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In mouse brain profilin I and profilin II associate with regulators of the endocytic pathway and actin assembly

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Profilins are thought to be essential for regulation of actin assembly. However, the functions of profilins in mammalian tissues are not well understood. In mice profilin I is expressed ubiquitously while profilin II is expressed at high levels only in brain. In extracts from mouse brain, profilin I and profilin II can form complexes with regulators of endocytosis, synaptic vesicle recycling and actin assembly. Using mass spectrometry and database searching we characterized a number of ligands for profilin I and profilin II from mouse brain extracts including dynamin I, clathrin, synapsin, Rho-associated coiled-coil kinase, the Rac-associated protein NAP1 and a member of the NSF/sec18 family. *In vivo*, profilins co-localize with dynamin I and synapsin in axonal and dendritic processes. Our findings strongly suggest that in brain profilin I and profilin II complexes link the actin cytoskeleton and endocytic membrane flow, directing actin and clathrin assembly to distinct membrane domains.

Keywords: actin cytoskeleton/endocytosis/MALDI/phosphoinositides/profilin ligands

Introduction

The actin-binding protein profilin is thought to be a key regulator of actin polymerization in cells. Profilin binds to G-actin in a 1:1 complex and thereby sequesters monomeric actin. Upon binding to actin monomers, profilin acts as a nucleotide exchange factor, charging actin with ATP (Mockrin and Korn, 1980; Goldschmidt *et al.*, 1992). The only known physiological compounds able to release actin from the profilin–actin complex are phosphoinositides (Lassing and Lindberg, 1985). Profilin binds $PtdInsP_2$ with high affinity and is able to inhibit the non-tyrosine-phosphorylated form of phospholipase C-γ suggesting that profilin might also play a role in signal transduction through tyrosine kinases and phospholipids (Goldschmidt *et al.*, 1990).

In recent years it has become clear that *in vivo* profilin does not solely act as a sequestering protein for G-actin. Kinetic studies of actin polymerization have shown that profilin can actually accelerate actin filament growth if new free barbed filament ends are formed (Pantaloni and Carlier, 1993). This finding suggests that *in vivo* profilin might be essential to promote actin polymerization. Overexpression of human profilin I in CHO cells leads to an increase of filamentous actin in cells further supporting a role of profilin for promoting actin polymerization rather than inhibiting it (Finkel *et al.*, 1994). Besides actin, profilin binds with high affinity to poly-L-proline stretches. This suggested that *in vivo* profilin binds to proline-rich proteins and serves other functions beside actin binding. Recently, the proline-rich ligands VASP (Reinhard *et al.*, 1995), MENA (Gertler *et al.*, 1996), diaphanous (Watanabe *et al.*, 1997) and the ARP2/3 complex (Machesky *et al.*, 1994) were shown to associate with profilin. VASP, MENA and ARP2/3 (Welch *et al.*, 1997) have been found in the listeria actin tails and focal contacts and are thought to play a role in recruiting profilin–actin to the site of actin polymerization at the bacteria as well as in the cell.

However, it is not clear what the functions of profilins are in mammalian tissues. In mammals two profilins are encoded on different genes (Kwiatkowski and Bruns, 1988; Honore *et al.*, 1993). Based on RNA levels the expression of human profilin I and profilin II appears to be somewhat complementary with profilin I being highly expressed in all tissues except skeletal muscle, heart and brain, while profilin II is highly expressed in brain, skeletal muscle and kidney (Honore *et al.*, 1993). The biochemical properties of recombinant human profilin I and profilin II are very similar with respect to $PtdInsP_2$ and poly-L-proline-binding but the affinity for actin is 4- to 5-fold increased for human profilin I compared with human profilin II (Gieselmann *et al.*, 1995). Using different binding assays, Lambrechts and co-workers (1997) found a higher affinity of bovine profilin II for $PtdInsP_2$ compared with profilin I. Furthermore, bovine profilin II appears to have a higher affinity for the profilin ligand VASP than bovine profilin I (Lambrechts *et al.*, 1997).

The study presented here aims to elucidate the general functions of profilins in mammalian tissues and profilin II-specific activities in brain. Using profilin I- and profilin II-specific antibodies we demonstrate that profilin II is highly expressed in mouse brain while profilin I is expressed ubiquitously in mouse tissues. We report that mouse profilin I and profilin II can form different complexes with proteins from mouse brain extracts. The components of the complexes were identified by high accuracy MALDI mass spectrometry followed by database searching. The components of these complexes are proteins involved in endocytosis and synaptic recycling and effector proteins of the Rac and Rho signaling pathway. *In vivo*, profilin II and dynamin I co-localize at vesicular structures in primary hippocampal neurons, supporting the idea that *in vivo* the profilin complexes exist and might play a role

in recruiting actin during endocytosis and synaptic vesicle recycling.

Results

Profilin II is mainly expressed in mouse brain

The tissue distribution of profilin I and profilin II protein has not been investigated yet. Northern blot analysis with RNA from human tissues showed that profilin I and profilin II have a somewhat complementary expression pattern, with profilin II expression being high in brain, skeletal muscle and kidney (Honore *et al.*, 1993). We raised antibodies specific for profilin I and profilin II and compared the relative expression of protein in different mouse tissues. Using these antibodies we can show that profilin I is expressed at high levels in most tissues except skeletal muscle while profilin II is expressed predominantly in brain, and at lower levels in skeletal muscle, uterus and kidney (Figure 1). Profilin I is expressed in brain as well at relatively high level. Therefore, profilin II is not just the brain profilin isoform but might rather serve a yet unknown brain-specific function which involves other ligands beside actin.

We decided to approach the question of the brain-specific functions of profilins by identifying the major profilin I- and profilin II-binding proteins from mouse brain. The isolated profilin complexes should give us some clues about the actin-dependent processes in which profilins are involved, and how these functions might be regulated.

Profilin I and profilin II affinity chromatography yields two different complexes from mouse brain extracts

We used profilin affinity chromatography to isolate proteins from brain extracts which bind specifically to profilin I and profilin II. To test the specificity of a direct binding assay with solid phase bound profilin, we passed extracts prepared from mouse brains over profilin II–Sepharose and eluted bound proteins with poly-L-proline, 1 M KCl and 8 M urea (Figure 2A). Poly-L-proline released seven major proteins that were recovered in larger quantity by increasing the salt concentration. Final elution with 8 M urea yielded mainly actin. As a control we passed brain extract over a mock column which did not bind any detectable protein (Figure 2A). DNase I binds monomeric actin and was used to control for proteins which bind to the columns via actin. DNase I–Sepharose bound actin and several other proteins, most of which were different from the constituents of the profilin II complex (Figure 2A). These results demonstrate that we can isolate proteins which bind specifically to profilin II by affinity chromatography and that the majority of ligands can be eluted with high salt. We then isolated the profilin I- and profilin II-binding complexes from brain using the respective affinity matrix (Figure 2B). The pattern of the profilin I and profilin II complexes from mouse brain was highly reproducible in all experiments. An important parameter for the isolation of the complex was the presence of low concentrations of divalent cations (see Materials and methods) while the presence of chelators like EGTA or EDTA disrupted the interactions.

Although profilin I and profilin II are biochemically similar they bind different sets of proteins from mouse brain. The predominant profilin I-binding proteins are in the range of 45, 56, 70, 100 and 190 kDa. The most prominent profilin II-binding proteins are 45, 70, 100 and 130–150 kDa in size. Relatively few proteins smaller than 45 kDa were found in the profilin I and profilin II complexes. After silver staining minor protein bands could be detected around 30 kDa which were not further characterized.

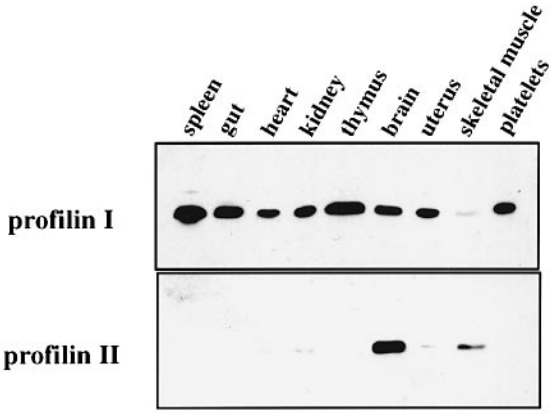

Fig. 1. Expression of profilin I and profilin II protein in mouse tissues. Equal amounts (40 μg/lane) of total protein extracts from several tissues and total platelet lysate (20 μg/lane) were subjected to SDS–PAGE, transferred to Immobilon-P membrane and probed with anti-profilin I- and anti-profilin II-specific antibodies. The antibodies are isoform-specific as shown by their reactivity against recombinant mouse profilin I and profilin II (data not shown).

Characterization of the profilin complexes by MALDI

A rapid analysis of the profilin I and profilin II complexes by conventional microsequencing would have been difficult. Methods for the efficient microanalysis of proteins by mass spectrometry are now available and their large-scale use in characterizing multi-protein complexes has been suggested (Lamond and Mann, 1997; Winter *et al.*, 1997). For the analysis, the profilin I- and profilin II-binding complexes were separated by conventional one-dimensional SDS–PAGE, the most prominent protein bands excised after Coomassie Blue staining, 'in-gel'-digested with trypsin, and analyzed by MALDI, followed by database searching with the resulting peptide masses. Using delayed extraction reflector MALDI, very high mass accuracy and unambiguous protein identification can be obtained (Jensen *et al.*, 1996; Shevchenko *et al.*, 1996). As an example, Figure 2C shows the MALDI peptide mass map of the 100 kDa band of the profilin II complex. A search of these masses in a comprehensive sequence database showed that 35 matched to calculated tryptic peptide masses of mouse dynamin I (accession number P21575) with an accuracy of >50 p.p.m., covering 44% of its sequence. The complete results of the MALDI analyses are summarized in Table I.

Interestingly, the protein pattern in our complexes (Figure 2B) is completely different from profilin-associated proteins that have been described in lower eukaryotes (Machesky *et al.*, 1994) and in human platelets (Reinhard *et al.*, 1995). We cannot exclude that we missed the platelet-specific profilin-binding protein VASP because of

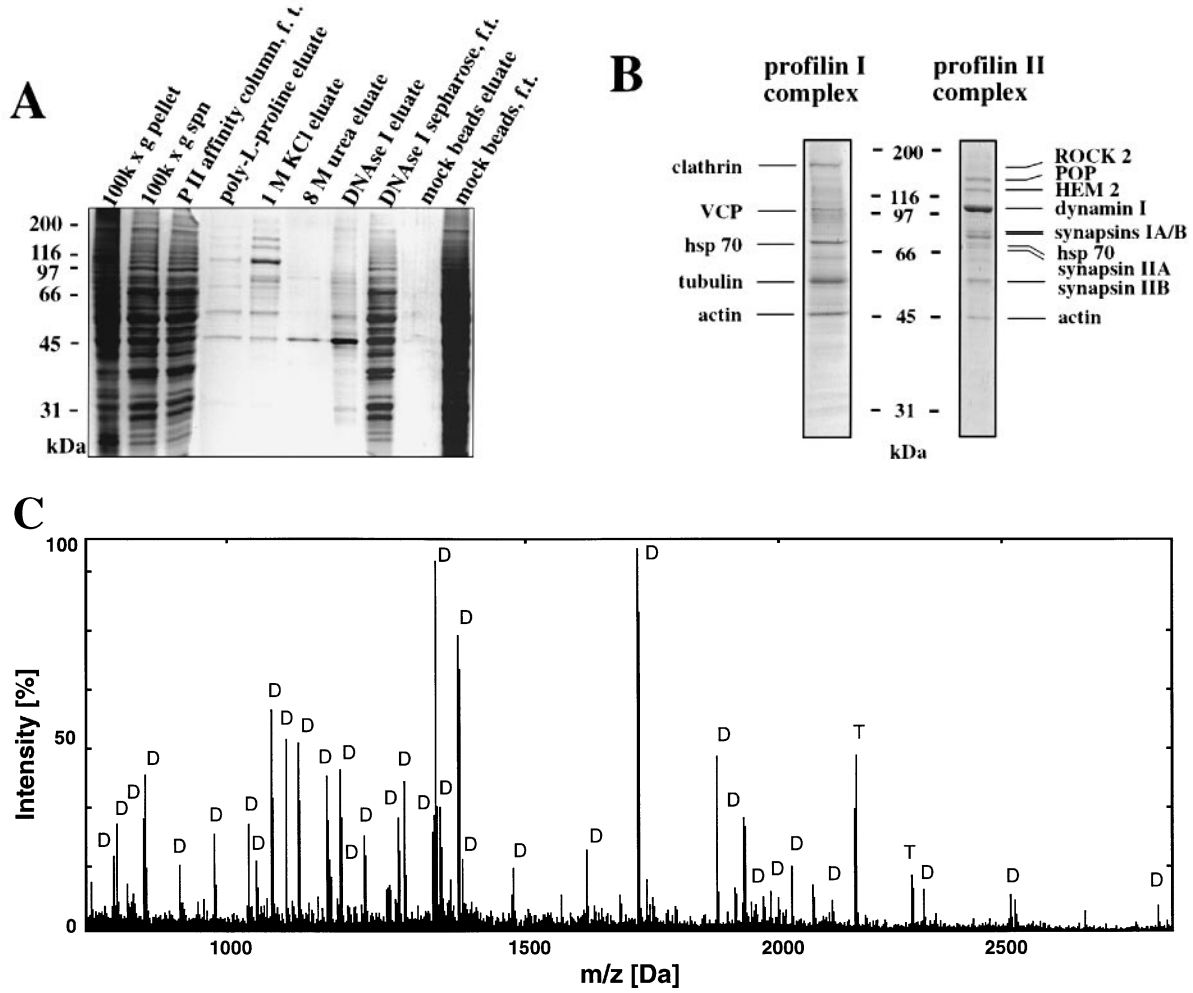

Fig. 2. Elution profile of profilin II-binding proteins (**A**). The high speed supernatant of brain extracts (100k×g spn) were passed over a profilin II column (f.t., flow through) and bound proteins eluted with ~50 μM poly-L-proline (mol. wt >30 000), 1 M KCl, and 8 M urea. As controls, extracts were passed over a mock column and a DNase I column. DNase I is known to bind G-actin. Note that most proteins eluted from the DNase I column differ from the proteins eluted from profilin II–Sepharose demonstrating that the binding of proteins to profilin II is not simply mediated by G-actin. Equivalent amounts were loaded for each fraction. Profilin I- and profilin II-binding complexes from brain (**B**). Brain extracts were loaded onto profilin I and profilin II columns and the bound proteins eluted by a high salt step elution with 1 M KCl. Profilin I- and profilin II-binding proteins were identified by MALDI. Peptide mass map of the prominent 100 kDa protein in the profilin II complex (**C**). The map was produced of the peptide supernatant obtained after 'in-gel' digestion of the band with trypsin. Database search of the measured tryptic peptide masses uniquely identified dynamin I (accession number P21575 in SWISSPROT). The peaks labeled by D match calculated tryptic peptide masses from dynamin I within 50 p.p.m. Trypsin autolysis products are marked by a capital T. The other proteins isolated in the profilin complexes were identified in the same way.

its low abundance in brain compared with the other ligands. However, using an antibody against the VASP homolog MENA we could detect complete recruitment of MENA to the brain profilin II complex (data not shown).

Proteins involved in signal transduction and endocytosis are the major components of the profilin complexes

The major components in the profilin I complex are clathrin, valosine containing protein (VCP), hsp 70, tubulin and actin. Clathrin assembles at membranes to form coated pits and subsequently coated vesicles. VCP is an ATP-binding protein with homology to the cdc48/sec18/NSF family involved in vesicle transport (Frohlich *et al.*, 1991). Interestingly, it has been shown that VCP is tyrosine phosphorylated upon T-cell receptor activation and that it can form a complex with clathrin and hsp 70 (Pleasure *et al.*, 1993). Large amounts of tubulin were found tightly bound in the profilin I complex, while no tubulin could be identified in the profilin II complex. Since tubulin by itself does not bind to profilin I (data not shown) one of the components in the complex must provide multivalent tubulin-binding sites, or the intact complex itself is required for efficient tubulin recruitment.

The most prominent component of the profilin II complex is dynamin I, a protein with an important role in clathrin-mediated vesicle formation (Damke *et al.*, 1994). Dynamin I is a GTPase that has been shown to localize to the neck of budding vesicles and to form a collar-like structure (Takel *et al.*, 1995). The other abundant components in the complex are HEM 2 (Baumgartner *et al.*, 1995), synapsin IA/B, synapsin IIA/B, Rho-associated coiled-coil kinase (ROCK 2) (Nakagawa *et al.*, 1996), hsp 70 and a protein of unknown function which we named POP (partner of profilin). Mammalian HEM 2 is identical to rat NAP1 (Kitamura *et al.*, 1996), a protein

Table I. Proteins identified by MALDI mass spectrometry

Accession No.	Protein	Mol. wt (kDa) cal	Mol. wt (kDa) gel	Peptide matched	Sequence coverage (%)
	Profilin I complex				
P11442	clathrin heavy chain	193.3	170	42	32
Q01853	transitional endoplasmic reticulum ATPase (VCP)	90.0	98	29	42
M19141	heat shock protein 70	71.1	71	23	38
P04691	tubulin B-chain	50.4	54	21	39
X13055	actin	41.4	43	12	34
	Profilin II complex				
U58513	Rho-associated coil-coiled forming protein kinase, ROCK 2	161.7	160	18	18
D38549	POP, human ORF[a] (KIAA0068)	148.8	140	33	22
P55161	membrane-associated protein HEM 2	130.2	125	19	17
P21575	dynamin I	96.3	100	35	44
P09951	synapsins IA and IB	74.1	82/78	19	35
M19141	heat shock protein 70	71.1	71	20	29
M27925	synapsin IIA	63.7	67	14	35
D30411	synapsin IIb	52.9	55	13	28
M12481	actin	39.6	43	7	25

[a]The protein POP was found as a homolog of a human ORF (accession number D38549) with unknown function. Sequence comparison of the human ORF with the mouse EST database identified a homologous EST clone (accession number W64430).

that has been identified as an nck ligand. Recently, it has been shown that NAP1 is associated with the activated G protein Rac (Kitamura *et al.*, 1997). ROCK 2 is a downstream effector of Rho, containing a pleckstrin homology domain and a proline-rich C-terminus (Nakagawa *et al.*, 1996).

Dynamin I binds directly to profilin II

To analyze the interactions of the individual proteins with profilins we partially purified dynamin I from mouse brain, removing the other profilin ligands, and performed binding studies with this fraction. Dynamin I, the major constituent of the profilin II complex, binds directly and with high affinity to profilin II (Figure 3A). Compared with profilin II, profilin I has a much lower affinity for dynamin I (Figure 3B). The high affinity of dynamin I for profilin II is also demonstrated by the Western blot shown in Figure 3C. More than 80% of dynamin I could be depleted from total brain extracts by profilin II–Sepharose (Figure 3C, top). The profilin II column completely depleted synapsin from total mouse brain extracts (Figure 3C, bottom). Synapsin was also partially retained on the DNase I column because of its affinity for actin, nevertheless, the elution of synapsin from the profilin II column by poly-L-proline suggests that at least a portion of the synapsin is bound directly to profilin II via its proline-rich domain.

We further addressed the question of direct binding of profilin II to the complex components by performing blot overlays with recombinant profilin II. After separation by SDS–PAGE and transfer to Immobilon-P membrane, several but not all proteins in the profilin II complexes were labeled by profilin II (Figure 3D). It is impossible to predict which of the membrane-bound proteins renature to an extent that allows profilin II binding. Labeling therefore indicates a specific interaction with this protein on the membrane while no labeling in the blot overlay does not necessarily rule out direct binding to the native protein. The best example is actin which does not bind to profilin II in blot overlays because actin does not renature readily on filters. In our blot overlay experiments dynamin I was the most prominent protein that could be labeled with profilin II in total brain extracts, the profilin II complex, and the enriched dynamin I fraction (Figure 3D). Western blot confirmed that the profilin-binding protein is dynamin I. This result further supports that dynamin I can bind directly to profilin II and shows that the binding of dynamin I to profilin II–Sepharose (Figure 3A) is not mediated by some other protein. It is notable that even in a blot overlay on total brain extract only a limited number of proteins are labeled by profilin II, with dynamin I being again the most prominent. In blot overlays of the profilin II complex several other proteins can be labeled with profilin II. Judged by size, the labeled proteins are most likely the synapsin isoforms, hsp 70 and POP, indicating that these proteins are able to bind directly to profilin II (Figure 3D).

Since dynamin's GTPase activity is important for its function in endocytosis we asked whether the binding of dynamin to profilin II might be nucleotide dependent. In total brain lysate binding of dynamin I to profilin II–Sepharose was inhibited by about 80% in the presence of 2 mM GTP-γ-S and 2 mM ATP while GMP had no effect on binding. This result suggests that either dynamin I binding to profilin II is inhibited in its GTP form or another protein in the cytosol regulates the binding of dynamin in a nucleotide-dependent fashion. We have no explanation for the ATP effect but it is tempting to speculate that the nucleotide bound in the profilin–actin complex might influence the binding of dynamin I.

Dynamin I and profilin II co-localize in hippocampal neurons

One question is whether the interaction of dynamin I and profilin II can be observed *in vivo*. To address this question we performed double immunofluorescence labeling of polarized rat primary hippocampal neurons which were differentiated for 14 days (Dotti *et al.*, 1988) with antibodies against dynamin I and mouse profilin II. In mature cultured neurons dynamin I localized to vesicular structures in the cell body, the axon and the dendrites (Figure 4e and f). Similar dynamin I localization has been observed *in situ* (Noda *et al.*, 1993). Most dynamin I containing

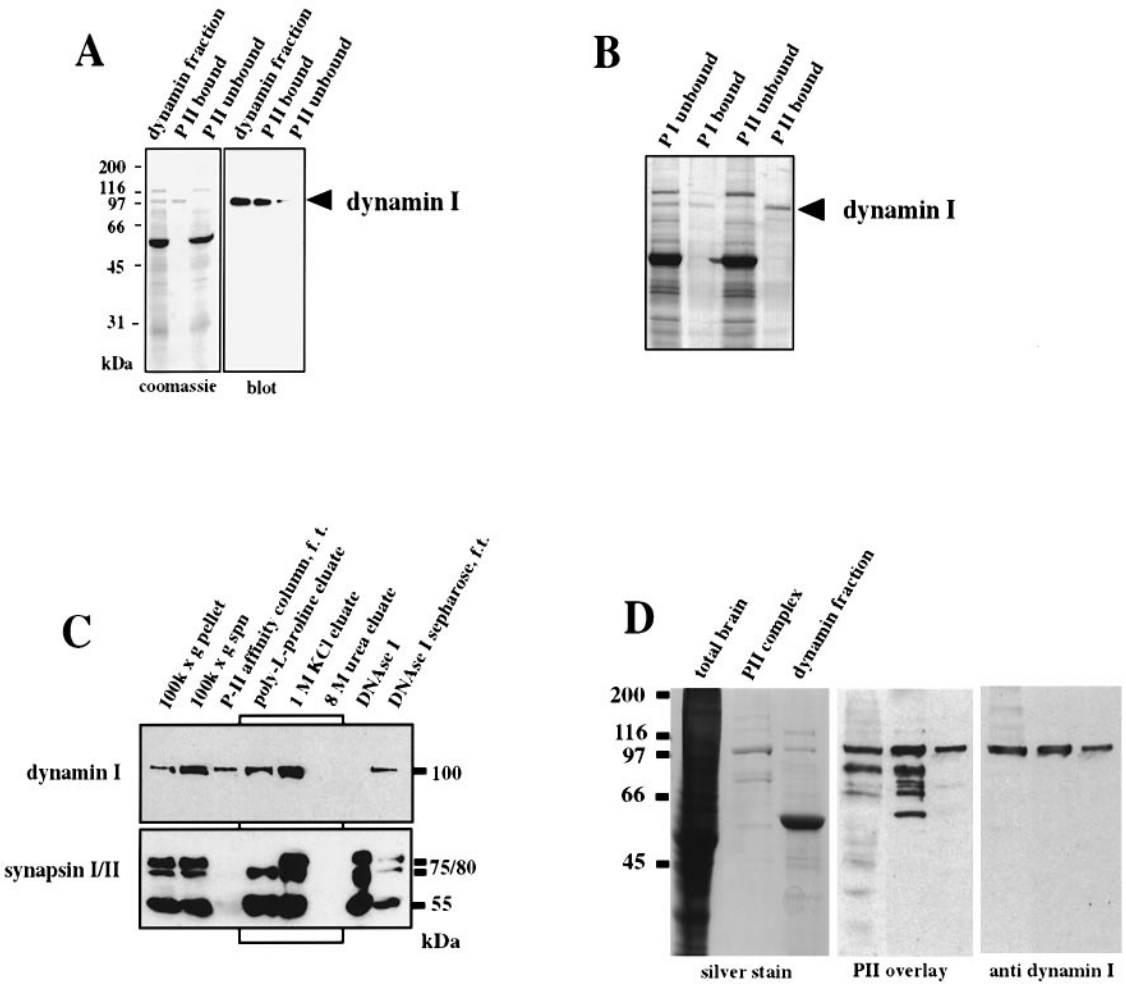

Fig. 3. Dynamin I binds directly to profilin II (**A**). Dynamin I was partially purified from mouse brain to yield a visible band on a Coomassie Blue-stained gel. The enriched fraction was passed over profilin II–Sepharose, and bound proteins eluted with SDS sample buffer. Approximately 90% of the dynamin I bound to profilin II. Comparison of dynamin I binding to profilin I and profilin II (**B**). The dynamin-enriched fraction was passed over profilin I– and profilin II–Sepharose, and bound proteins released with SDS sample buffer. Equivalent amounts were subjected to SDS–PAGE and the gel silver stained. Quantitative analysis of dynamin I and synapsin I/II binding to profilin II–Sepharose (**C**). Fractions from the profilin II binding experiment from Figure 2A were analyzed by Western blot with different antibodies. Most of the dynamin I and all of the synapsin I/II present in brain extract was bound to profilin II–Sepharose. No dynamin I bound to DNase I–Sepharose. Synapsin I/II was also recruited to DNase I–Sepharose via actin. Note that the synapsin antibody recognizes all synapsin isoforms. Blot overlay with profilin II (**D**). Total brain extract, the profilin II complex and the dynamin I enriched fraction were subjected to SDS–PAGE and the filters used for a blot overlay with profilin II (see Materials and methods). The left panel shows the corresponding silver stained gel, the middle panel the overlay with profilin II and the right panel the staining with the dynamin I-specific antibody.

vesicles also labeled for profilin II indicating that dynamin I and profilin II bind to the same structures *in vivo* (Figure 4c, f and i). Profilin I staining showed a similar distribution as profilin II, and both partially co-localized with synapsin-containing vesicles (data not shown). Dynamin I as well as clathrin are enriched in regions of endocytosis. Therefore, it is not surprising that we find a similar distribution for profilin I and profilin II at these sites. Even though co-localization by immunofluorescence does not prove physical interactions of molecules it clearly shows recruitment of the complex components to the same sites of early endocytic vesicle formation in neuronal cells and suggests a function of the profilin–ligand interaction in the endocytic process.

Discussion

Here we describe the isolation and characterization of profilin I- and profilin II-binding complexes in mouse brain extracts. Because the object of our study was the functional characterization of profilins via their protein interaction partners, we focused on the proteins detectable by Coomassie stain which were more likely to represent stoichiometric binding partners. As the analysis method we employed MALDI mass spectrometry followed by database searching. The use of MALDI peptide mapping, which does not involve any sequencing and uses robotics in the sample preparation, allows large scale identification of proteins, so that complexes such as the profilin complexes dscribed here, can be analyzed in a short time. We identified five proteins in the profilin I complex and a total of nine proteins in the profilin II complex.

Profilin-binding proteins from mouse brain are different from the Arp2/3 complex

An interesting feature of the brain profilin complexes is that they differ from the complexes which have been isolated from lower eukaryotes (Machesky *et al.*, 1994).

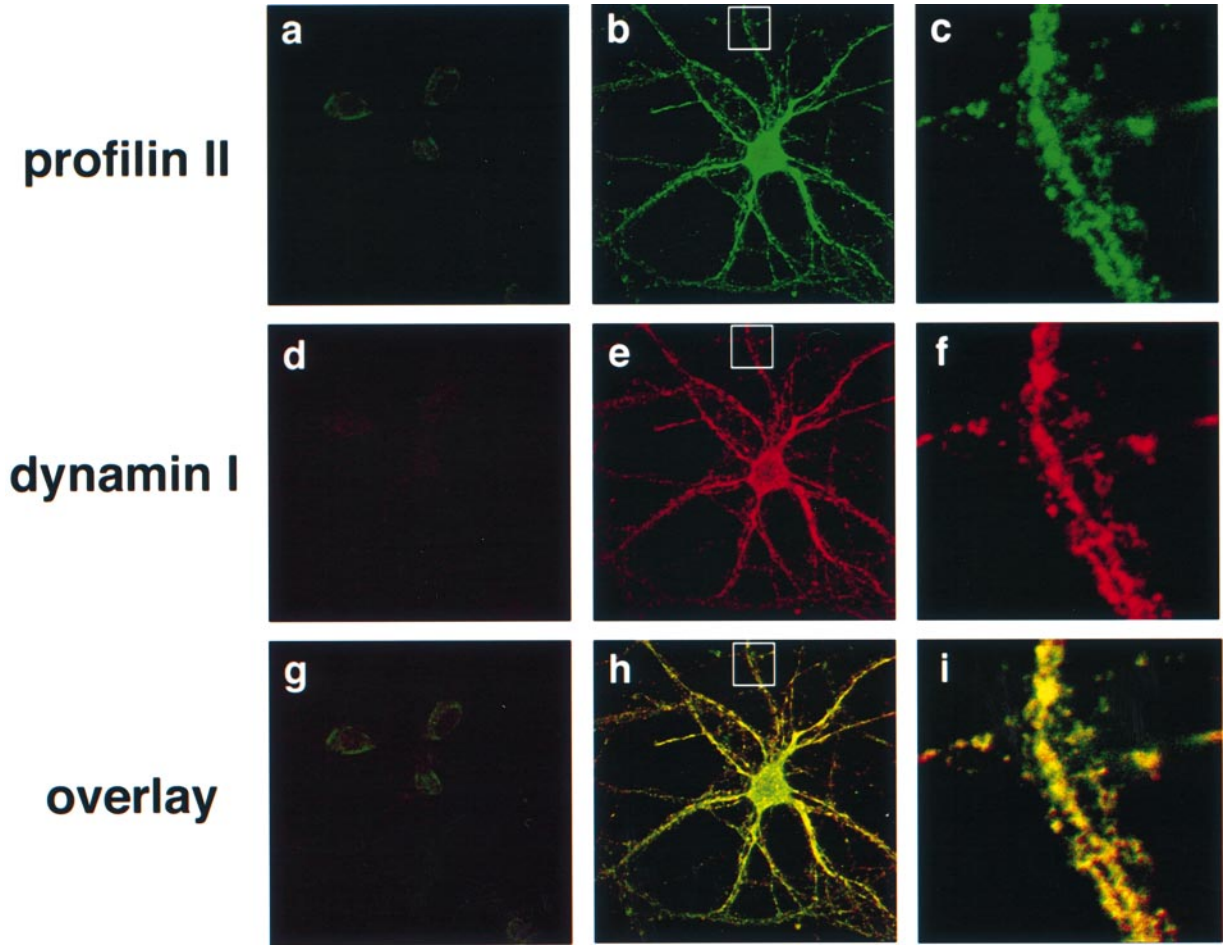

Fig. 4. Double immunofluorescence of cultured hippocampal neurons with profilin II and dynamin I-specific antibodies. Rat hippocampal neurons were cultured for 14 days to allow them to polarize and form axonal and dendritic processes. Cells were fixed and stained using the affinity purified polyclonal profilin II antibody and the mouse monoclonal dynamin I antibody, Hudy-1. Profilin II was detected using a FITC labeled secondary antibody, dynamin I was detected using a TRITC labeled secondary antibody. The left row shows the background staining of the secondary antibodies alone (**a**, **d** and **g**), the middle and right rows show the specific staining for profilin II (**b** and **c**) and dynamin I (**e** and **f**). The right row shows a process at higher magnification (c, f and **i**). Co-localization of profilin II and dynamin I is shown in (**h**) and (i). Note that dynamin I and profilin II co-localize at vesicular structures.

In Acanthamoeba the Arp2/3 complex has been found to bind to profilin affinity columns. The function of the complex is not clear yet but yeast mutants for the Arp2 protein show a defect in endocytosis (Moreau *et al.*, 1996) and Arp3 mutants display disorganized actin patches (McCollum *et al.*, 1996) suggesting a function of the Arp2/3 complex in endocytosis. It is tempting to speculate that the profilin–Arp2/3 complex and the brain complex described here have analogous functions during the early steps of endocytosis even though the proteins in the complexes are very different. The Arp2/3 complex can be isolated through several columns as a stable complex but interestingly this complex does not contain profilin and only a certain fraction of this complex can rebind to profilin (McCollum *et al.*, 1996; Mullins *et al.*, 1997). Whether the profilin-binding proteins from mouse brain described here form a stable multi-protein complex or whether most of the components interact individually with profilin cannot be answered yet. We can partially purify dynamin I from mouse brain which removes the other profilin-binding proteins which would argue against a stable multi-protein complex. However, we would certainly disrupt such a complex during several column runs if the interactions between the components are of low affinity, regulated, or sensitive to dilution. *In vivo* and *in vitro* crosslinking will be an alternative method to investigate further the question of who interacts with whom.

The interaction of profilin II with dynamin I are particular interesting. Not only is profilin II preferred over profilin I but in addition, the interaction appears to be nucleotide dependent. GTP-γ-S and ATP greatly diminish the binding of only dynamin I in the complex while all the other components are apparently not affected (not shown). Whether this is due to the exchange of the nucleotide on dynamin I or some other regulatory factor in the cytosol needs to be shown. The ATP effect is puzzling and needs further analysis. Although we have no evidence so far, one possible explanation might be that the nucleotide bound in the profilin–actin complex plays a role in regulating dynamin I binding. Profilin is known to accelerate the nucleotide exchange on actin (Mockrin and Korn, 1980; Goldschmidt *et al.*, 1992).

Another interesting observation which we would like to stress but which we cannot explain at the moment is the finding of large amounts of tubulin in the brain profilin

I complex. Some of the tubulin can be eluted from the profilin I–Sepharose column with high salt but most of it can only be eluted under denaturing conditions (not shown). We never observed binding of tubulin in the profilin II complex. We also could not detect any direct binding of purified tubulin to profilin I or profilin II. These findings would suggest that one of the tight binding proteins in the profilin I complex recruits tubulin in non-stoichiometric amounts.

A proline-rich motif but not poly-L-proline stretches appear to be required for profilin binding

Profilin I and profilin II bind to different proteins although biochemically profilin I and profilin II are very similar. This raises the question about the nature of profilin–ligand interactions. Traditionally it was thought that only proteins with continuous proline stretches of more than eight prolines are able to bind to profilins and that the poly-L-proline interaction is the sole basis for the binding. This criteria might be true for some of the known binding proteins like VASP and MENA, however this concept does not explain our findings and might not reflect the actual *in vivo* situation. MENA has been shown to bind to profilin (Gertler *et al.*, 1996), and in our experiments MENA was almost quantitatively depleted from brain extracts by profilin II–Sepharose. In contrast, ASP56/CAP (Gieselmann and Mann, 1992), a protein which contains a stretch of 12 prolines interrupted by one glycine, did not bind to profilin II–Sepharose although one would expect binding if the poly-L-proline criteria would be the only valid one (data not shown).

Most of the proteins we find in the profilin complexes have proline-rich domains but do not contain long proline stretches. Comparing the sequence of the profilin-binding proteins we have identified, we searched for a common proline-containing motif which could explain the binding to profilin. The only consensus sequence we could identify was a ZPPX motif (where Z generally is proline, glycine, alanine, or occasionally a charged amino acid, and X preferentially a hydrophobic residue). This short motif alone is unlikely to be sufficient for selective profilin binding since thousands of proteins in the database contain the ZPPX motif. However, POP, ROCK 2, VCP, clathrin, synapsin and dynamin contain two or more copies of the ZPPX motif in a short amino acid stretch of not more than 100 residues. Interestingly, the ZPPX amino acid motif, is similar to the SH3-binding motif which has been identified by peptide-binding studies (Ren *et al.*, 1993). From sequence comparison profilin cannot be recognized as an SH3 domain-containing protein but the structural folds in profilin very much resemble an SH3 domain (Schutt *et al.*, 1993). Dynamin I and HEM2/NAP1 are known to bind to the SH3 domains in Grb2 and Nck respectively. In this context it is noteworthy that we did not find Grb2 or Nck enriched in the profilin complexes even though they are expressed in mouse brain. One possible explanation for this finding is that profilin mimics an SH3 domain which binds to the SH3-binding site on dynamin I and HEM2/NAP1, thereby competing for the binding of Grb2 and Nck.

The profilin complexes in brain suggest a role of profilins in endocytosis and signal transduction

The membrane is the key structure to regulating actin polymerization in cell protrusions as well as in endocytosis. How actin polymerization is nucleated at specific membrane sites and how membrane flow is regulated during cell motility is a fundamental problem that has not been solved yet but there is growing evidence that phospholipids might trigger these events *in vivo* (Stossel, 1993; De Camilli *et al.*, 1996). There is also strong evidence that the early steps of endocytosis involve actin and are dependent on actin polymerization (Salisbury *et al.*, 1980; Gottlieb *et al.*, 1993). Whether there are completely actin independent endocytosis mechanisms and whether intracellular vesicle trafficking also depends on the actin cytoskeleton is controversial. A deeper understanding of the relation of endocytosis and actin nucleation might hold important answers to how membranes regulate and catalyze these processes.

The profilin complexes described here physically bring together cytoskeletal elements and proteins which are known to be important in endocytosis and synaptic recycling as well as signaling molecules which play a role in regulating the actin cytoskeleton. One class of molecules in the brain profilin complexes appears to play a role in signaling through the small G proteins Rac and Rho. ROCK 2, which binds in the profilin II complex, is a Rho-associated kinase which is activated by Rho and acts downstream. The targets of this kinase are not well characterized. Interestingly, Rac and Rho appear not only to function in regulating the actin cytoskeleton but have also been shown to play a role in regulating endocytosis (Lamaze *et al.*, 1996). HEM2/NAP1 which also associates with the profilin II complex was recently shown to interact with Rac (Kitamura *et al.*, 1997). NAP1 was originally identified as a ligand for Nck, a linker molecule with three SH3 and one SH2 domains (Kitamura *et al.*, 1996). This suggests that profilin II could be part of the Rac/Rho, tyrosine kinase pathway which signals to the actin cytoskeleton, however, the exact mode of action is not clear at the moment. It is noteworthy that VCP which is found in the profilin I complex is one of the major tyrosine phosphorylated proteins upon stimulation in T cells (Egerton *et al.*, 1992). Whether VCP is also tyrosine phosphorylated in brain, what the kinase, and what VCP's function is, remains to be shown. How could one picture the role of profilin in signal transduction? One explanation could be that profilin plays the role of a negative regulator, buffering certain effector molecules which would otherwise constitutively be recruited and activated through SH3-domain containing linkers (Grb2, Nck). Alternatively, profilins might directly influence tyrosine phosphorylation pathways.

The second class of profilin-binding proteins are molecules involved in endocytosis. We showed that dynamin I is able to bind directly and selectively to profilin II and must therefore be considered as a new profilin II ligand. Synapsins, clathrin and VCP are other proteins found in the profilin complexes which play a role in endocytosis. How could profilins be involved in the endocytic process and what might be the relation to actin polymerization? Profilins and most of the binding partners are phospholipid binding proteins. Profilin–ligand interaction could serve to increase the avidity for phospholipids, to target the complex to the membrane, and induce the clustering of phospholipids at the inner leaflet of the membrane. Thereby, profilin could recruit monomeric actin to specific

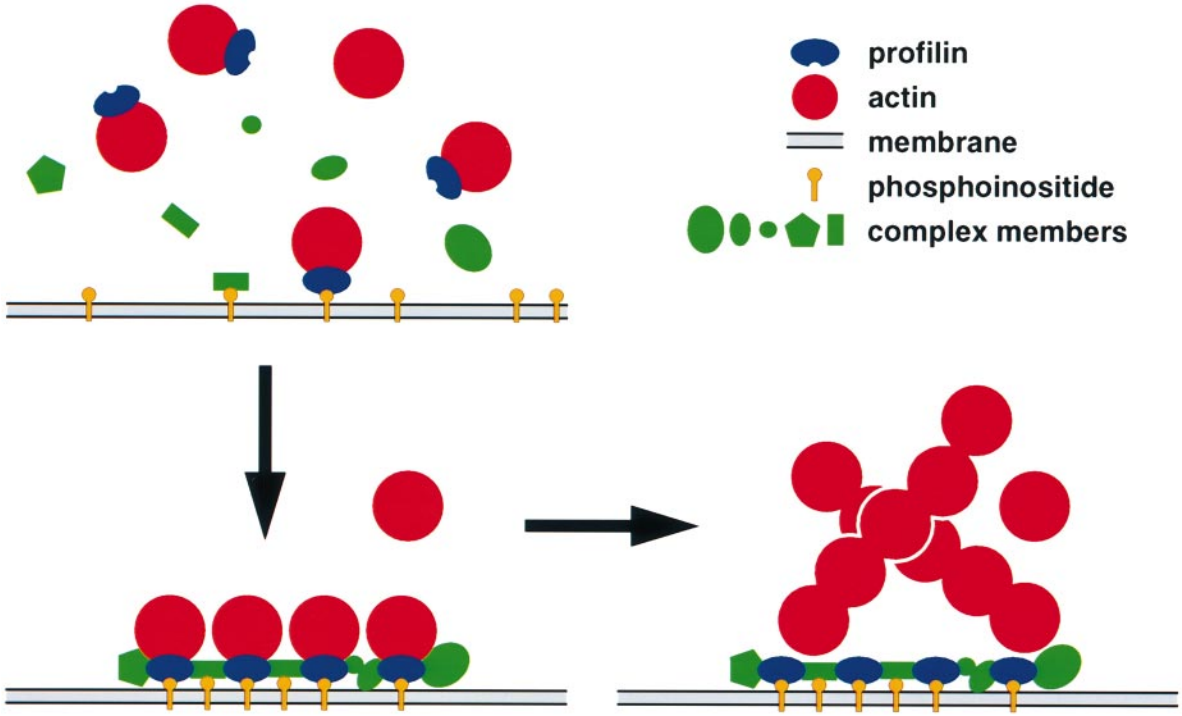

Fig. 5. Hypothetical model for the function of the profilin complexes. Profilin–actin can interact with phosphoinositides and the respective ligands to form larger complexes at specific membrane sites. The interaction of the complexes with the membrane causes clustering of phosphoinositides into micro domains leading to the formation of a 'signaling membrane site'. This signaling membrane site could be a transient aggregation of a number of signaling molecules including receptors, kinases and phospholipids. Phosphoinositide islands at this spot could trigger the release of actin from the profilin–actin complexes as well as local uncapping of actin filaments, leading to actin polymerization. Actin polymerization and endocytosis might act in concert in such a way that actin polymerization and network formation at the site of endocytosis stabilizes and prolongs the lifetime of the 'signaling membrane site' necessary in the early stage of endocytosis.

sites at the membrane and temporarily increase the monomer concentration sufficiently for nucleation and polymerization (Figure 5). These 'signaling membrane sites' would represent a transient aggregation of a number of signaling molecules which could include receptors, kinases and phospholipids. According to this model, the actin cytoskeleton would function as a temporary organizer of the membrane allowing signaling to persist for a period necessary to induce endocytosis. This hypothesis would explain the only transient association of actin at sites of endocytosis and would predict that the actin-dependent endocytosis is highest in areas of the cell with high actin dynamics such as ruffles or the leading edge.

In conclusion, actin polymerization and endocytosis have many features in common. They both appear to be regulated by small G proteins, phosphoinositides modulate proteins involved in both processes, and nucleation always takes place at the membrane. We favor the idea, that this close relation of endocytosis and actin polymerization reflects the use of a common mechanism to alter the membrane topology, employed for both processes. Since profilin I, profilin II, clathrin, ROCK 2, dynamin I and synapsin are all able to interact with phospholipids (Lassing and Lindberg, 1985; Seppen *et al.*, 1992; Benfenati *et al.*, 1993; Liu *et al.*, 1994; Gieselmann *et al.*, 1995; Nakagawa *et al.*, 1996), it is tempting to speculate that clustering of phospholipids, particularly phosphoinositides, into local domains by the profilin complexes could be a common mechanism to regulate actin assembly as well as membrane flow.

Materials and methods

Antibody production and Western blot analysis

Recombinant human profilin I and a 15 amino acid peptide from mouse profilin II were used to raise polyclonal antibodies in rabbits. Polyclonal antibodies were affinity-purified using recombinant profilin I or the profilin II-specific peptide coupled to Sepharose. The antibodies were isoform-specific as shown by their reactivity against recombinant mouse profilin I and profilin II. Mouse tissue extracts were prepared as follows. Individual frozen mouse organs were directly lysed in 1 ml cold protein extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EGTA, 5 mM EDTA, 0.5% TritonX-100, 1 mM benzamidine, 0.5% PMSF) by 20 strokes in a tight-fitting Dounce homogenizer. The lysate was cleared by a 30 min spin at 4°C at 70 000 r.p.m. in a TLA100.2 rotor. Supernatants were mixed with SDS sample buffer, heated to 95°C for 5 min and stored frozen. Equal amounts of tissue extracts from mouse were subjected to SDS–PAGE (15% acrylamide), transferred to Immobilon-P and probed with the two specific antibodies. Signals were detected using HRP-coupled secondary antibodies and ECL reagent (Amersham).

Isolation of profilin I- and profilin II-binding complexes

Recombinant mouse profilin I and profilin II, or DNase I was coupled to CNBr-activated Sepharose (Pharmacia) at a concentration of 2 mg protein per ml beads. Brains from ten mice were prepared freshly and lysed in 20 mM HEPES pH 7.2, 50 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween-20 by 20–30 strokes in a tight-fitting Dounce homogenizer. The lysates were centrifuged for 1 h at 150 000 *g* and equivalent amounts of the supernatant cycled over the profilin, DNase I or mock column. After extensive washing of the profilin I or profilin II column with lysis buffer, proteins were subsequently eluted with ~50 µM poly-L-proline (mol. wt >30 000), 1 M KCl and 8 M urea. Since the majority of proteins could be eluted with high salt, the subsequent elutions were performed with 1 M KCl only. The DNase I and mock column were washed extensively and the bound proteins eluted with SDS sample buffer.

Protein identification by MALDI analysis

The profilin complexes were subjected to one-dimensional SDS–PAGE (10% acrylamide), gels stained with Coomassie Brilliant Blue R-250, and bands excised. Gel pieces were washed, 'in-gel' reduced, S-alkylated, and proteins enzymatically degraded with trypsin using a robot system (Ashman *et al.*, 1997). After 3 h, digest products were analyzed by a Bruker Reflex Matrix Assisted Laser Desorption Ionization 'Time-of-Flight' mass spectrometer equipped with delay ion extraction (Bruker-Franzen, Bremen, Germany). Matrix and samples were prepared using the 'thin film' method as described (Vorm *et al.*, 1994). Measured tryptic peptide masses were searched with PeptideSearch software developed at EMBL against a non-redundant protein sequence database (maintained by C.Sander EBI, Hinxton Park, England).

Dynamin binding experiment

Dynamin I was partially purified from mouse brains by DE52 and P11 chromatography essentially as described (Gout *et al.*, 1993), followed by a hydroxyapatite chromatography step. The enriched fraction was cycled over profilin I or profilin II–Sepharose, the column washed with three column volumes of PBS and the bound protein eluted with SDS sample buffer. Equivalent amounts were subjected to SDS–PAGE, and the gel silver stained or transferred for Western blot in order to analyze the binding to the two profilins.

To test the nucleotide dependence of dynamin binding, cleared brain extracts were prepared as described above and incubated with 2 mM GTP-γ-S, 2 mM GMP or 2 mM ATP for 10 min on ice. The binding to profilin II–Sepharose was performed in the presence of the respective nucleotides as described. Equal amounts of bound proteins were separated by SDS–PAGE and proteins visualized by silver staining. The intensities of the dynamin bands were determined by densitometry.

Profilin blot overlay

Protein fractions were subjected to SDS–PAGE and proteins transferred to Immobilon-P membrane. The membrane was rinsed briefly in NCP buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.02% Na azide) and incubated overnight at 4°C in NCP, 5% fetal calf serum, 0.5 mM DTT. Filters were incubated with 30 μg/ml profilin for 4 h at 4°C, washed 3× 10 min with NCP, incubated with profilin antisera for 45 min, washed again 3× 10 min, and incubated with HRP-coupled secondary antibody for 30 min. After 5× 5 min wash the filters were developed using ECL reagent (Amersham). As a control, the secondary HRP-coupled antibody alone did not yield any signal in the blot overlay with profilin II.

Immunofluorescence

Rat hippocampal neurons were cultured for 14 days to allow them to polarize and form axonal and dendritic processes (Dotti *et al.*, 1988). Cells were fixed in freshly prepared paraformaldehyde and stained following standard protocols, using the affinity purified polyclonal profilin II antibody and the mouse monoclonal dynamin I antibody, Hudy-1 (Upstate Biotechnology Inc.).

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