Mammals Have Two Twinfilin Isoforms Whose Subcellular Localizations and Tissue Distributions Are Differentially Regulated*

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Twinfilin is a highly conserved actin monomer-binding protein that regulates cytoskeletal dynamics in organisms from yeast to mammals. In addition to the previously characterized mammalian twinfilin-1, a second protein with ~65% sequence identity to twinfilin-1 exists in mouse and humans. However, previous studies failed to identify any actin binding activity in this protein (Rohwer, A., Kittstein, W., Marks, F., and Gschwendt, M. (1999) Eur. J. Biochem. 263, 518–525). Here we show that this protein, which we named twinfilin-2, is indeed an actin monomer-binding protein. Similar to twinfilin-1, mouse twinfilin-2 binds ADP-G-actin with a higher affinity ($K_D = 0.12 \mu M$) than ATP-G-actin ($K_D = 1.96 \mu M$) and efficiently inhibits actin filament assembly in vitro. Both mouse twinfilins inhibit the nucleotide exchange on actin monomers and directly interact with capping protein. Furthermore, the actin interactions of mouse twinfilin-1 and twinfilin-2 are inhibited by phosphatidylinositol (4,5)-bisphosphate. Although biochemically very similar, our Northern blots and in situ hybridizations show that these two proteins display distinct expression patterns. Twinfilin-1 is the major isoform in embryos and in most adult mouse non-muscle cell-types, whereas twinfilin-2 is the predominant isoform of adult heart and skeletal muscles. Studies with isoform-specific antibodies demonstrated that although the two proteins show similar localizations in unstimulated cells, they are regulated by different mechanisms. The small GTPases Rac1 and Cdc42 induce the redistribution of twinfilin-1 to membrane ruffles and cell-cell contacts, respectively, but do not affect the localization of twinfilin-2. Taken together, these data show that mammals have two twinfilin isoforms, which are differentially expressed and regulated through distinct cellular signaling pathways.

Actin is a conserved protein that is necessary for a large number of cellular processes including cell division, movement, polarized growth, secretion, and endocytosis. In cells, actin filaments form dynamic structures that rapidly assemble and disassemble in a coordinated fashion. Actin dynamics are tightly regulated by an array of actin-binding proteins that interact with actin filaments and/or monomeric actin. The pool of unpolymerized actin in non-muscle cells can constitute up to 50% of the total cytoplasmic actin, and thus the various actin monomer-binding proteins play an important role in actin dynamics by regulating the size, localizations, and dynamics of the cellular actin monomer pool (1).

Three families of small actin monomer-binding proteins are conserved in evolution, existing in organisms as diverse as yeast and mammals. These are profilins, ADF/cofilins, and twinfilins. In addition to these evolutionarily conserved proteins, a fourth class of small actin monomer-binding proteins, the $\beta$-thymosins, exists in vertebrates. $\beta$-Thymosins are small peptides that bind specifically to ATP-G-actin, prevent actin filament assembly, and thus function as actin monomer-sequestering agents in certain specialized vertebrate cell types (2).

Profilins are ubiquitous actin monomer-binding proteins with a molecular weight of 12–16 kDa. Profilins have a higher affinity for ATP-actin monomers than they do for ADP-G-actin, and they also interact with phospholipids and polyproline (1). These ubiquitous proteins can affect several different aspects of actin dynamics. In the absence of free filament-barbed ends, profilins function as actin monomer-sequestering proteins, whereas in the presence of free barbed ends, profilins promote actin filament assembly (3, 4). Most profilins also enhance the nucleotide exchange on actin monomers, and at least in yeasts, Saccharomyces cerevisiae and Schizosaccharomyces pombe, this activity is essential in vivo (5, 6).

ADF/cofilins are small (molecular mass = 15–19 kDa) essential proteins composed of a single ADF-H domain. They interact with both actin monomers and filaments and bind ADP-actin with a higher affinity than they do to ATP-actin (7, 8). ADF/cofilins enhance actin filament dynamics by depolymerizing filaments from their pointed ends (8) and by severing actin filaments, thereby increasing the amount of filament ends (9). Furthermore, ADF/cofilins inhibit the nucleotide exchange on actin monomers (10, 11).

Twinfilin was originally identified from the yeast S. cerevisiae by its sequence homology to ADF/cofilin proteins (12, 13). Twinfilin is composed of two ADF/cofilin-like domains (ADF-H domains) that are separated by a short linker region and flanked by a short tail region. The two ADF-H domains are 20%
homologous to ADF/cofilin and to each other (12). Although twinfilin shares similar building blocks to ADF/cofilins, these two proteins have profound differences in their biochemical properties. Whereas ADF/cofilins bind both actin monomers and filaments, twinfilins interact only with monomeric actin and do not promote filament depolymerization (13–15). Similar to ADF/cofilins, twinfilins bind ADF-G-actin with a higher affinity than they do to ATP-G-actin and have slower $k_{cat}$ rates from actin monomers and prevent filament assembly much more efficiently than ADF/cofilins (16, 17). Twinfilins also interact with heterodimeric capping proteins, and their physiological function may be to localize actin monomers to the cellular sites of rapid actin dynamics (16, 18).

In multicellular organisms, there are typically several isoforms of both ADF/cofilin and profilin. The three mammalian ADF/cofilin isoforms (cofilin-1, cofilin-2, and ADF) are expressed in distinct tissues, and their affinities for actin monomers and their effects on actin filament polymerization and nucleation differ. This finding suggests that the three mammalian ADF/cofilin isoforms have evolved to fulfill the specific requirements for actin dynamics in differentiated cell types (19, 20). All the three profilin isoforms in mammals (profilin I, profilin IIa, and profilin IIb) show cell-type specific expression patterns (21, 22) and display differences in their affinities for actin and their other ligands: polyproline and phospholipids (21–24). To examine whether there are also several twinfilin isoforms in mammals, we carried out searches on mouse and human sequence databases. These searches revealed one mouse and one human protein with ~65% sequence identities to previously characterized mouse and human twinfilin-1. Here, we show that the mouse protein, which we named twinfilin-2, is an actin monomer-binding protein with similar biochemical activities to other known twinfilins.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The DNA fragment corresponding to mouse twinfilin-2 cDNA was amplified by PCR from an embryonic day (E)7–20 mouse embryo cDNA library. The oligonucleotides introduced Neol and HindIII sites at the 5’ and 3’ ends of the PCR product, respectively. The PCR fragment was digested with Neol and HindIII and ligated into the pGAT2 vector (25) to create plasmid pPL68. The plasmid for expressing mouse twinfilin-1 as a glutathione S-transferase (GST) fusion protein (pPL78) has been described previously (14). For expression of His-tagged versions of twinfilin-1 and twinfilin-2 in Escherichia coli, the plasmids pPL78 and pPL68 were digested with Neol and HindIII and the cDNA fragments were cloned into the pGAT2 vector and transformed into Bl2(DE3) cells. The plasmids pPL144 (twinfilin-1) or pPL182 (twinfilin-2) were grown in Luria broth medium to an optical density of 0.6 at 600 nm and expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 3 h after induction, washed once with 20 mM Tris (pH 7.5), resuspended to 40 ml of 20 mM imidazole, 50 mM NaCl, 10 mM Tris (pH 7.5), lysed by sonication, and centrifuged for 30 min at 30,000 × g. The supernatant was loaded onto a nickel-nitriilotrietactic acid-agarose column (Qiagen) and eluted with a linear 20–250 mM imidazole gradient. The peak fractions containing the His-tagged twinfilins were concentrated to 1.5 ml in Centricron 10-kDa cutoff devices and loaded onto a Superdex-75 HiLoad gel filtration column (Amersham Biosciences), which had been equilibrated with 10 mM Tris (pH 7.5), 50 mM NaCl. The peak fractions containing twinfilins were pooled, concentrated in Centricron 10-kDa cutoff devices to a final concentration of 100–300 μM, frozen in liquid N2, and stored at −70°C. Chicken αβ3 and mouse αβ3-capping proteins were produced in E. coli BL2(DE3) cells transformed with pET expression plasmids containing the cDNAs for these capping proteins. Cells were grown in Luria broth medium to an optical density of 0.6 at 600 nm, and expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 3 h after induction, washed once with 20 mM Tris (pH 7.5), resuspended in 20 ml Tris (pH 7.5), and lysed by sonication. The cell lysate was centrifuged in a Beckmann SS-34 rotor at 10,000 rpm for 30 min at 4°C, and the supernatant was further centrifuged in a Beckman Ti45 rotor at 30,000 rpm for 30 min at 4°C. The supernatant from the second centrifugation was applied onto a Q-Sepharose Fast Flow anion exchange column (Amersham Biosciences) equilibrated with 20 mM Tris (pH 7.5), and the proteins were eluted with a 0–1 M NaCl gradient. The fractions containing the capping protein were pooled, concentrated to a volume of 0.5 ml with a 20 mM KH2PO4 (pH 7.0), and applied onto a hydroxyapatite column (Bio-Rad) that had been equilibrated in 20 mM KH2PO4 (pH 7.0). Proteins were eluted with a linear 20–250 mM KH2PO4 gradient, and the fractions containing capping protein were pooled, concentrated to 1.5 ml, and applied onto a Superdex-75 column equilibrated in 10 mM Tris (pH 7.5), 50 mM NaCl. The peak fractions containing the capping protein were concentrated to 100–300 μM and stored at −70°C. Rabbit muscle actin was prepared from acetone powder as described previously (27), and the recombinant mouse cofilin-1 was purified as described by Vartiainen et al. (19). The human platelet actin and pyrene-labeled actin were from the Cytoskeleton Inc.

Actin Assembly Assays—Assays were carried out in 4 ml hybridizations at 30°C. The change in the fluorescence of 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled G-actin was used to monitor the binding of twinfilins to actin monomers. Actin was labeled by NBD-Cl as described previously (28, 29). ADP-actin was prepared by incubating NBD-actin with hexokinase-agarose beads (Sigma) and glucose for 2 h at 4°C. The final concentration of actin in these assays was 0.2 μM, and the twinfilin concentration was varied from 0.05 to 14 μM. Experiments were carried out at room temperature in F-buffer (2 mM Tris-HCl (pH 8.0), 0.1 mM CaCl2, 0.1 mM DTT, 0.2 mM ADP or ATP, 1 mg/ml bovine serum albumin, 2 mM MgCl2, 0.1 mM EDTA). The data were analyzed as described previously (17). The rate of nucleotide exchange on actin monomers in the absence and presence of twinfilin was measured as described previously.

Actin Assembly Assays—Kinetics of actin filament assembly were monitored by pyrene fluorescence with excitation at 365 nm and emission at 407 nm using a BioLogic MOS-250 fluorescence spectrophotometer. 54 μl of 3.9 mM actin (1.6 pyrene actin:human platelet actin) in G-buffer (5 mM Tris (pH 7.5), 0.2 mM ATP, 0.2 mM DTT, 0.2 mM CaCl2) was mixed with 7 μl of G-buffer or 15/30 μl twinfilin-1 or twinfilin-2 in the presence or absence of P1 (4,5,6-P1, Matreya Inc). Polymerization was induced by the addition of 7 μl of 10 X initiation mixture (1X KCl, 20 mM MgCl2, 5 mM ATP).

Native Gel Electrophoresis—The interaction of mouse twinfilins with PI(4,5)P2 was examined by native gel electrophoresis as described for profilins (16). Final concentrations of twinfilins (600 μM) were 4 μM, and the PI(4,5)P2 concentrations were 0, 20, or 30 μM. Twinfilin-capping protein interactions were studied on 6% native polyacrylamide gels. Purified mouse twinfilins and chicken αβ3-, or mouse αβ3-capping protein (either alone or in a mixture with each other) was diluted to desired concentrations in 10 mM Tris (pH 7.5), 50 mM NaCl, 100 mM DTT, and incubated for 60 min at room temperature. DTT aliquots of the protein samples were then mixed with 5 μl of loading buffer (125 mM Tris (pH 8.8), 250 mM NaCl, 2.5 mM DTT, 50% glycerol) and loaded onto a 6% polyacrylamide gel. The gel was run for 150 min at 100 V by using buffer (25 mM Tris, 194 mM glycine (pH 8.5), 0.5 mM DTT), and the proteins were detected either by Coomassie Blue staining or by Western blotting with anti-twinfilin antibodies.

Northern Blotting—Twinfilin-1 and twinfilin-2 cDNA probes were prepared as described previously (14). Both twinfilin probes were hybridized to commercial mouse embryo and adult mouse tissue Northern blots (Clontech) according to manufacturer’s instructions.
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Mouse Twinfilin-2 Is an Actin Monomer-binding Protein—To examine the biochemical activities of mouse twinfilin-2, we expressed it as a GST fusion protein in E. coli cells. GST was subsequently removed by thrombin digestion, and the recombinant protein was further purified by gel-filtration chromatography. The purified recombinant mouse twinfilin-2 was fully soluble and monomeric according to its elution position from a gel-filtration column.

We recently showed that the fluorescence of muscle NBD-G-actin is modulated upon binding to mouse twinfilin-1, thereby providing a means to determine the affinity of twinfilin-1/actin monomer complex (17). Therefore, we examined whether mouse twinfilin-2 would also affect the fluorescence of NBD-G-actin. The assays were carried out under physiological ionic conditions according to manufacturer instructions. Immunofluorescence microscopy was performed as described previously (14).

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concentrations and by using NBD-labeled platelet actin. The binding of twinfilin-2 resulted in a 50% enhancement in the fluorescence of NBD-G-actin, indicating that twinfilin-2 also interacts with actin monomers (Fig. 2). The $K_D$ values of twinfilin-2 for ADP-G-actin and ATP-G-actin were 0.12 and 1.96 $\mu$M, respectively. A similar assay with twinfilin-1 provided $K_D$ values of 0.09 $\mu$M (ADP-G-actin) and 1.77 $\mu$M (ATP-G-actin). Therefore, both mouse twinfilins bind ADP-G-actin with ~20-fold higher affinity than the ATP-G-actin, and the affinities of both proteins for actin monomers are very similar to each other. Although both twinfilins appear to bind platelet ADP-G-actin with similar affinities ($K_D = 0.09–0.12 \mu M$) than previously reported for muscle ADP-G-actin/twinfilin-1 complex ($K_D = 0.05 \mu M$) (17), the affinities of these proteins for platelet ATP-G-actin ($K_D = 1.77–1.96 \mu M$) (Fig. 2B) are significantly lower than the affinity of twinfilin-1 for muscle ATP-G-actin ($K_D = 0.47 \mu M$) (17).

We also used an actin co-sedimentation assay to examine the possible interactions of mouse twinfilins with actin filaments. Neither twinfilin-1 nor twinfilin-2 co-sedimented with actin filaments, providing further support for the previous conclusions that twinfilins do not interact with F-actin (13, 15). However, both twinfilin-1 and twinfilin-2 increased the amount of monomeric actin in F-actin co-sedimentation assays, indicating that they inhibit actin filament assembly in vitro (data not shown).

Previous studies on yeast twinfilin (13) showed that this protein decreases the rate of nucleotide exchange of G-actin. The ability of mouse twinfilin-1 and twinfilin-2 to inhibit the exchange of $\epsilon$-ATP to ATP on actin monomers was measured by using 1 $\mu$M G-actin and 0–3 $\mu$M twinfilins. The results showed that both twinfilin-1 and twinfilin-2 efficiently inhibit the spontaneous nucleotide exchange on G-actin in a concentration-dependent manner (Fig. 3).

**Interactions of Mouse Twinfilin Isoforms with PI(4,5)P2 and Capping Protein—Phosphoinositides, especially PI(4,5)P2, modulate the activities of many actin-binding proteins such as ADF/cofilin (34) and profilin (35). Previous studies showed that yeast twinfilin also interacts with phosphoinositides and that PI(4,5)P2 inhibits its actin monomer-sequestering activity in vitro (16). To examine whether mouse twinfilins also interact with PI(4,5)P2, we carried out a native gel electrophoresis assay (Fig. 4A). In this assay, 4 $\mu$M mouse twinfilin-1 or twinfilin-2 were loaded onto a native gel either alone or in a mixture with 20 or 30 $\mu$M PI(4,5)P2, and run toward the anode by their own charges. As shown in Fig. 4A, purified twinfilins migrate as a single sharp band, but when mixed with PI(4,5)P2 before loading on a gel, twinfilins migrate as a smear. This shows that both mouse twinfilins directly interact with PI(4,5)P2.

To examine the effects of PI(4,5)P2 on the actin binding activity of mouse twinfilins, we carried out a pyrene-actin assembly assay (Fig. 4B). Both mouse twinfilin-1 and twinfilin-2 inhibit the nucleation/polymerization of pyrene-actin in a concentration-dependent manner. However, when twinfilins were preincubated with PI(4,5)P2 before the assay, they were significantly less effective in inhibiting the pyrene-actin assembly. 3 $\mu$M twinfilin-1 and twinfilin-2 with 30 $\mu$M PI(4,5)P2 inhibit actin assembly as efficiently as 1.5 $\mu$M twinfilin without PI(4,5)P2, suggesting that 30 $\mu$M PI(4,5)P2 down-regulates the activity of twinfilin by ~50% (Fig. 4B). It is important to note that higher concentrations of PI(4,5)P2 decreased twinfilin activity even further, but they also affected the pyrene-actin signal (data not shown).

In addition to actin and phospholipids, yeast twinfilin binds capping protein (Cap1/2p). This interaction appears to be essential for the correct subcellular localization of twinfilin in yeast cells (16). Furthermore, mouse twinfilin-1 was shown to also interact with $\alpha_1\beta_2$-capping protein in vitro (16). To examine whether mouse twinfilin-2 also interacts with capping protein and whether the two mouse twinfilins would have any specificity toward certain capping protein isoforms, we carried out a native gel electrophoresis assay with purified twinfilins and chicken $\alpha_1\beta_1$ and mouse $\alpha_1\beta_2$-capping proteins. $\alpha_1\beta_1$ is the
major capping protein isoform in striated muscle cells, whereas $\alpha_1\beta_2$ is the predominant isoform of non-muscle cells (36, 37). In these assays, we used His-tagged versions of twinfilin-1 and twinfilin-2, because thrombin cleavage of the GST-twinfilin inhibited the interaction with capping protein. 2

Native polyacrylamide gel, purified $\alpha_1\beta_1$-capping protein runs as a single band below the migration positions of twinfilin-1 and twinfilin-2. However, when mixed with each other before loading on the gel, a complex of twinfilin and capping protein emerges, running between the migration positions of twinfilin and capping protein (Fig. 5). Similar native gel electrophoresis assays were also carried out with lower protein concentrations, and the twinfilin/capping protein complexes were subsequently visualized by Western blotting. These assays provided approximate $K_D$ values of 0.5 $\mu$M for both twinfilin-$\alpha_1\beta_1$-capping protein and twinfilin-$\alpha_1\beta_2$-capping protein complexes (data not shown). This indicates that both mouse twinfilin isoforms interact with chicken $\alpha_1\beta_1$-capping protein with similar affinities to each other. Furthermore, based on a native gel electrophoresis assay, both mouse twinfilins also bind mouse $\alpha_1\beta_2$-capping protein with similar affinities, suggesting that they do not have any isoform specificity, at least toward the $\beta$-subunit of capping protein (data not shown).

Expression Patterns of Mouse Twinfilins—The expression patterns of twinfilin-1 and twinfilin-2 genes in developing mouse embryos and adult mouse tissues were examined by Northern blot (Fig. 6) and RNA in situ hybridization analyses (Fig. 7). Northern blot analysis with twinfilin-1 and twinfilin-2 cDNA probes showed that twinfilin-1 is the major isoform during embryonic development and is expressed at a constant level during embryonic days (E) 7–17. In contrast, twinfilin-2 was only weakly expressed in embryos and its mRNA levels appeared to increase during development (Fig. 6A). These results were further confirmed by in situ hybridization analysis of mouse embryos. Twinfilin-1 expression was widespread throughout the embryonic stages analyzed (E10.5, E12.5, E14.5, and E18.5) (Fig. 7A and data not shown). At E14.5, strongest expression was observed in the developing central and peripheral nervous system (CNS and PNS, respectively) and in the olfactory sensory epithelium (Fig. 7, C and D). In the CNS, the proliferating neuronal precursors in the ventricular zone expressed twinfilin-1 more than the postmitotic neurons (Fig. 7C). At E18.5, highest expression levels were detected in the mechanosensory hair cells of the inner ear (Fig. 7G) and in the differentiated keratinocytes of the skin (Fig. 7I). Twinfilin-2 expression was relatively weak during all of the embryonic stages (Fig. 7B and data not shown). At E14.5, a slight increase in the expression could be observed in heart, CNS, and PNS (Fig. 7D and data not shown). At E18.5, twinfilin-2 was strongly expressed in the inner ear hair cells and in the head muscles (Fig. 7H). Expression of both genes could be detected in the differentiating bone, whereas in cartilage, very little mRNA, if any, was present (Fig. 7, C, E, G, and H). In contrast to twinfilin-1, no expression of twinfilin-2 was detected in the nasal epithelium or in the skin keratinocytes (Fig. 7, F and J).

Northern blot analysis of adult mouse tissues indicated that twinfilin-1 is found in most tissues and is strongly expressed in liver and kidney (Fig. 6B). However, no twinfilin-1 expression could be detected in skeletal muscles. This expression pattern agreed well with our previous data where we examined the expression of twinfilin-1 mRNA by using a similar Northern blot filter (14). Interestingly, the expression pattern of twinfilin-2 was different from the one of twinfilin-1. Northern blot analysis showed that twinfilin-2 mRNA is expressed strongly only in the heart and is found at much lower levels in other tissues, whereas twinfilin-1 mRNA is expressed in all tissues tested (Fig. 6B). This suggests that the expression patterns of twinfilin-1 and twinfilin-2 are different from each other, indicating that these two isoforms may have different roles in the development of the mouse embryo and adult tissues.

2 S. Falck, M. A. Wear, J. A. Cooper, and P. Lappalainen, unpublished data.
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Fig. 6. Northern blot analysis of twinfilin expression in mouse embryos and adult mouse tissues. A, expression of twinfilin-1 (upper panel) and twinfilin-2 (lower panel) in mouse embryos. Note that the twinfilin-2 blot has been exposed five times longer than the twinfilin-1 blot. Twinfilin-1 mRNA is expressed at constant levels throughout the development. Twinfilin-2 mRNA is present at much lower levels than twinfilin-1, and its expression increases during development. B, Northern blot analysis of adult mouse tissues. Twinfilin-1 (upper panel) is expressed at high levels in the liver and kidney and at moderate levels in heart, brain, spleen, lung, and testis. No twinfilin-1 expression is detected in the skeletal muscle. Twinfilin-2 (lower panel) is expressed at high levels only in the heart and at lower levels in the brain, spleen, lung, skeletal muscle, kidney, and testis. Twinfilin-2 is not expressed in the liver.

It is important to note that twinfilin-2 is expressed in skeletal muscle where twinfilin-1 mRNA is absent and that twinfilin-1 is expressed at high levels in liver where no twinfilin-2 expression could be detected (Fig. 6B).

The in situ hybridization analyses on adult tissue sections confirmed the results obtained with the Northern blot analysis. Twinfilin-1 was the only isoform expressed in adult mouse liver, and twinfilin-2 was the only one in skeletal muscle (data not shown). Both genes were expressed in all of the brain areas but at different levels. Whereas twinfilin-1 expression was especially strong in the septum and the ependymal cells lining the ventricles, twinfilin-2 was highly expressed in the cerebral cortex (data not shown).

Twinfilins Are Abundant Proteins in Cultured Mouse Cells—To study the cell biological properties of mouse twinfilins, we generated isoform-specific antibodies against these two proteins. We had previously generated a polyclonal antibody against mouse twinfilin-1 in rabbit, but a Western blot analysis revealed that this antibody recognizes both twinfilin-1 and twinfilin-2 (data not shown). We further purified this antibody by adsorbing the cross-reactive fraction by a twinfilin-2 affinity column and obtained an antibody that is at least 500-fold more specific for twinfilin-1 than for twinfilin-2 (Fig. 8A, upper panel). Similarly, we generated antibodies against twinfilin-2 in hen (Agrisera Inc.), affinity-purified the IgY-fraction with recombinant mouse twinfilin-2, and cross-reacted it against twinfilin-1. After these purification steps, this antibody was at least 500-fold more specific for twinfilin-2 than for twinfilin-1 (Fig. 8A, lower panel).

We next used these specific antibodies to determine the abundances of twinfilin-1 and twinfilin-2 in cultured mouse cells. We carried out a Western blot analysis with different dilutions of NIH 3T3 and Neuro2A cell extracts and several concentrations of purified mouse twinfilin-1, twinfilin-2, and mouse coflin-1 (Fig. 8B). On Western blots, the affinity-purified twinfilin-1 and coflin-1 antibodies recognize a single band with mobilities identical to the recombinant proteins, whereas the twinfilin-2 antibody recognizes a double band that migrates at the position of the purified recombinant mouse twinfilin-2. Because this affinity-purified antibody is highly specific for twinfilin-2 (Fig. 8A) and because a similar double band is also recognized by our other twinfilin-2 antibody generated in guinea-pig (data not shown), these two bands most probably represent different posttranslationally modified (e.g., phosphorylated) forms of twinfilin-2. A comparison of the intensities of twinfilin staining in the recombinant protein and cell lysate samples shows that in these cell lines twinfilin-2 is slightly more abundant than twinfilin-1, being present at molar ratio of 1.3:1 to twinfilin-1. It is important to note that the twinfilin-1/2:cofilin-1 molar ratio in these cells is ~1.7 (Fig. 8B). Because coflin-1 is a major ADF/cofilin in NIH 3T3 and Neuro2A...
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Fig. 8. Both twinfilins are abundant proteins in NIH 3T3 and Neuro2A cells. A, three different concentrations of recombinant mouse twinfilin-1 and twinfilin-2 proteins were run on polyacrylamide gels, and the proteins were visualized by Western blotting using anti-twinfilin-1 and anti-twinfilin-2 antibodies. These antibodies are isoform-specific, because each of them recognizes only one mouse twinfilin isoform. B, mouse cell extracts and known concentrations of recombinant mouse twinfilin-1, twinfilin-2, and cofilin-1 were run on polyacrylamide gels, and the proteins were visualized by Western blotting using isoform-specific twinfilin-1/2 and cofilin-1 antibodies. The twinfilin-1:twinfoin-2:cofilin-1 molar ratio in these cells is ~1:1.3:15. It is important to note that twinfilin-2 from NIH 3T3 and Neuro 2A cells runs as a double band on this gel, suggesting that a posttranslationally modified form of this protein exists in these cells (see "Results" for further details).

Fig. 9. Regulation of twinfilin-1 and twinfilin-2 localization in cultured mammalian cells. A–C, localization of twinfilin isoforms in Neuro2A cells. Twinfilin-1 (B) and twinfilin-2 (C) visualized with anti-twinfilin-1 and anti-twinfilin-2 antibodies show punctate cytoplasmic staining but are also localized to the actin filament-rich filopodia visualized by rhodamine-phalloidin (A). D–I, effects of Rho family GTPases on mouse twinfilin localization in NIH 3T3 cells. Cells were transfected with the active Cdc42(V12) (D–F) or Rac1(V12) (G–I). Localization of the Cdc42(V12) and Rac1(V12) green fluorescent fusion proteins are shown in panels D and G, respectively. Cells were stained with anti-twinfilin-1 antibody (E and H) and anti-twinfilin-2 antibodies (F and I). Twinfilin-1 co-localizes with the active Cdc42 at the cell-cell contacts (arrows in panel E), whereas twinfilin-2 (panel F) is not found at these sites. The activated form of Rac1 induces the localization of twinfilin-1 to the membrane ruffles (arrows in panel H), but it does not affect the localization of twinfilin-2 (panel I).

This family are RhoA, Cdc42, and Rac1 (38). Previous studies with an antibody that recognized both twinfilin isoforms suggested that the localization of twinfilins in NIH 3T3 cells may be regulated by the small GTPases Rac1 and Cdc42 (14). Therefore, we examined the effects of these Rho GT Pases on the localization of twinfilin-1 and twinfilin-2 by using the isoform-specific antibodies. Surprisingly, we observed that Rac1 and Cdc42 have distinct effects on the localization of twinfilins. Twinfilin-1 co-localizes very strongly with the activated Cdc42(V12) at the cell-cell contacts, whereas twinfilin-2 did not concentrate at these sites in NIH 3T3 cells (Fig. 9, D–I). Furthermore, in cells expressing the activated form of Rac1(V12), twinfilin-1 concentrated strongly to the Rac1-induced membrane ruffles, whereas twinfilin-2 was localized equally throughout the lamellipodium (Fig. 9, G–I). We further confirmed that the twinfilin-1-rich sites induced by Cdc42(V12) are cell-cell contacts and not dividing cells by β-catenin staining (data not shown). In conclusion, these results suggest that the subcellular localizations of the two mammalian twinfilin isoforms are regulated by different signaling pathways.

**Discussion**

Twinfilin is a ubiquitous regulator of actin dynamics found in organisms from yeast to mammals (18). Here, we identified and characterized a second mouse twinfilin isoform, which shows 65% sequence identity to previously characterized mouse twinfilin-1. The protein, which we named twinfilin-2, is a mouse orthologue of human “A6-related protein” that was recently identified by Rohwer et al. (33). Interestingly, these authors reported that the recombinant GST fusion of human A6-related protein did not have any actin binding activity and therefore

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3 M. K. Vartiainen, P. Hilpela, and P. Lappalainen, unpublished data.
suggested that despite high sequence homology to twinfilins, this protein would exhibit a different function. Because our direct actin monomer binding, filament sedimentation, actin assembly, and G-actin nucleotide exchange assays clearly demonstrate that mouse twinfilin-2 binds actin monomers with a high affinity that is similar to those of mouse twinfilin-1 and other known twinfilins, we conclude that twinfilin-2 (A6-related protein) is indeed a functional member of the twinfilin protein family. We speculate that the lack of actin binding activity in the study by Rohwer et al. (33) may have resulted either from the lack of proper actin monomer-binding assays or from the inactivity of their recombinant GST fusion protein. It is possible that the GST at the N terminus of the recombinant protein in the study by Rohwer et al. (33) inhibits the actin binding activity of twinfilin-2 (in our studies the GST was removed from the recombinant proteins before biochemical assays).

Our Northern blot and in situ hybridization analyses suggested that either twinfilin-1 or twinfilin-2 is expressed in most cell types of mouse embryos and adult mice. Therefore, twinfilins appear to be involved in some central cellular processes that are common to all of the mammalian cell types. Similar to the mammalian ADF/cofilin and profilin isoforms, the two twinfilin isoforms show distinct expression patterns. Twinfilin-1 is the major isoform during development and in adult non-muscle cells, whereas twinfilin-2 is the predominant isoform of heart and skeletal muscles. Similarly, one of the mammalian ADF/cofilin isoforms, cofilin-2, is predominant in muscle, whereas the two other isoforms, cofilin-1 and ADF, are expressed strongly in non-muscle cells (19, 39). It is important to note that both mouse twinfilins are especially abundant in the mechanosensory hair cells of the inner ear. These cells have actin-based apical projections called stereocilia that display structural and functional similarity to Drosophila mechanosensory bristles. In a Drosophila strain that carries a mutation in the twinfilin gene, the actin bundles forming the bristles are severely misorganized (15). Therefore, high levels of twinfilins in mammalian inner ear hair cells may be necessary to maintain proper stereocilia structure and function.

Although the two mammalian twinfilin isoforms have distinct expression patterns, we could not detect any significant differences in their affinities for actin monomers or capping protein. Furthermore, our studies did not reveal any specificity toward certain actin or capping protein isoforms for either of the mouse twinfilins (data not shown). Our previous studies (13) demonstrated that yeast twinfilin inhibits the nucleotide exchange on actin monomers and that the activity of yeast twinfilin can be down-regulated by PI(4,5)P2 in vitro (16). Here, we show that both mouse twinfilins inhibit the nucleotide exchange on actin monomers and bind PI(4,5)P2 similarly to yeast twinfilin, suggesting that these functions are universal for all of the members of the twinfilin family. Although we could not detect any significant biochemical differences between mouse twinfilin-1 and twinfilin-2, it is possible that the two mouse proteins have differences in some other aspects of actin dynamics that can not be measured by currently available methods (e.g. the kinetics of actin monomer dissociation from twinfilin after interactions with capping protein). Furthermore, at least one of these proteins may have other ligands than actin, capping protein, and PI(4,5)P2. For example, it is possible that twinfilin-2 may interact with certain sarcomere proteins to promote its specific function in the muscle cells.

Our quantitative Western blotting analysis showed that twinfilins are abundant proteins, at least in cultured mouse Neuro-2A and NIH 3T3 cells. In these cells, twinfilins are found in ~1:7 ratio to the major ADF/cofilin isoform, cofilin-1. In yeast cells, the twinfilin:cofilin ratio was reported to be 1:2.5 (16). Therefore, the high abundance of twinfilins in these two mammalian cell lines, together with their high affinities for ADP-G-actin, suggest that twinfilins may interact with a relatively large proportion of actin monomers in cells and thus have a profound effect on the localization and dynamics of the cellular actin monomer pool. Although the two twinfilin isoforms show relatively similar localizations in unstimulated cells, only the subcellular localization of twinfilin-1 appears to be regulated by Rac1 and Cdc42. These two small GTPases are central regulators of polarized growth and motility in non-muscle cells (38), suggesting that twinfilin-1 may be involved in these cellular functions. The role of twinfilin-1 in polarized growth is further supported by its strong expression in developing neurons, skin, and olfactory sensory epithelium. In contrast, the predominant isoform in muscle cells, twinfilin-2, is not regulated by the small GTPases Cdc42 and Rac1. Twinfilin-2 may therefore be involved in the maintenance of sarcomere structure and perhaps does not promote any Rac1 and Cdc42-induced morphological and motile processes in muscle cells.

In conclusion, our data shows that mammals have two isoforms of an actin monomer-binding protein, twinfilin. Twinfilin-1 is the major isoform in non-muscle cells, whereas twinfilin-2 is strongly expressed in heart and skeletal muscle cells. In addition, high levels of both proteins seem to be required in the inner ear sensory hair cells. Although biochemically similar, the subcellular localizations of twinfilin-1 and twinfilin-2 appear to be regulated through different intracellular signaling pathways. In the future, it will be important to elucidate the in vivo roles of these two mammalian twinfilin isoforms by genetic methods and to reveal the molecular mechanisms by which the activities and localizations of these two abundant actin-binding protein isoforms are regulated in mammalian cells.

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