Alternative splicing of the mouse profilin II gene generates functionally different profilin isoforms

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SUMMARY

Profilins are a conserved family of proteins participating in actin dynamics and cell motility. In the mouse, two profilin genes are known. Profilin I is expressed universally at high levels, while profilin II is expressed mainly in the brain. Here we describe the occurrence of two mouse profilin II isoforms, A and B, which are derived by alternative splicing. They are identical through residue 107 of the protein, but then have distinct C-terminal sequences. Profilin IIA binds to poly-L-proline and actin with high affinity similar to profilin I. Profilin IIB on the other hand does not bind to actin and the affinity for poly-L-proline is greatly diminished. However, tubulin was found to bind to GST-profilin IIB, and in vivo GFP-profilin IIB was recruited to spindles and asters during mitosis in HeLa cells. Our results indicate unexpected diversity in the functions of the profilin family of proteins, and suggest that in mouse profilin IIA is intimately involved in actin dynamics, while profilin IIB associates with other cytoskeletal components.

Key words: Mouse profilin II, Poly-L-proline binding, Microtubule, Alternative splicing, Actin cytoskeleton

INTRODUCTION

Profilin is a ubiquitous 15 kDa actin binding protein found in eukaryotes. In all mammals, two profilin genes have been found to date. In human, profilin I (Kwiatkowski and Bruns, 1988) and profilin II (Honore et al., 1993) cDNA sequences have been isolated and in mouse, the profilin I cDNA has been sequenced (Widada et al., 1989). No information on the mouse profilin II gene is available yet. In general, the sequences of profilin from different species are not well conserved. For example, the human profilin I and profilin II genes are 65% identical on amino acid level but only 35% identical to profilins from lower eukaryotes and about 20% identical to plant profilins. In spite of the sequence differences, the biochemical properties of profilin are extremely well conserved (Gieselmann et al., 1995; Lambrechts et al., 1997). Even the in vivo functions appear to be conserved to a certain extent, as demonstrated by the ability of plant profilin to rescue Dictyostelium profilin null mutants (Karakesisoglou et al., 1996).

Biochemically, profilin was identified by its ability to bind to poly-L-proline and shown to form a 1:1 complex with monomeric actin (Carlsson et al., 1977) which can be dissociated by PtdlnP₂ micelles (Lassing and Lindberg, 1985). Upon binding to an actin monomer, profilin increases the rate of nucleotide exchange on the actin monomer, thereby charging the monomer with ATP, and possibly enhancing actin filament dynamics (Goldschmidt-Clermont et al., 1992). In vivo, profilin is thought to be essential in promoting actin polymerization upon cell stimulation by facilitating the addition of actin monomers to the fast growing end of actin filaments (Pantaloni and Carlier, 1993). Profilin might also play an important role in signal transduction since it binds PtdlnP₂ with high affinity and is able to inhibit its cleavage by phospholipase-C under certain conditions (Goldschmidt-Clermont et al., 1991).

Actin binding of profilin is only one side of profilin function. Profilin ligands such as the Arp2/3 complex (Machesky et al., 1994), VASP (Reinhard et al., 1995), Mena (Gertler et al., 1996), SMN (Giesemann et al., 1999), gephyrin (Mammoto et al., 1998) and diaphanous (Watanabe et al., 1997) have been characterized and implicated in regulating actin polymerization. We have shown recently that in mouse brain profilin I and profilin II interact with a large number of ligands – including dynamin I, synapsin, ROCKII, VCP and others – suggesting a function of profilin in membrane trafficking and signal transduction (Witke et al., 1998). Interestingly, the ligands are specific for either mouse profilin I or profilin II.

Here we report that in mice the diversity of profilin isoforms is extended by alternative splicing of the profilin II transcript which produces two profilin II isoforms – the major profilin IIA isoform which is expressed in brain and the less abundant profilin IIB isoform which differs in the C-terminal 32 amino acids.

MATERIALS AND METHODS

Isolation of cDNAs and genomic clones

A Balb/c mouse brain cDNA lambda Zap library (Stratagene) was
screened with the full length human cDNA fragment (Honore et al., 1993). Plaque purified clones were rescued as phagemids and the longest clone sequenced by primer walking with T7-polymerase (USB, Sequenase). These sequence data have been submitted to the EMBL database under accession number AJ272203.

Genomic clones were isolated from a 129/Sv, EMBL3 phage library. Briefly, genomic DNA was isolated from 129Sv embryonic stem cells grown without feeder layer and the DNA partially digested with Sau3A. The restriction digest was size fractionated on a sucrose gradient and the 10-15 kb range ligated into BamHI cut EMBL phage arms. The library was packaged using Giga-Gold packaging extracts (Stratagene) and amplified once in the recombination deficient SSB E. coli host strain. Concurrent screening was performed in LE392 host strain. The average insert size of the library ranges from 12 kb to 18 kb.

Expression of recombinant proteins

For protein expression, profilin IIA and profilin IIB cDNAs were cloned in pGEX-2T vectors and expressed in E. coli strain JM105 as glutathione-S-transferase (GST) fusion protein (Pharmacia, Piscataway, NJ).

Bacterial cultures were grown overnight in LB/Ampicillin medium, diluted 1:100 into fresh medium for 3-4 hours at 37°C. Induction of recombinant proteins was carried out at room temperature for 5 hours with 0.5 mM IPTG. Bacteria were pelleted and resuspended in 10 mM Tris-HCl, pH 7, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 30% sucrose and lysed by mild sonication on ice. Lysates were cleared by centrifugation at 35,000 g for 1 hour at 4°C, and the supernatants incubated overnight with glutathione Sepharose 4B beads with gentle rocking. The beads were washed with PBS, 0.1% Triton X-100 and bound proteins eluted with 20 mM glutathione in 50 mM Tris-HCl, pH 8.0. Cleavage of eluted fusion proteins was carried out at 16°C, overnight using thrombin. The homogeneity of purified GST, GST-profilin IIA and GST-profilin IIB was analyzed by SDS-PAGE and Coomassie staining (Laemmli, 1970).

Detection of profilin splice forms by RT-PCR

Total tissue RNA was reverse transcribed and aliquots from this first strand synthesis were subjected to two rounds of PCR amplification with profilin II specific primers.

Two primers spanning the alternatively spliced region (5'-GAGTCAAGGTGGGAGCCCAAC-3'; 5'-CGAAGTGATGCCCAA-CCTGGC-3') were used in the first PCR round. The same 3’ primer and an internal 5’ primer (5’-TACAACGTTCGTGGCAGG-3’) were used in the second round of PCR reaction. The 201 bp product after the second round of PCR indicates the presence of the alternatively spliced profilin IIB mRNA. The 468 bp product corresponds to the profilin IIA mRNA. The 201 bp and 468 bp amplification bands were isolated and their identity confirmed by cycle sequencing. Also the nonspecific products from the control reactions were sequenced, but no homology to profilin was found.

Poly-L-proline binding assays

Poly-L-proline binding experiments were performed in batch in 1.5 ml Eppendorf tubes. GST, cleaved GST-profilin IIA, and GST-profilin IIB were incubated with 50 µl of poly-L-proline beads in PBS for 3 hours. Supernatants containing the unbound proteins were collected after centrifugation and after extensive washes of the poly-L-proline beads with PBS, bound proteins were eluted in 1× SDS sample buffer (Laemmli, 1970). Equivalent amounts of supernatant and bound material were subjected to SDS-PAGE and analyzed after Coomassie staining.

Actin polymerization assays

Pyrene actin was prepared according to Cooper et al. (Cooper et al., 1983) and polymerization assays in the presence of profilin were carried out essentially as described by Korenbaum et al. (Korenbaum et al., 1998). 5 µM G-actin containing 5% pyrene labeled actin was mixed with different amounts of recombinant profilins and polymerization was started by adding polymerization buffer to a final concentration of 10 mM imidazol, pH 7.6, 2 mM MgCl2, 1 mM EGTA. Polymerization was monitored at 25°C with a fluorescent plate reader (Fluoroskan Ascent II, Labsystems) at 10 second intervals. The results were plotted as relative fluorescence versus time using the programme ‘GraFit’ (Erithacus Software Ltd).

Binding assay and isolation of profilin complexes

GST-profilin IIA and GST-profilin IIB proteins were coupled to glutathione Sepharose 4B beads at a concentration of 2 mg of protein per ml of beads. Brains were prepared freshly from two mice and lysed in 2 ml HEPES lysis buffer (20 mM HEPES, pH 7.2, 5 mM EGTA 50 mM NaCl, 5 mM MgCl2, 0.05% Tween-20) by 20-30 strokes in a tight-fitting Dounce homogenizer. The lysates were centrifuged for 1 hour at 100,000 g at 4°C and incubated in batch with GST, GST-profilin IIA and GST-profilin IIB beads for 4 hours at 4°C. Supernatants were collected, the beads extensively washed in HEPES lysis buffer and the bound proteins eluted by boiling the beads in 1× SDS sample buffer. Equivalent amounts of protein were subjected to SDS-PAGE, transferred to Immobilon membrane, and probed with antibodies against actin, tubulin, and VASP.

Expression of GFP-tagged and epitope tagged profilins in HeLa cells

Mouse profilin IIA and profilin IIB cDNAs were subcloned into the EcoRI/SalI sites of the EGGF-C2 vector (Clontech Laboratories, Inc., USA) which puts the GFP moiety at the N terminus of profilin. To obtain better expression of GFP-profilin IIB, the GFP-profilin IIB fragment was released from the EGGF-C2 vector and subcloned into the pIREs1 neo reactor (Clontech Laboratories, Inc., USA) which allows more stringent selection and higher expression levels. HeLa cells were transfected in DMEM (Dulbecco’s modified Eagle’s medium, GibcoBRL) and electroporated in DMEM, 20 mM HEPES with the respective plasmids at 250 V/500 µF. After initial selection in 1 mg/ml G418 (geneticin) single clones were picked and propagated in 0.5 mg/ml G418. HeLa cells were maintained in DMEM containing 10% FCS, glutamine and non-essential amino acids.

For expression of epitope tagged profilin IIA and profilin IIB, the respective cDNAs were fused at their N-termini to a 13 amino acid epitope derived from Sendai virus coat protein which is recognized by the monoclonal antibody 7E-VII (Eibenberger et al., 1990). The fusion proteins were expressed in HeLa cells using the pIREs1 neo vector. The Sendai epitope has been shown previously not to interfere with protein function (Witke et al., 1991). Protein lysates were prepared from transfected HeLa cells after hypotonic shock followed by mild sonication in HE buffer (10 mM HEPES, pH 8.0, 5 mM EGTA) spun at 100,000 g to pellet debris, and used for polyl-proline binding assays as described above.

Miscellaneous

Poly-L-proline beads were prepared according to published methods (Janney, 1991). Actin was purified from rabbit skeletal muscle (Spudich and Watt, 1971). VASP polyclonal antibodies were raised in rabbits against mouse VASP (generous gift of Dr Frank Gertler).

For detecting actin on western blots hybridoma supernatant from clone 224-236 was used (Westphal et al., 1997). This monoclonal antibody recognizes a wide range of actins from different species. The anti α-tubulin antibody DM1A was purchased from Sigma.

RESULTS

Sequence of the mouse profilin II cDNA

Using the human profilin II cDNA (Honore et al., 1993) as a
We isolated overlapping genomic clones for mouse profilin II.

Genomic organization of the mouse profilin II gene

We examined the intron-exon structure of the profilin II gene. Sequential analysis led us to recognize a region in the 3′ untranslated sequence, the putative exon 4, which is in perfect agreement with the postulated alternative splicing mechanism. If exon 3 is skipped and splicing occurs at position +598 in the 3′-untranslated region, the putative exon 4 will be encoded on a separate exon which can be skipped.

Sequence analysis led us to recognize a region in the 3′ untranslated region of the mouse brain cDNA sequence suggested that in vivo alternative splicing might occur, generating a second profilin II isoform by replacing the C-terminal 32 amino acids of profilin II which is in perfect agreement with the postulated alternative splicing mechanism. If exon 3 is skipped and splicing occurs at position +598 in the 3′-untranslated region, the putative exon 4, for this to occur, the last 32 amino acids of the profilin II C terminus have to be encoded on a separate exon which can be skipped, followed by splicing onto the second ORF in the 3′-untranslated region – the putative exon 4. The presence of a splice acceptor site at the beginning of this putative exon 4 followed by splicing onto the second ORF in the 3′-untranslated region will give rise to a mRNA with the alternative exon 4 encoding the last 32 amino acids of the profilin II C terminus.

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Two open reading frames are found on the cDNA indicated by the translated amino acid sequences. The first ORF starts at position +7 and ends at position +426, encoding a protein of 139 amino acids. The second short ORF from position +599 to +712 with a preceding, potential splice acceptor site is marked by the filled diamond.
Profilin IIB mRNA is generated by tissue specific alternative splicing

To show that the profilin IIB mRNA is expressed in vivo, we performed an RT-PCR based analysis on RNA samples from different mouse tissues which allowed us to distinguish the profilin IIA and profilin IIB splice products. RNA from mouse brain, heart, skeletal muscle, kidney, embryonic stem cells, and day 13 mouse embryos was reverse transcribed and the presence of the two isoforms relative to each other analysed by a nested PCR approach using primers which flank exon 3 (Fig. 3B). We could clearly distinguish the alternatively spliced profilin IIB, which misses exon 3, as a 201 bp PCR product and the profilin IIA as a 468 bp PCR product (Fig. 3A). The PCR products were isolated and directly sequenced by cycle sequencing in order to confirm the identity of the PCR products. Both, the 468 bp and the 201 bp products yielded exactly the sequence we predicted for the alternative splicing. None of the non-specific bands from the control reactions showed any homology to profilin.

Although the quantitation of the profilin IIA and profilin IIB transcripts by PCR is not absolute, we can estimate the relative amount of the isoforms in the different tissues. In brain tissue we estimate that more than 95% of profilin II is the profilin IIA isoform, while in other tissues, mainly kidney and ES cells, the ratio of profilin IIA to profilin IIB is approximately 1:1.

Profilin IIA and profilin IIB have distinct biochemical properties and subcellular localization

Alignment of the amino acid sequence of mouse profilin IIA and profilin IIB shows that the C terminus is relatively conserved except the 6 amino acid extension in profilin IIB (Fig. 4). All the residues which have been shown to contact actin in the profilin I-actin complex (Schutt et al., 1993) are in fact conserved in both isoforms – profilin IIA and profilin IIB, suggesting comparable actin binding properties (Fig. 4).

To examine the biochemical properties of the profilin IIA and profilin IIB isoforms, we produced recombinant profilin IIA and profilin IIB as GST-fusion proteins in E. coli. All proteins were soluble and purified under native conditions after lysis of the bacteria.

We first examined the poly-L-proline binding of profilin IIB. Recombinant profilin IIA and profilin IIB were allowed to bind to poly-L-proline and the proteins were separated on a 12% SDS-PAGE gel and stained with Coomassie blue. The profiles of profilin IIA and profilin IIB are shown in Fig. 4A. The profilin IIA and profilin IIB proteins showed similar binding characteristics.

Fig. 3. Characterization of profilin IIA and profilin IIB splice forms in different mouse tissues using RT-PCR. Tissue RNA from brain, kidney, heart, skeletal muscle, embryonic stem cells, and e13 mouse embryos was reverse transcribed and first strand synthesis subjected to two rounds of PCR amplification with profilin II specific primers flanking the alternatively skipped exon 3 (+RT). As a control, RNA from brain and kidney was processed without reverse transcriptase and subsequently PCR amplified (−RT). The strategy is outlined below, and the primers used are indicated (B). The PCR products after the nested PCR reaction were separated on a 2% agarose gel and the DNA stained with ethidium bromide. The 201 bp PCR product clearly indicates the presence of the alternatively spliced profilin IIB mRNA while the 468 bp band represents the profilin IIA isoform. Highest amounts of profilin IIB RNA can be seen in kidney and embryonic stem cells. The 201 and 468 bp product bands were isolated and their identity confirmed by direct cycle sequencing. As a size marker (M) BstNI cleaved pBR322 DNA is shown.
to poly-L-proline beads, the beads washed, and binding assessed by SDS-PAGE. Binding of profilin IIB was not above background, indicating strongly diminished affinity for poly-L-proline, while profilin IIA bound to poly-L-proline with high affinity (Fig. 5A).

In order to exclude any adverse effect on the folding of profilin IIB by expressing it in E. coli, we also expressed an epitope tagged version of profilin IIA and profilin IIB in HeLa cells and tested the binding to poly-L-proline. As seen with the bacterially expressed profilin IIA and profilin IIB, also the profilin IIA expressed in HeLa cells bound to poly-L-proline (data not shown), while profilin IIB did not bind to poly-L-proline (Fig. 5B).

A number of ligands such as dynamin I, Mena, and VASP have been shown to bind through the poly-L-proline binding site to profilin. The poly-L-proline binding site in profilin is constituted by two aligning helices formed by the N- and the C-terminus (Mahoney et al., 1999). To investigate potential binding partners for profilin IIB we coupled GST-profilin IIB to glutathione beads and incubated the beads with brain extracts. As a control, we used GST-profilin IIA for which the ligands have been well characterized. After washing, bound proteins were eluted with SDS-sample buffer and analyzed by western blot using different antibodies. No VASP binding was detectable for profilin IIB compared to profilin IIA suggesting that profilin IIB is not only lacking the binding site for poly-L-proline but also for certain ligands (Fig. 5C).

In these binding assays, actin is expected to be the most abundant ligand. As expected, profilin IIA binds actin in the complex as shown in Fig. 5C, while no actin binding was detectable for profilin IIB. Since GST-fusion proteins were used in these assays, one cannot rule out interference of the GST moiety with profilin IIB which might affect actin binding. Therefore, we examined the actin binding properties of profilin IIB in more detail after thrombin cleavage of the GST portion, using the pyrene actin polymerization assay (Cooper et al., 1983). With this assay the effects of profilin IIA and profilin IIB on the kinetics of actin assembly can be studied. Increasing amounts of profilin IIA inhibited the polymerization kinetic by sequestration of actin monomers (Fig. 6, upper panel) while

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Fig. 4. Alignment of the mouse profilin IIA and profilin IIB amino acid sequences. Mouse profilin IIA and profilin IIB are aligned and identical residues printed in black while mismatches are highlighted in gray. Mouse profilin IIA is 139 aa long like all other mammalian profilin II forms. The mouse profilin IIB sequence is 145 aa long, with a 6 amino acid C-terminal extension. The splice site leading to the different isoforms is indicated by an arrow. The C-terminal domain of profilin is involved in actin binding and the amino acid residues known from the profilin I structure to make contact with actin are indicated in italic below the corresponding mouse profilin II sequence. Note that in profilin IIA and particular profilin IIB there is a perfect match of these residues suggesting that both molecules should bind to G-actin.

Fig. 5. Poly-L-proline and ligand binding by profilin IIA and profilin IIB. (A) Recombinant GST-profilin IIA and GST-profilin IIB were cleaved with thrombin in order to release the profilin moiety and binding assays performed on poly-L-proline beads. Samples were subjected to SDS-PAGE and proteins visualized by Coomassie staining. Profilin IIA binds with high affinity to poly-L-proline beads (bound fraction) while profilin IIB does not interact significantly with the beads. Note that some non specific binding to the beads is also observed with GST alone. (B) Epitope tagged profilin IIB (S-profilin IIB) was expressed in HeLa cells (S-HeLa) and tested for binding to poly-L-proline (PLP). S-profilin IIB cannot be depleted by passing extracts over poly-L-proline (PLP unbound). As an internal control for poly-L-proline binding, the binding of endogenous profilin I was tested in the same experiment confirming the activity of the poly-L-proline beads. (C) Recombinant GST-profilin IIA and GST-profilin IIB were immobilized on glutathione beads and brain lysate allowed to bind to the beads. After stringent washing, proteins bound to the beads were eluted with SDS-sample buffer and subjected to SDS-PAGE. Profilin ligands were identified by western blot using antibodies against VASP, actin and tubulin. VASP and actin interact with profilin IIA, while no binding to profilin IIB was detectable. Conversely, tubulin was found in the profilin IIB complex but not in the profilin IIA complex.
essentially no inhibition was detectable with profilin IIB (Fig. 6, lower panel). From these results we conclude that profilin IIB does not interact with G-actin with appreciable affinity. The lack of actin binding was also confirmed by immunoprecipitation of epitope tagged profilin IIB expressed in HeLa to exclude any adverse effects of expressing the recombinant protein in E. coli. Profilin IIB immunoprecipitated from HeLa lysates does not bind actin (data not shown).

The lack of poly-L-proline and actin binding raises the question whether virtually all binding properties are lost in profilin IIB or whether the protein has also gained new binding properties. Several lines of evidence support the latter. First, we can detect proteins from mouse brain binding to GST-profilin IIB which we have not characterized yet (data not shown). Second, testing the profilin IIB complexes with antibodies for different cytoskeletal elements, we noticed that tubulin was a component in the profilin IIB complex but not in the profilin IIA complex (Fig. 5B). Tubulin apparently binds indirectly to profilin IIB since purified tubulin did not show significant binding to GST-profilin IIB, neither did it bind in microtubule pelleting assays (data not shown). Most likely, tubulin binds in the profilin IIB complexes via another, as yet unidentified tubulin associated protein.

To further test profilin IIA and profilin IIB function and to see whether profilin IIB could interact with tubulin in vivo, we expressed GFP-profilin IIB in HeLa cells and studied the localization in comparison to GFP-profilin IIA.

GFP-profilin IIA localization in interphase cells suggests that the majority of the protein is diffusely distributed in the cytoplasm with occasionally stronger staining in active membrane areas such as ruffles, seemingly co-localizing with F-actin (Fig. 7, lower panel). However, a more detailed analysis showed that profilin IIA is rather excluded from F-actin but enriched in close vicinity at sites of actin polymerization at the plasma membrane. In dividing cells profilin IIA remains mostly cytoplasmic without particular localization.

The localization of profilin IIB is quite different from profilin IIA. In interphase HeLa cells, GFP-profilin IIB was evenly distributed throughout the cytoplasm and the nucleus, but no localization to actin rich domains was observed in agreement with our finding that profilin IIB does not bind to actin (Fig. 7, lower panel). Interestingly, in dividing cells, GFP-profilin IIB brightly decorated mitotic spindles and asters (Fig. 7, upper panel). The same structures could be counterstained with anti-tubulin antibodies (Fig. 7, upper panel). Notably, we could not detect microtubule co-localization in interphase cells (data not shown). This suggests that depending on the cell cycle, profilin IIB is recruited to microtubule containing structures.

**DISCUSSION**

Profilins are conserved from slime molds to humans. In mammals, two profilin genes have been found to date. Here we present the first genomic structure of a mammalian profilin I and profilin II gene, provide evidence for alternative splicing of the profilin II transcript in mouse and compare the functional differences of the two splice forms profilin IIA and profilin IIB. Based on sequence comparison it is difficult to judge whether both splice forms would display the ‘classical’ profilin binding properties or whether functions might have diverged in evolution. In fact, here we present evidence that the profilin II splice forms have diverged significantly, with profilin IIA behaving as a ‘classical’ profilin, while profilin IIB has acquired new properties.

The availability of the genomic organization gives some insight into the evolutionary origin of the profilin genes. From
Functionally different mouse profilin II isoforms

our current knowledge it is very likely that profilin II has been derived from the profilin I gene by duplication. Mouse profilin I is expressed ubiquitously in all tissues except skeletal muscle, while profilin IIA is mainly expressed in neuronal tissue (Witke et al., 1998). Thus profilin I seems to be the housekeeping gene important for vital functions while profilin II has been tailored later in evolution to serve a specific function, mainly in brain. This is supported by the finding that profilin I is essential in mouse (W. Witke et al., unpublished) while knockout mice devoid of functional profilin II develop specific neurological defects (A. Di Nardo, unpublished). Interestingly, both genes show absolutely conserved exon-intron structure and exon-intron boundaries. Introns are located at the exact same amino acid position – suggesting that the two genes are products of a duplication event. One of these changes is apparently the extension of the last exon in profilin II which opened the possibility for generating another variant of profilin II by alternative splicing.

What is the role and significance of mouse profilin IIA and profilin IIB?

Profilin IIA binds to actin and poly-L-proline with high affinity, comparable to profilin I. Hence, profilin IIA can be considered a ‘classical’ profilin isoform. However, recent results suggested that the actin binding properties of profilin IIA is only one aspect of its function and that the interaction with ligands such as dynamin I, Mena, VASP and other ligands might regulate a number of different cellular pathways such as membrane trafficking and signalling. It remains to be shown how these ligand interactions regulate signalling, membrane trafficking, and actin polymerization. Studies on cultured hippocampal neurons showed profilin IIA localization at synaptic densities, supporting the idea that profilin IIA plays a role in synapse function (Witke et al., 1998). GFP tagging of profilin can be used to address its in vivo localization (Geese et al., 2000). Here we show that GFP-profilin IIA expressed in HeLa cells in general does not co-localize with F-actin but instead profilin IIA is rather enriched in close vicinity to F-actin at the membrane. In interphase cells, GFP-profilin IIB does neither localize to F-actin nor to the plasma membrane.

Fig. 7. Localization of GFP-profilin IIA and profilin IIB in HeLa cells. In the upper panel mitotic stages of HeLa cells expressing GFP, GFP-profilin IIA, and GFP-profilin IIB are shown. Cells were counterstained with anti-tubulin and DAPI. Note that GFP and GFP-profilin IIA are mostly distributed throughout the cytosplasm while GFP-profilin IIB is recruited to asters (arrows). The lower panel shows the comparison of GFP-profilin IIA, and GFP-profilin IIB in interphase HeLa cells. Cells were counterstained with TRITC-phalloidin in order to visualize the cytoskeletal architecture. No localization to F-actin was observed for both profilin isoforms but a rather uniform diffuse staining throughout the cytosplasm with occasional concentration of GFP-profilin IIA at the plasma membrane and F-actin rich domains. However, at higher magnification (insert) it is apparent that there is no co-localization with F-actin, but instead profilin IIA is rather enriched in close vicinity to F-actin at the membrane. In interphase cells, GFP-profilin IIB does neither localize to F-actin nor to the plasma membrane.
II mRNA is profilin IIA, in other tissues such as kidney and embryonic stem cells the ratio of profilin IIA and profilin IIB mRNA is about 1:1. We should point out that in total lysates the absolute expression levels of profilin IIA are low in tissues other than brain. Given the fact that profilin IIA is very specific for neuronal cells we might underestimate its local expression since the majority of cells in tissues are not neuronal.

Profilin IIA and profilin IIB differ only in the last 32 amino acids and no antibodies are currently available which would allow to distinguish profilin IIB and to determine the protein levels in different tissues. Based on RT-PCR we estimate the expression levels to be low, but expression could be restricted to certain cell types and we might underestimate the amount by looking in total tissue extracts.

While we have good indications for profilin IIA function, the role of profilin IIB is somewhat more enigmatic. We have shown here that the protein does not bind to actin or poly-L-proline, however, we observed that profilin IIB interacts with tubulin in vitro, and with microtubules in vivo – possibly via tubulin associated proteins. Interestingly this interaction with microtubules can be observed only during cell division but not in interphase cells. Furthermore, modest expression of GFP-profilin IIB in HeLa cells leads to accumulation of cells in M-phase judged by the increased number of dividing cells identified by DAPI stain. On average, we counted twice as many cells in M-phase upon expression of GFP-profilin IIB compared to controls. Whether profilin IIB indeed plays a role in cell cycle control is suggestive but at this juncture speculative. Identification of other profilin IIB ligands will certainly shed more light on the role of this distinct profilin isoform.

The lack of actin binding in profilin IIB is surprising since the C terminus is very similar to the C terminus of mouse profilin IIA and human profilin I. Recent data on the crystal structure of human profilin II showed that the overall structure is well conserved between human profilin I and profilin II and that binding sites for actin match very well (Nodelman et al., 1999). Particular residues V118, H119, G121, M122, N124, K125, Y128, E129 in human profilin I have direct contact with profilactin. Such a conformational change would also explain the concomitant loss of poly-L-proline binding. This is an intriguing observation since it was shown previously that subtle changes in the poly-L-proline binding domain can also affect actin binding (Bjorkegren-Sjogren et al., 1997). Since we observed lack of poly-L-proline and actin binding not only for the bacterially expressed protein but also for profilin IIB expressed in HeLa cells, problems with folding or posttranslational modifications can be excluded.

The actin binding properties of profilin play an important role in cells (Kaiser et al., 1999), however, our results presented here together with previous findings suggest that other interactions of profilin might be equally important such as docking of ligands through the poly-L-proline binding site or other, yet not recognized binding sites.

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Note added in proof