VASP is located in the very edge of advancing lamellipodia, just outside the actin and Arp2/3-containing space. The results shown in Fig. 4, strengthen the conclusion that TM is located in the same region of the leading edge as Arp2/3 and actin.

The demonstration that all HA-tagged isoforms of TM have access to lamellipodia and filopodia (Fig. 5), and that lamellipodia similarly could be populated with the GFP-tagged TMs (Fig. 7) further supports the conclusion that TMs may be present in all domains of the leading edge of motile cells. In the lamellipodia of spreading cells, HA-TM-4 and HA-TM-5 appeared to be present at higher concentrations than the other isoforms. Both types of tagged TM isoforms gave rise to a marbled pattern in lamellipodia and a periodic staining of stress fibers. In the MTLn3 cells GFP-TM-5 was seen as randomly distributed punctae in the periphery of the lamellipodia, aligned in rows in the more proximal regions of the lamellipodia, and periodically positioned in stress fibers in the inner regions of the cells (Fig. 8). In live mouse embryo fibroblasts GFP-TM-5 was clearly seen as a diffuse staining close to the edge of the advancing lamellipodium, further into the lamellipodia as fibrous structures moving towards the center of the cell and coalescing with stress fibers (see Supplementary video).

Analysis of cells prepared to optimize the preservation of the arrangements of actin filaments in lamellipodia have revealed the presence of a dense organization of unbranched filaments, several micrometers long in this part of the cell (Hoglund et al., 1980; Small et al., 1982). In the advancing edge of lamellipodia, small numbers of long filaments appear to converge at special structures in the plane of the membrane at the advancing cell edge. There are also bundles of actin filaments in the lamellipodia, some of which protrude outside the main cell edge as filopodia (Hoglund et al., 1980; Small et al., 1982; Svitkina et al., 1995). The density of filaments in the lamellipodium precludes the direct observation of the presence of branched filaments. However, using a novel method (Svitkina and Borisy, 1999; Svitkina et al.,

1997), involving detergent extraction and brief treatment with gelsolin, elaborate branching patterns of actin are seen in the most distal 1–2 μm region of the lamellipodia. It is plausible that resulting short actin filaments could be transformed into the type of long filaments that dominate the lamellipodial scene, but there is no experimental evidence for this, as yet.

There is no question that Arp2/3 has the capacity to form branched filaments in vitro, that cofilin can sever actin filaments, and that TM inhibits these two activities. However, it is unclear how these proteins cooperate in the productive formation of lamellipodia and filopodia during cell migration. As shown earlier, TM can anneal gelsolin-capped actin oligomers (Nyakern-Meazza et al., 2002), i.e. TM displaces gelsolin from the (+)-end of actin oligomers and facilitates the annealing of the released product. It was shown that TM is very efficient in releasing actin from even the smallest gelsolin:actin complexes, and that there is a cooperativity in this process. Finally, gelsolin at increased concentrations is found associated with the TM-decorated filaments in a particular ratio (2:2:14 of gelsolin:TM:actin, respectively), suggesting that gelsolin and TM together are involved in the control of the activity of assembled filaments (Nyakern-Meazza et al., 2002).

During the preparation of this paper, it was reported (Gupton et al., 2005) that after microinjection of skeletal muscle TM into PtK_1 epithelial cells, there is an intensified motile activity in the advancing cell edge into which the skeletal TM had entered together with myosin II. The lamellipodia appeared to disappear, but the cells gained in migratory speed, which seemed to suggest that lamellipodia are dispensable in the process of cell migration. However, it is possible that formation of cell surface protrusions and their retraction is greatly speeded up by the injected skeletal TM, such that the normal morphology of the lamellipodia is lost, but their function retained (Gupton et al., 2005). It was also reported that introduction of skeletal TM led to an increased formation of filopodia. These observations are in agreement with the results presented here, in the sense that even skeletal TM microinjected into cultured cells has access to the lamellipodia and affects their function. Interestingly, cells transfected with HA-TM-5 often had a morphology suggesting an increased migratory activity as compared to non-transfected cells. In the case of fibroblasts expressing HA-TM-5, there was also an increased number of distinct filopodia, a feature also seen in the epithelial cells injected with skeletal TM.

Actin filaments assemble in vivo in the presence of an abundance of severing proteins. Therefore, the need for stabilizing factors like TM is obvious. Observations concerning the assembly of actin cables in budding yeast illustrate this (Evangelista et al., 2002). In this system, it was shown that TM must be acetylated for the actin cables to assemble. A similar situation could be at hand in mammalian cells. It is even plausible that TM is involved in the control of the early stages of actin filament assembly as an uncapping factor (Nyakern-Meazza et al., 2002), and subsequently as an assembly factor annealing actin oligomers formed in the early stages of polymerization. Finally TM most likely is involved in the control of force generation by stabilized long actin filaments interacting with myosin. This could be the case regardless of the mechanism responsible for actin polymerization. It will be of great interest to investigate these aspects of the functioning of TM and its regulation in vivo, as well as in the available in vitro systems (Le Clainche et al., 2003; Romero et al., 2004).

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2005.12.005.

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