Profilin-I-ligand interactions influence various aspects of neuronal differentiation

Anja Lambrechts[1,*], Veronique Jonckheere[1], Christa Peleman[1,‡], Debby Polet[1], Winnok De Vos[2], Joël Vandekerckhove[1] and Christophe Ampe[1]

[1]Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, and Department of Medical Protein Research, Flanders Interuniversity Institute for Biotechnology (VIB09), Albert Baertsoenkaai 3, 9000 Ghent, Belgium
[2]Department of Molecular Biotechnology, Faculty of Bio-engineer sciences, Ghent University, 9000 Ghent, Belgium

*Author for correspondence (e-mail: anja.lambrechts@Ugent.be)
‡Present address: Subfaculty of Medicine, Interdisciplinary Research Center, Katholieke Universiteit Leuven-Campus Kortrijk, 8500 Kortrijk, Belgium

Accepted 4 January 2006
Journal of Cell Science 119, 1570-1578 Published by The Company of Biologists 2006
doi:10.1242/jcs.02884

Summary

Differentiating neurons extend membrane protrusions that develop into growing neurites. The driving force for neurite outgrowth is the dynamic actin cytoskeleton, which is regulated by actin-binding proteins. In this study, we describe for the first time, the role of profilin I and its ligand interactions in neuritogenesis of PC12 cells. High-level overexpression of wild-type profilin I had an inhibitory effect on neurite outgrowth. Low levels of profilin I did not disturb this process, but these cells developed many more filopodia along the neurite shafts. Low-level overexpression of mutant forms of profilin I changed one or more aspects of PC12 differentiation. Expression of a profilin I mutant that is defective in actin binding (profilin I^{R74E}) decreased neurite length and strongly inhibited filopodia formation. Cells expressing mutants defective in binding proline-rich ligands (profilin I^{W3A} and profilin I^{R136D}) differentiated faster, developed more and longer neurites and more branches. The profilin I^{R136D} mutant, which is also defective in phosphatidylinositol 4,5-bisphosphate binding, enhanced neurite outgrowth even in the absence of NGF. Parental PC12 cells treated with the ROCK inhibitor Y27632, differentiate faster and display longer neurites and more branches. Similar effects were seen in cells expressing profilin I^{WT}, profilin I^{W3A} and profilin I^{R74E}. By contrast, the profilin I^{R136D}-expressing cells were insensitive to the ROCK inhibitor, suggesting that regulation of profilin I by phosphatidylinositol 4,5-bisphosphate metabolism is crucial for proper neurite outgrowth. Taken together, our data show the importance of the interaction of profilin I with actin, proline-rich proteins and phosphatidylinositol 4,5-bisphosphate in neuronal differentiation of PC12 cells.

Key words: Profilin mutants, Actin, PC12, Neuritogenesis, PtdIns(4,5)P_2

Introduction

During differentiation, neurons extend membrane protrusions (sprouts) at the onset of neuritogenesis, some of which develop into elongating neurites, characterized by a guiding growth cone and branch formation. These morphological changes depend largely on a dynamic actin cytoskeleton (reviewed by da Silva and Dotti, 2002), which is regulated by a plethora of actin-binding proteins.

Profilins are monomeric actin (G-actin)-binding proteins with a complex regulatory function on actin dynamics (Witke, 2004). Profilin was originally described as an actin-sequestering molecule (Carlsson et al., 1977), but was more recently found to enhance actin polymerization by adding monomers to the fast-growing ends of actin filaments (Pantaloni and Carlier, 1993; Kang et al., 1999) and catalyzing nucleotide exchange (Goldschmidt-Clermont et al., 1992; Selden et al., 1999). Thereby, profilins contribute to actin polymerization at the leading edge of lamellipodia and in the tips of filopodia. It is thought that accumulation of actin-profilin complexes at these sites may be achieved by the interaction of profilin with the proline-rich domains of Ena/VASP proteins, Diaphanous-related formins and N-WASP family members (Reinhard et al., 1995; Watanabe et al., 1997; Suetsugu et al., 1998). These proteins have been shown to bind to actin and to directly or indirectly increase the polymerization rate (Laurent et al., 1999; Lambrechts et al., 2000b; Yang et al., 2000; Moseley et al., 2004).

In addition, profilin was the first actin-binding protein to be identified as a ligand for phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] (Lassing and Lindberg, 1985). Profilin I contains two PtdIns(4,5)P_2-binding sites of which one overlaps with the actin-binding site, the other with the poly-proline-binding site (Lambrechts et al., 2002) and thus PtdIns(4,5)P_2 competes with both actin and proline-rich proteins for binding to profilin (Lassing and Lindberg, 1985; Lambrechts et al., 1997). Therefore, PtdIns(4,5)P_2 may be considered as a master regulator of profilin function. Extracellular stimuli cause fluctuations in the PtdIns(4,5)P_2 level, which then, depending on the stimulus, inhibit profilin or allow profilin to exert its function.

In mammals, profilin I and IIa are expressed in neurons (Lambrechts et al., 2000a). In differentiated neurons both isoforms are targeted to synaptic sites, where they interact with pre- and post-synaptic scaffolding proteins, consistent with an emerging view that profilin contributes to actin turnover or microfilament organisation at these sites (Faivre-Sarrailh et al.,

1993; Kneussel and Betz, 2000; Ackermann and Matus, 2003; Neuhoff et al., 2005). Although mammalian profilin I is known to interact in vitro with several proteins involved in actin-driven neuronal differentiation, its role and the importance of its ligand interactions in this process have not yet been addressed.

Here we have used rat pheochromocytoma cells (PC12) as a model system to investigate the role of profilin I in neurite outgrowth. We address the questions: (1) whether overexpression of wild-type human profilin I influences neurite outgrowth of PC12 cells; (2) how the disruption of one or more ligand interactions affects various aspects of neurite formation; and (3) how PtdIns(4,5)P_2 levels may change profilin function downstream of the Rho-associated kinase ROCK during neuritogenesis.

Results

Dose-dependent effect of profilin I^{WT} on PC12 differentiation

Before we investigated the role of profilin I in neurite formation and elongation, we probed the subcellular localization of EYFP-profilin-I fusion protein in differentiating PC12 cells using confocal microscopy. Although EYFP-profilin-I is mainly found in the cytoplasm of the cell body and the neurite shafts (Fig. 1A), the protein is enriched in protruding areas close to the substrate: the tips of neuronal sprouts, in growth cones and in branch points (Fig. 1B,C). These data are consistent with immunostaining of profilin in differentiating cortical neurons (Faivre-Sarrailh et al., 1993). Therefore, profilin is present in regions that actively protrude during neuritogenesis and neuronal differentiation.

To determine whether increased profilin I expression levels influence neurite outgrowth, we created PC12 cell lines stably overexpressing wild-type human profilin I (profilin I^{WT}) and selected clones with different expression levels of the protein. After plating the cells on collagen, we added NGF and forskolin to induce neuronal differentiation. We observed no differences in the percentage of differentiated cells over a broad range of profilin levels (3- to 36-fold excess over the endogenous levels) (Fig. 2). When profilin I was overexpressed approximately 80-fold, however, the percentage of differentiated cells decreased from 79% in the parental PC12 cells to 56% after 76 hours of differentiation. Similar results were obtained after 122 hours (data not shown).

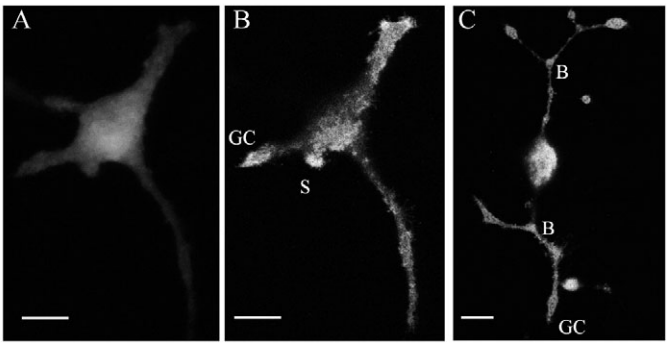

Fig. 1. Subcellular localisation of EYFP-profilin I^{WT} in PC12 cells. (A) Projection of a confocal image stack series. (B,C) Selected stacks at a position close to the substrate. EYFP-profilin I^{WT} is seen enriched in growth cones (GC), neuronal sprouts (S), and branch points (B). Bars, 10 μm.

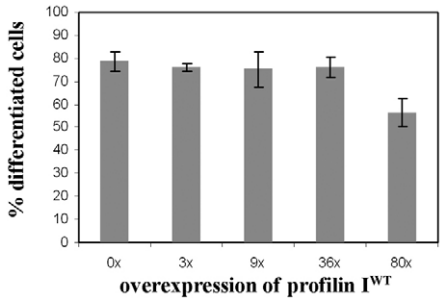

Fig. 2. Dose-dependent effect of profilin I^{WT} on PC12 cell differentiation. The percentage of differentiated cells 76 hours after the addition of NGF, is shown as a function of the overexpression level of human profilin I^{WT}. The cells were scored as differentiated when they had at least one neurite that is longer than the cell diameter. Results are presented as means ± s.e.m. with n=177-278 cells.

In order to study in more detail the effects of wild-type and mutant profilin I on neuritogenesis, we chose cell lines with comparable low levels of overexpression, to observe dominant effects of the mutants. We obtained different cell lines with two- to threefold more wild-type or mutant protein compared with the endogenous protein level (Fig. 3A). We analyzed profilin I^{WT} cells in more detail and compared them with the parental PC12 cells. Since we were investigating the effect of profilin I on neuritogenesis, we determined the following aspects of this process: (1) the percentage of differentiated cells 24 hours and 96 hours after the start of differentiation with NGF/forskolin (a cell is considered differentiated when it contains at least one neurite that exceeds the cell diameter); (2) the percentage of differentiated cells bearing two or more neurites; (3) the percentage of neurites that developed branches; and (4) the total neurite output, which is the sum of all neurite lengths of a cell.

The profilin I^{WT} cells show only a slight decrease in differentiation rate over the first 96 hours when compared with parental PC12 cells (Fig. 3C). In addition, the threefold increased concentration of profilin I^{WT} did not significantly alter the number of neurites or branches in differentiated cells (Fig. 3D,E). The profilin I^{WT} cells did, however, have a significantly larger neurite output than the parental PC12 cells at 96 hours (Table 1). Taken together, these data indicate that a moderate change in profilin I level has no dramatic effect on

Table 1. Mean neurite output of PC12 cells expressing WT and mutant profilin I

	Neurite length (μm)		
	NGF/F for 24 hours	NGF/F for 96 hours	NGF/F/Y27632 for 24 hours
PC12	34.65±2.30	73.96±7.16	64.30±3.86***
Profilin I^{WT}	38.21±3.14	116.31±12.11**	68.43±5.13***
Profilin I^{W3A}	29.91±2.83	203.40±18.74*	71.35±5.71***
Profilin I^{R74E}	29.59±2.23	96.07±14.00	48.34±3.43***
Profilin I^{R136D}	52.58±3.46*	184.62±17.99*	46.25±3.57

Results of three experiments are presented as means ± s.e.m. (n=50 cells). *P<0.005 compared with neurite lengths of profilin I^{WT} cells; **P<0.01 compared with neurite lengths of parental PC12 cells; ***P<0.0001 compared with neurites in the same cell line without Y27632.

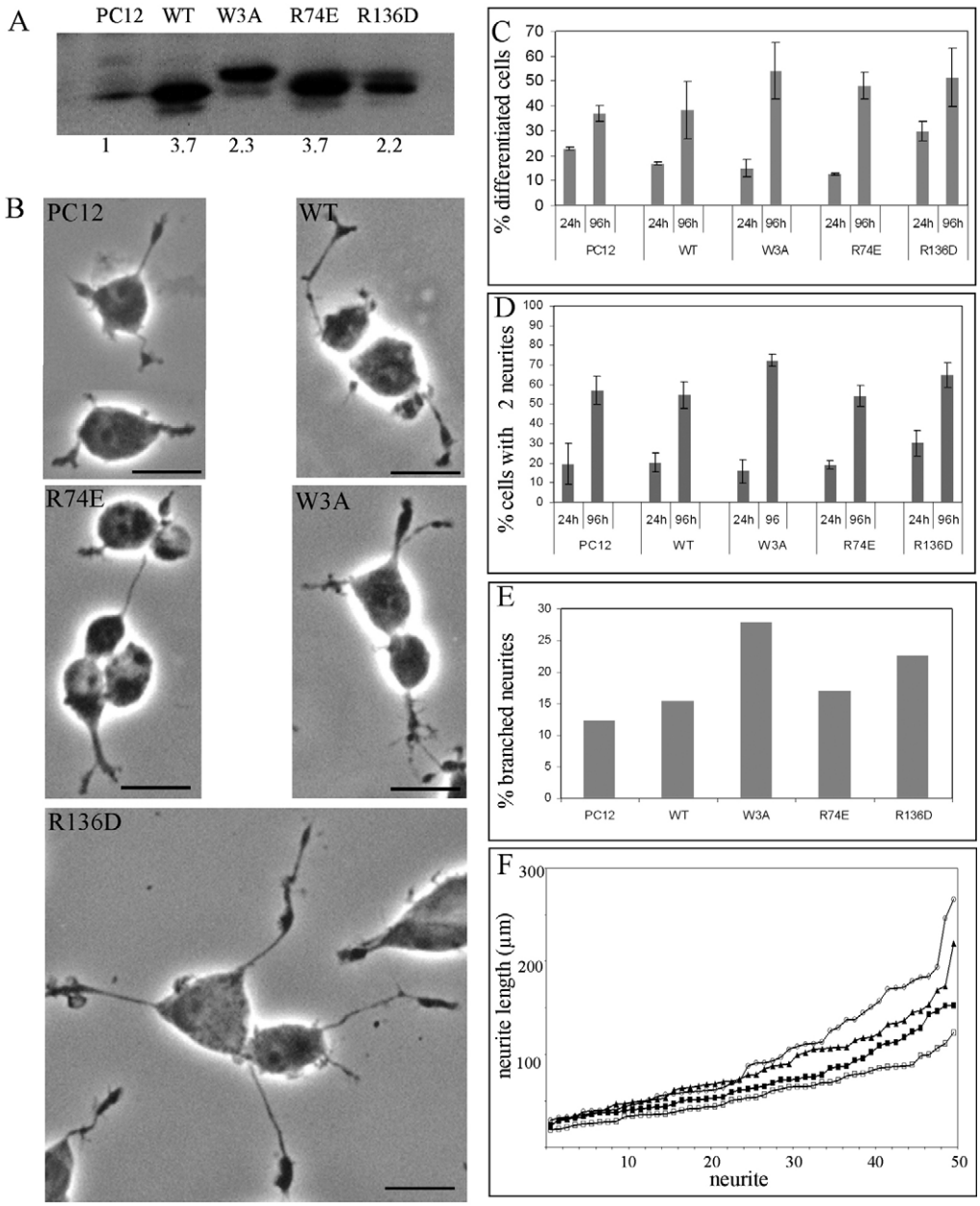

Fig. 3. Expression of WT and mutant human profilin I in PC12 cells: a quantitative analysis of neuronal differentiation. (A) Western blot showing the expression levels of human WT and mutant profilins in the stably transfected PC12 cells. The levels were quantified by densitometry of the blot. The apparent higher molecular weight of the profilin I^{W3A} mutant is also observed for the purified recombinant protein and is probably due to the introduced substitution (data not shown). (B) Representative images of the cell lines used in this study. The cells were grown on collagen-coated plastic and stimulated with NGF/forskolin for 24 hours. The profilin I^{R136D}-expressing cells are visibly more differentiated. Bars, 20 μm. (C) Percentage of differentiated cells at 24 and 96 hours after NGF/forskolin stimulation. Results of two independent experiments are presented as means ± s.e.m.; *n*=317-355 cells. (D) Percentage of differentiated cells with two or more neurites at different time points after the addition of NGF/forskolin. Results of three independent experiments are presented as means ± s.e.m.; *n*=355-404 cells. (E) Percentage of neurites with branches, 96 hours after the addition of NGF/forskolin. *n* ranges from 136 to 159 cells, covering two independent experiments. (F) Profilin I^{R136D} and profilin I^{W3A} cells have longer neurites and profilin I^{R74E} cells have shorter neurites from profilin I^{WT}. The length of the longest neurite per cell (*n*=50 cells) was measured 96 hours after the start of differentiation and these lengths were plotted from short to long. The neurites can be divided into two populations: short – newly formed neurites up to 80 μm; and long – established neurites longer than 80 μm. The difference between the cell lines is most obvious in the population of longer neurites. ■, profilin I^{WT}; ▲, profilin I^{W3A}; □, profilin I^{R74E}; ○, profilin I^{R136D}.

PC12 differentiation, except that these cells have an increased neurite output.

Disrupting different ligand interactions in profilin I influences different aspects of neurite outgrowth

We previously described point mutations of human profilin I disrupting the actin, poly-proline and/or PtdIns(4,5)P_2 binding sites (Lambrechts et al., 2002). The profilin I^{W3A} mutant lacks polyproline binding, whereas the profilin I^{R74E} mutation abolishes the interaction with actin. The third mutant used in this study (profilin I^{R136D}), has reduced affinity for polyproline and strongly reduced affinity for PtdIns(4,5)P_2. We created PC12 cell lines stably expressing each of these mutants to levels comparable with the reference profilin I^{WT} cell line (Fig. 3A). The subcellular distribution of fluorescent fusion proteins of the mutants was not significantly different from profilin I^{WT} (Fig. 1 and data not shown).

The cells were plated on collagen-coated plastic dishes and stimulated with NGF and forskolin. Pictures were taken 24 and 96 hours after the addition of NGF and forskolin. Representative images for each of the cell lines are shown in Fig. 3B. After 24 hours, we did not observe much difference between the parental PC12 cells and cells expressing WT, W3A and R74E profilin I. By contrast, the profilin I^{R136D}-expressing cells were visibly more differentiated with more and longer neurites (Fig. 3B and Table 1). Quantitative analysis of the 24-hour time point confirms that the profilin I^{R136D} cells are more differentiated and revealed that the profilin I^{R74E} cells

were slightly less differentiated than profilin I^{WT} cells (Fig. 3C). After 96 hours, the profilin I^{W3A} cells were also more differentiated than profilin I^{WT} cells (Fig. 3C). Similar but more pronounced differences are observed when we determined the percentage of differentiated cells having two or more neurites (Fig. 3D). Whereas only 8% of the profilin I^{WT} cells have two or more neurites, more than 18% of the profilin I^{R136D} cells develop at least two neurites after 24 hours of NGF/forskolin stimulation. After 96 hours of NGF/forskolin addition, 30% of the profilin I^{WT} cells develop two or more neurites. By contrast, 53% and 47% of the profilin I^{W3A} and profilin I^{R136D} cells, respectively, display two or more neurites per cell.

In later stages of the differentiation process, neurites develop branches. We scored and calculated the percentage of neurites that were branched at least once 96 hours after NGF/forskolin addition (Fig. 3E). Whereas overexpression of WT or R74E profilin I had no significant effect on branching, both the profilin I^{W3A} and profilin I^{R136D} mutants strongly increased the percentage of branched neurites (Fig. 3E) and the number of branches per neurite (data not shown).

Since it is clear from the images that the profilin I^{R136D} cells develop more and longer neurites, we determined the mean neurite output (i.e. total neurite length per soma). Indeed, 24 hours after the addition of NGF, the profilin I^{R136D} cells have a significantly longer neurite output than the profilin I^{WT} cells (Table 1). By 96 hours after NGF/forskolin addition, the profilin I^{W3A} and profilin I^{R136D} cells also showed significant longer neurite output than the profilin I^{WT} cells. By contrast, neurites of the profilin I^{R74E}-expressing cells are systematically shorter than the neurites of the profilin I^{WT}-expressing cells (Fig. 3F), suggesting that actin-profilin interaction is necessary for neurite elongation.

To summarize, we do not observe major defects in the cells expressing the profilin I^{R74E} actin-binding mutant, except that the neurites of these cells are shorter. The profilin I^{W3A} mutant, defective in binding proline-rich ligands, stimulated neurite outgrowth and maturation at later stages (96 hours). A similar stimulatory phenotype was observed for the profilin I^{R136D} mutant that is partially defective in binding to poly-L-proline and strongly defective in PtdIns(4,5)P_2 binding. The phenotype of the profilin I^{R136D} cells was, however, much stronger as these cells showed more and longer neurites and branches already after 24 hours of differentiation. These data suggest that the interaction of profilin I with both proline-rich proteins and PtdIns(4,5)P_2 are crucial for correct neuritogenesis.

Profilin I^{R136D} facilitates neurite outgrowth even in the absence of NGF

The fact that PC12 cells expressing profilin I^{R136D} differentiate more quickly after NGF addition, led us to ask whether the profilin I^{R136D}-expressing cells develop neurites in the absence of NGF stimulation. We plated profilin I^{WT}- and profilin I^{R136D}-expressing cells on collagen. Fig. 4 shows representative images of both cell lines. The profilin I^{R136D} cells are more spread than the profilin I^{WT} profilin cells and many cells extend short neurites. Although a few of the profilin I^{WT} cells also show extensions, these do not seem to elongate further. These data suggest that the profilin I^{R136D} mutant facilitates neurite outgrowth in the absence of NGF, possibly because it is not fully regulated by PtdIns(4,5)P_2 and proline-rich ligands.

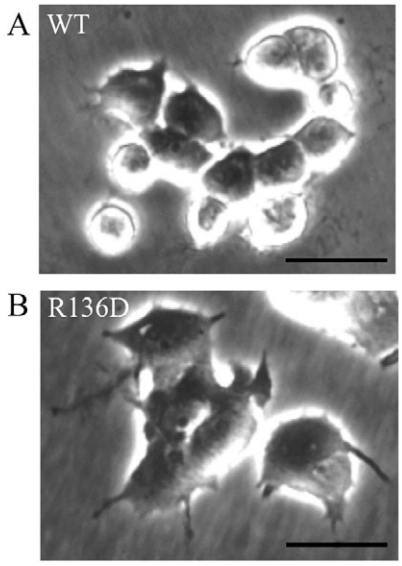

Fig. 4. Profilin I^{R136D} facilitates neurite outgrowth without NGF/forskolin stimulation. Profilin I^{WT} (A) and profilin I^{R136D} (B) cells were plated on collagen for 36 hours without adding NGF/forskolin to the medium. The profilin I^{WT} cells are less spread and do not extend neurites. The profilin I^{R136D} cells are well spread and several of them extend neurites. Bars, 25 μm.

A role for profilin I in filopodia formation

Next, we analyzed whether overexpression of WT and mutant profilin I has an effect on the F-actin organization. PC12 cells were plated on poly-D-lysine-coated coverslips, differentiated with NGF and then stained with fluorescently labeled phalloidin. The parental PC12 cells have several F-actin-rich sprouts around the cell body that may eventually become new neurites, and some thin filopodia along the neurite shafts (Fig. 5A). The profilin I^{WT} cells, however, have long, thin filopodia over the entire cell body and along the neurite shaft. Similar, but less-pronounced phenotypes were seen when the cells were plated on collagen (data not shown). The profilin I^{W3A}- and profilin I^{R136D}-expressing cells have multiple sprouting neurites around the cell body, consistent with the fact that these cells develop more neurites than the profilin I^{WT} cells. The profilin I^{W3A} and profilin I^{R136D} cell lines extend filopodia, however, the network of filopodia appears less dense compared with the profilin I^{WT} cells, and individual filopodia are visibly thicker. Interestingly, the profilin I^{R74E} mutant displayed few sprouting sites and few or no filopodia along the neurite shafts, consistent with its lower ability to differentiate and develop neurites.

CAD cells transiently expressing WT and mutant profilin I, show similar phenotypes (Fig. 5B). Overexpression of profilin I^{WT} seems to increase the F-actin-rich spots in the soma and these cells develop more irregularly arranged filopodia. By contrast, the profilin I^{R74E} cells show a decrease in cortical F-actin around the cell body and the appearance of only small F-actin-rich spots. Filopodia formation is decreased in these cells. Cells expressing profilin I^{R136D} and profilin I^{W3A} develop very long filopodia along the neurite shafts. In some cells expressing profilin I^{W3A}, we observed extensive regions along the neurite shafts that develop a ridge of filopodia close to each other.

These data demonstrate that profilin I, together with its ligands, is involved in the regulation of filopodia formation in neuronal cells.

Profilin I^{R136D} mutant cells do not respond to inhibition of ROCK activity

Changes in actin dynamics during neurite outgrowth are governed by multiple signal transduction pathways, many of which converge on the Rho GTPases (Sebok et al., 1999; Nikolic, 2002). Constitutive active RhoA causes neurite retraction, while inhibition of RhoA enhances neurite extension (Jalink et al., 1994; Kozma et al., 1997). A downstream effector of RhoA is ROCK, which is responsible for many of the effects of RhoA on neuritogenesis, since ROCK controls many downstream proteins, such as LIM-Kinase, PtdIns 5-kinase (Yamazaki et al., 2002) and profilin IIa (Da Silva et al., 2003), in turn regulating the actin cytoskeleton. Inhibition of ROCK activity with Y27632, results in an increased level of differentiation and longer neurites (Ishizaki et al., 2000). This is reminiscent of the phenotype we observed for the profilin I^{R136D} cells. Since this mutant is defective in PtdIns(4,5)P_2 binding and since ROCK may regulate PtdIns(4,5)P_2 levels through PtdIns 5-kinase (Yamazaki et al., 2002), we investigated the effect of ROCK inhibition on neurite outgrowth and differentiation in the parental cells and cells overexpressing WT and mutant profilin I. Cells were plated on collagen in duplicate. We added 20 μg/ml Y27632 to one dish, 15 minutes prior to supplementing NGF and forskolin to both dishes. The cells were allowed to differentiate for 24 hours, the time period in which the inhibitor is most active. Representative images of the different cell lines are shown (Fig. 6A). ROCK inhibition of PC12 cells increases the percentage of differentiated cells (Fig. 6B) as well as neurite length and total neurite output (Fig. 6C and Table 1). In addition, branching is increased fivefold (Fig. 6D). Thus, ROCK inhibition with Y27632 strongly stimulates neuronal differentiation of PC12 cells. Next we examined the effect of ROCK inhibition on the profilin-overexpressing cell lines. All cells, except for the profilin I^{R136D} cell line, were significantly more differentiated when treated with the ROCK inhibitor Y27632 (Fig. 6B). ROCK inhibition also strongly increased neurite length (Fig. 6C and Table 1) except for the profilin I^{R136D} cell line where the increases were statistically not significant. Also branching, which at this time point is hardly observable in the absence of ROCK inhibitor, augments dramatically for the profilin I^{WT} and profilin I^{W3A} cells, whereas the increase is moderate but still significant for the cells expressing profilin I^{R74E}. By contrast, no significant increase in branching is observed for the profilin I^{R136D} cells. These data indicate that the profilin I^{R136D} mutant inhibits the effects of the ROCK inhibitor or that these cells have lost their responsiveness to inhibition of the ROCK-signaling pathway in PC12 cells, suggesting that the profilin-I-PtdIns(4,5)P_2 interaction downstream of ROCK is important in neurite formation.

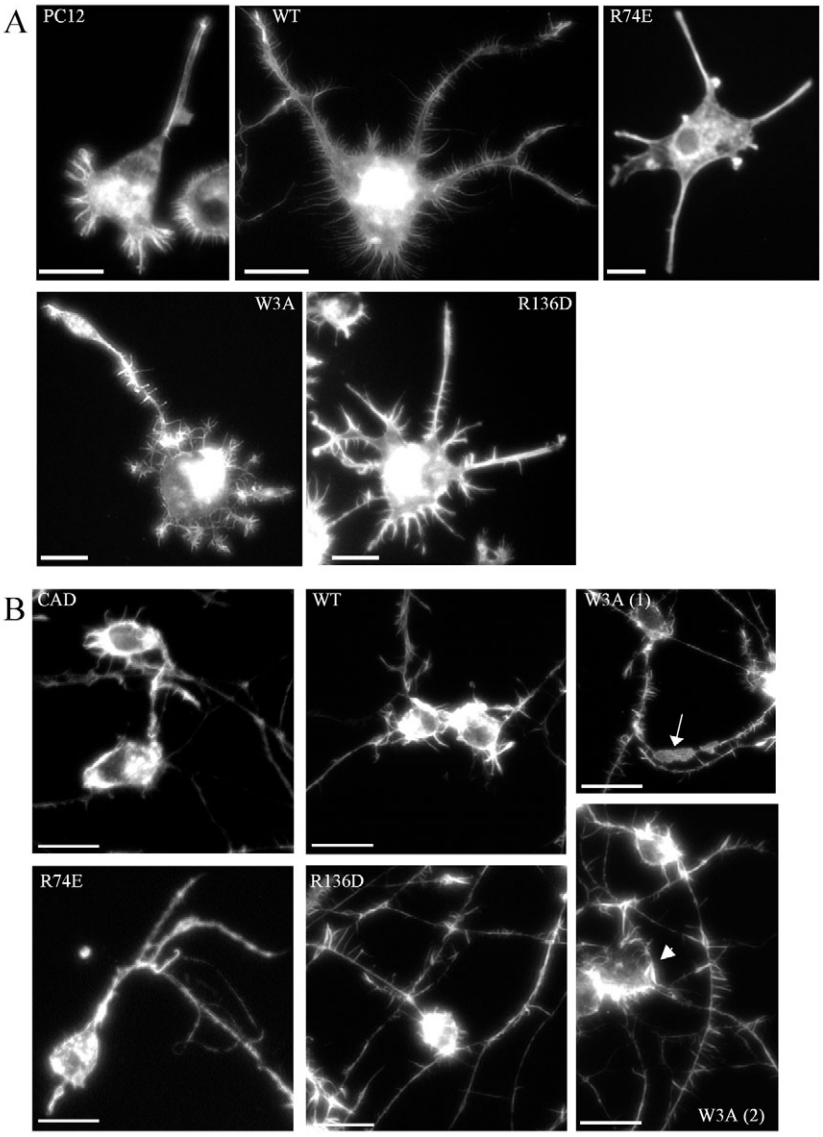

Fig. 5. Phalloidin staining of cells expressing WT and mutant profilin I. (A) PC12 cells were plated on poly-D-lysine-coated coverslips and F-actin was stained 60 hours after treatment with 20 ng/ml NGF and 10 μM forskolin. Note that neurite outgrowth on poly-D-lysine is slower than on collagen, resulting in fewer and shorter neurites (compare with Fig. 3A). (B) CAD cells were transiently transfected with IRES-GFP constructs of profilin I WT and mutants. All cells shown are transfected based on the GFP signal (not shown), except for one non-transfected cell in W3A(2), which is indicated with an arrowhead. The arrow in W3A(1) indicates a ridge of densely packed filopodia. Bars, 25 μm (A); 50 μm (B).

Discussion

The presence of profilin I and IIa in neuronal tissues and differentiated cells has been demonstrated (Faivre-Sarrailh et al., 1993; Kneussel and Betz, 2000; Lambrechts et al., 2000a; Ackermann and Matus, 2003; Neuhoff et al., 2005). The neuronal-specific isoform, profilin IIa, was recently shown to have a role in neurite formation (Da Silva et al., 2003). Genetic

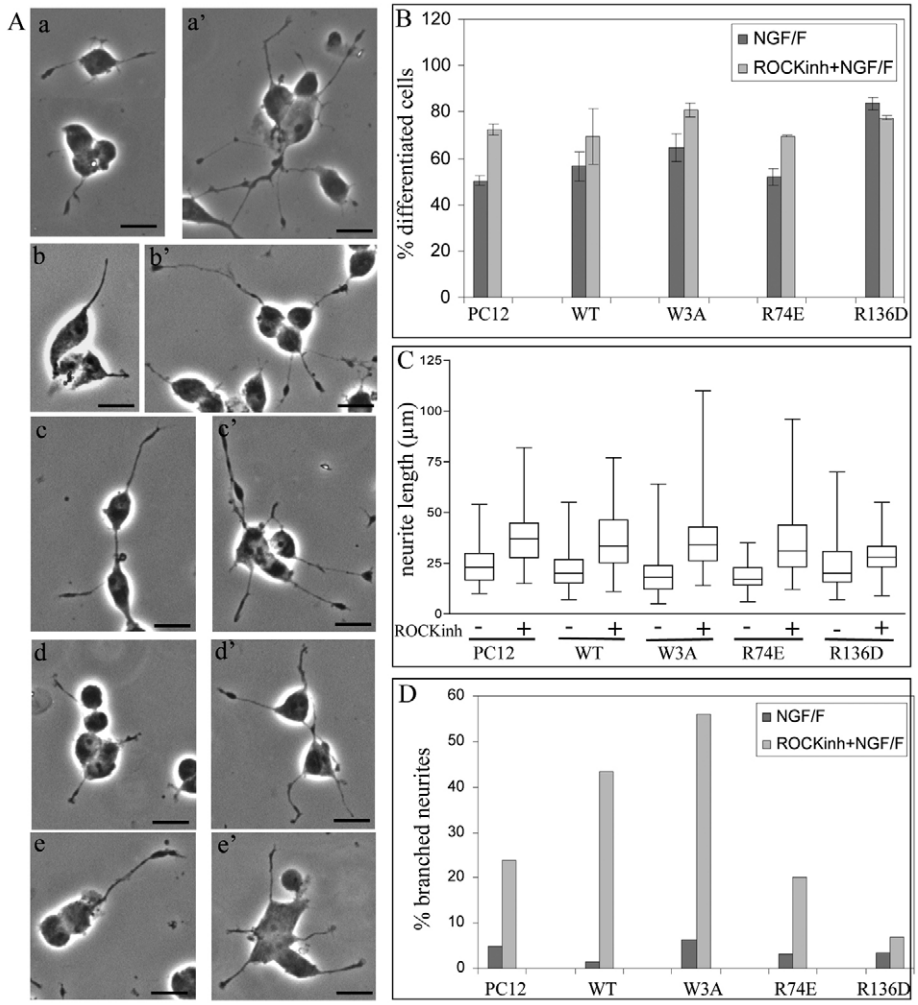

Fig. 6. Modulation of neurite outgrowth by inhibition of ROCK. (A) Cells were grown on collagen-coated coverslips and stimulated with NGF/forskolin for 24 hours without (a-e) or with prior incubation of 20 μg/ml ROCK inhibitor Y27632 for 15 minutes (a'-e'). a,a', parental PC12 cells; b,b', profilin I^{WT}; c,c', profilin I^{W3A}; d,d', profilin I^{R74E}; e,e', profilin I^{R136D}. Bars, 20 μm. The percentage of differentiated cells (B), the neurite length (C) and the percentage of branched neurites (D) after 24 hours were determined for cells treated with NGF/forskolin with or without Y27632. (B) All cell lines, except profilin I^{R136D} cells, have increased percentages of differentiated cells when treated with the ROCK inhibitor. Results of two independent experiments are presented as means ± s.e.m. (n=300 cells). (C) Treatment with the ROCK inhibitor enhances neurite elongation except for profilin I^{R136D} cells. The lengths of the longest neurite per cell (n=60) are displayed in box-and-whisker plots. + and – indicate the presence or absence, respectively, of Y27632 in the medium. (D) There was a strong increase in the percentage of branched neurites in PC12, profilin I^{WT}, profilin I^{W3A} and profilin I^{R74E} cells, but not in profilin I^{R136D}, in the presence of Y27632 (n=65-70 neurites).

evidence implies that profilin I functions in neuronal outgrowth in mice (Lanier et al., 1999) and combined knockdown of both profilin I and IIa in PC12 cells inhibits neurite outgrowth (Sharma et al., 2005), suggesting that profilin I also has a role in neuritogenesis. The subcellular localization of profilin I in PC12 cells shows that the protein localizes to neuronal sprouts and growth cones, regions that are actively protruding during neuritogenesis and neuronal differentiation (Fig. 1). The contribution of profilin I to various aspects of neurite formation, however, remained unclear. We therefore investigated whether profilin I contributes to PC12 differentiation and which ligand interactions are involved in this process. Only when human profilin I levels were increased to 80-fold over the endogenous rat profilin I level, was a significant decrease in the percentage of differentiated cells observed. We assume that at this high concentration profilin mainly functions as a sequestering agent, preventing actin polymerization and subsequently neurite outgrowth. By contrast, a threefold overexpression level of profilin I^{WT} does not increase the extent of differentiation of the PC12 cells, nor the number of neurites developed per cell, nor the number of branches formed per neurite (Fig. 3 and Table 2). Total neurite output is, however, increased in these cells, as well as filopodia formation. These results strongly contrast with those obtained for overexpression of profilin IIa in primary rat hippocampal neurons. This isoform reduces neurite number and length (Da

Table 2. Summary of results obtained with the various profilin mutants

	% Differentiation		Number of neurites		% Branched neurites	Neurite output		
	24 hours	96 hours	24 hours	96 hours	96 hours	24 hours	96 hours	Neurite length
Profilin I^{WT}*	=	=	=	=	=	=	↑	NT
Profilin I^{W3A}†	=	↑↑	↓	↑↑	↑↑	=	↑↑	↑
Profilin I^{R74E}†	=	=	=	=	=	=	=	↓
Profilin I^{R136D}†	↑	↑↑	↑	↑↑	↑	↑	↑↑	↑↑

*Profilin I^{WT}-expressing cells are compared with the parental PC12 cells. †The mutant profilin I cell lines W3A, R74E and R136D are compared with the Profilin I^{WT}-expressing cells. NT, not tested; =, no change; ↑↑, greatly increased; ↑, slightly increased; ↓, slightly decreased.

Silva et al., 2003), suggesting that profilin I and IIa have (partly) opposing roles in neuritogenesis. Similar to our results in PC12 cells, profilin I overexpression in NIE-115 cells did not significantly change the rate of differentiation (Suetsugu et al., 1998). Apparently, moderately increased profilin levels do not dramatically disturb the dynamic actin turnover, required for efficient neurite extension. One possible mechanism for compensation could be by changing actin levels, to restore the balance between profilin, actin and the profilin-actin complex. Quantification of total and F-actin levels, however, did not reveal consistent changes (data not shown).

Remarkably, overexpressing the mutant profilins at low levels does affect one or more aspects of differentiation (Table 2). The profilin I^{R74E} cells extend shorter neurites and form fewer neurite sprouts than the profilin I^{WT}-expressing cells. The cells also display greatly reduced numbers of filopodia along the cell body and neurite shafts. The latter observations are consistent with the role of N-WASP-profilin-I complexes in microspike formation in COS7 cells, where a profilin mutant that lacks actin binding suppresses microspike formation (Suetsugu et al., 1998). When treated with the ROCK inhibitor, the profilin I^{R74E} cells develop fewer branches than the profilin I^{WT} and profilin I^{W3A} cells, suggesting that the actin-profilin complex is necessary to drive this aspect of neuronal differentiation. It is, however, surprising that for the profilin I^{R74E} mutant we did not observe a dominant-negative effect on neurite outgrowth. The main reason for this may be that there is still a significant amount of endogenous profilin I^{WT} in these cells. We performed RNA interference (RNAi) of the endogenous rat profilin I in the profilin I^{R74E} cells. The cells, however, detached from the substrate (data not shown), indicating that the profilin-actin interaction is crucial for maintenance of cell adhesion and perhaps even cell survival and that profilin I^{R74E} cannot rescue this. Therefore the detailed direct contribution of the profilin-actin interaction on early neuritogenesis remains to be elucidated.

The profilin I^{W3A} and profilin I^{R136D} mutants are entirely and partly defective in poly-L-proline binding, respectively. In addition, the profilin I^{R136D} mutant displays a strongly reduced interaction with PtdIns(4,5)P_2 (Lambrechts et al., 2002). Both mutants exert dominant effects on neuritogenesis. The cells are more differentiated and develop more and longer neurites. In addition, the neurites of both profilin I^{W3A} and profilin I^{R136D} cells develop significantly more branches. There is, however, an important difference between the two cell lines. While the effects in the profilin I^{W3A} mutant are observed only at later time points (96 hours), differentiation of the profilin I^{R136D} mutant cells is already visible after 24 hours (Fig. 3) and even occurs in the absence of NGF (Fig. 4). These data indicate that interaction with both PtdIns(4,5)P_2 and proline-rich partners are involved in neurite formation and that the former interaction is important at earlier phases of the process.

Withdrawal of the proline-rich Ena/VASP proteins to the mitochondria decreases the number of neurites formed (Lebrand et al., 2004), suggesting that these proteins have a positive effect on neurite formation. Similarly, it is interesting to note that another proline-rich profilin partner mDia1, localizes at the emerging neurite bud of cerebellar granule cells (Arakawa et al., 2003). It was, however, unexpected to discover that disrupting the profilin poly-L-proline interaction results in longer neurites. This observation appears to disagree with the general notion that polyproline proteins serve as a sink for profilin-actin complexes to make them readily available for elongation of actin filaments (Holt and Koffer, 2001). Our results suggest that profilin I has an inhibitory modulatory role on its polyproline-containing ligands in an environment where profilin-actin complexes are available for elongation. Alternatively, the proline-rich ligand may place a restriction on profilin-actin activity. Although at present it is unknown which proline-rich ligand is involved in which particular process, it is clear that when the poly-proline interaction is broken, both the profilin-actin complexes and the proline-rich ligands are free to promote actin polymerisation and neurite elongation.

Interestingly, the profilin I^{R136D} cells develop multiple neurites earlier than profilin I^{W3A} cells. Upon NGF stimulation of PC12 cells, phospholipase C (PLC) activation results in PtdIns(4,5)P_2 hydrolysis, an event thought to free profilin from the membrane and allowing profilin to interact with actin (Goldschmidt-Clermont et al., 1990) and its proline-rich ligands (Lambrechts et al., 1997). Since the profilin I^{R136D} mutant will be much less inhibited by PtdIns(4,5)P_2 than profilin I^{WT} this mutant may have more opportunity to interact with actin and to increase actin polymerization, which is required for neurite outgrowth. Therefore profilin I^{R136D} cells may have an advantage at the onset of neuritogenesis. Our observation that these cells form neurites without NGF stimulation, supports this.

On the other hand, the profilin I^{R136D} mutant will be less capable of protecting PtdIns(4,5)P_2 from activated PLCγ1-mediated hydrolysis, which has also been linked to a decrease in the apparent membrane tension in fibroblasts and a resulting increase in the lamellipodial extension rate (Raucher et al., 2000). Also in neuronal cells PtdIns(4,5)P_2 hydrolysis, downstream of NGF, may be necessary to lower membrane tension locally and to facilitate neurite sprouting. The latter process depends on actin polymerization and may be (part of) the reason why the profilin I^{R136D} cells differentiate to some extent without NGF. Our observation that profilin I^{R74E}, incapable of actin binding, decreases sprouting, suggests actin-profilin complexes are actively involved in this process.

The Rho-ROCK pathway is known as a repulsive pathway for neurite outgrowth. Constitutive active Rho or ROCK inhibits neurite formation (Katoh et al., 1998) and inhibition of ROCK with its specific inhibitor Y27632 results in increased neuronal differentiation of PC12 cells (Ishizaki et al., 2000). We propose a model for how profilin I and IIa action may be regulated by PtdIns(4,5)P_2 and the Rho-ROCK pathway (Fig. 7). In the early phase of NGF stimulation, PLCγ1 is activated, causing a local drop in PtdIns(4,5)P_2 levels, and the Rho-ROCK pathway is inhibited (Kim et al., 1991; Sebok et al., 1999). Profilin I and IIa, amongst many other proteins, are free to interact with actin (and their proline-rich ligands), thereby stimulating neurite formation. At later stages, Rho becomes active again, creating a balance between neurite outgrowth and retraction, and forms a complex with ROCK and profilin IIa (Da Silva et al., 2003). Recently it was shown that PtdIns 5-kinase increases PtdIns(4,5)P_2 levels upon ROCK activation, resulting in a stabilized cortical actin network and increased membrane tension and subsequently inhibition of neurite outgrowth (Yamazaki et al., 2002). It is tempting to speculate that this PtdIns(4,5)P_2 increase may be a signal to inactivate part of the profilin I population locally, by recruiting profilin I

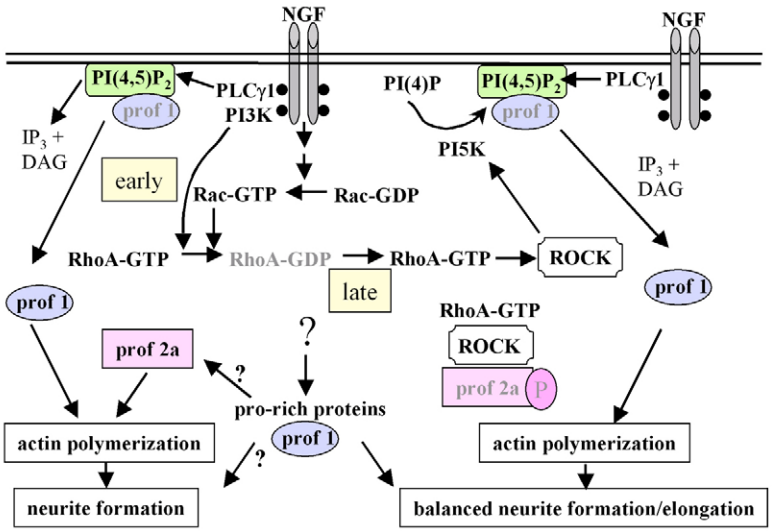

Fig. 7. Model for the regulation of profilin-I-actin interaction by PtdIns(4,5)P_2 during neurite formation and growth. NGF stimulation locally activates PLCγ1, causing PtdIns(4,5)P_2 hydrolysis into inositol 3-phosphate (IP_3) and diacylglycerol (DAG), and release of profilin I (and perhaps also profilin IIa), that can now interact with actin and stimulate neurite formation. In this early stage, RhoA is inactivated through Rac and phosphoinositide 3-kinase (PI3K). At later stages, RhoA becomes active again, thereby activating ROCK and forming a complex with profilin IIa, which is now inactivated. ROCK also activates PtdIns 5-kinase (PI5K), which again increases the local PtdIns(4,5)P_2 levels, resulting in inactivation of profilin I. Continuous stimulation of the NGF-receptor starts the PtdIns(4,5)P_2 hydrolysis cycle again, and creates a balanced level of free profilin I, which may then contribute to neurite elongation. Where inactive, proteins are shown in grey lettering.

molecules again to the membrane. Under continuous stimulation with NGF, this cycle of PtdIns(4,5)P_2 hydrolysis and formation is continuously repeated, constantly creating a level of active profilin I and a balance between neurite sprouting and elongation. Because the profilin I^{R136D} mutant has low affinity for PtdIns(4,5)P_2 it may be ineffective to respond to this signaling pathway and to the changing PtdIns(4,5)P_2 levels. Thus, the profilin I^{R136D} cell line may have more actin-profilin complexes to fuel actin polymerization and this may explain why they have longer neurites and more branches. The regulation of neurite elongation and branching, however, is probably more complex because the poly-L-proline-binding capacity of profilin I is also required for these aspects of neurite formation.

In conclusion, the data presented here provide evidence for multiple roles of profilin I in neurite outgrowth and for its requirement to interact with its three types of ligands: actin, proline-rich proteins and PtdIns(4,5)P_2. The profilin-PtdIns(4,5)P_2 interaction is of major importance in the regulation of early neurite formation, whereas this ligand and, as yet unidentified, proline-rich ligands appear implicated in balanced elongation and branching of neurites at later stages. In addition, our data suggest that profilin I functions downstream of the Rho-activation pathway in neuritogenesis but may do so in a different way than profilin IIa, which was shown to interact directly with ROCK.

Materials and Methods

Cell culture and transfection

PC12 teton cells (Clontech, Palo Alto, CA) were grown in suspension in DMEM supplemented with 1% horse serum and 100 μg/ml neomycin. For creation of stable cell lines, wild-type and mutant human profilin I cDNAs (Lambrechts et al., 2002) were cloned in the pTRE2-hyg plasmid and electroporated in the PC12 teton cells. After selection we observed leaky profilin expression independently of addition of tetracycline in all cell lines. Therefore we did not use this inducing agent, but rather selected cell lines with appropriate expression levels. The profilin expression levels of hygromycin-resistant clones were quantified by western blotting using an affinity-purified chicken polyclonal profilin I antibody (#1941; produced by Aves Lab, Oregon) and an anti-chicken IgY secondary antibody (Jackson Immunoresearch Lab, UK).

Culturing of the CNS catecholaminergic CAD cells was performed as described (Qi et al., 1997). For transient expression in PC12 or CAD cells, profilin cDNA was cloned in either pEYFP-C1 (BD-Biosciences, Palo Alto, CA) or pIRES2-EGFP (Clontech, Palo Alto, CA), respectively. Lipofection was carried out using Fugene6 (Roche, Belgium).

Differentiation and analysis

PC12 cells respond to nerve growth factor (NGF) with dramatic morphological changes including long, branched neurites and acquire a number of properties characteristic of mature sympathetic neurons (Greene and Tischler, 1976). For neuronal differentiation, PC12 cells were plated on 10 μg/cm^2 collagen type I (BD Biosciences, Palo Alto, CA) or 6 μg/cm^2 poly-D-lysine (Sigma, St Louis, MO) in RPMI1640 with 1% horse serum. When the cells were adherent, differentiation was induced by adding 10-20 ng/ml NGF (rat recombinant NGF, R&D systems, Minneapolis) and 10 μM forskolin (Sigma, St Louis, MO). The cells were analyzed by phase-contrast microscopy at different time points after the start of differentiation. Cells were considered differentiated when they had at least one neurite that is longer than the diameter of the cell. Statistical analysis of neurite lengths was performed with Graphpad Prism. When required, the ROCK inhibitor Y27632 (Calbiochem, VWR Belgium) was added 15 minutes prior to the addition of NGF/forskolin. Since the inhibitor is not active over longer periods, cells were in this case only monitored after 24 hours.

Confocal fluorescence microscopy

Cells were plated on poly-D-lysine- or collagen-coated coverslips, allowed to differentiate and fixed with 4% paraformaldehyde in phosphate-buffered saline, blocked with 10% BSA and permeabilized with 0.1% Triton X-100. F-actin was visualized with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, OR). Images were captured with a Nikon Eclipse TE300 epifluorescence microscope, equipped with a Bio-Rad Radiance 2000 confocal laser system. Image acquisition was performed using the Lasersharp2000 software and images were processed further with ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/).

We are very grateful to Lorene Lanier for helpful comments on the manuscript and Patrick Van Oostveldt for the use of his confocal microscopy facility (Ghent University, Faculty of Bio-engineer Sciences). A.L. is post-doctoral fellow of F.W.O.-Vlaanderen. This work was supported by BOF-GOA 2051401 to J.V. and C.A., F.W.O. grant G.0007.03 and a grant of the 'Geneeskundige Stichting Koningin Elisabeth' to C.A.

References

Ackermann, M. and Matus, A. (2003). Activity-induced targeting of profilin and stabilization of dendritic spine morphology. *Nat. Neurosci.* **6**, 1194-1200.

Arakawa, Y., Bito, H., Furuyashiki, T., Tsuji, T., Takemoto-Kimura, S., Kimura, K., Nozaki, K., Hashimoto, N. and Narumiya, S. (2003). Control of axon elongation via an SDF-1alpha/Rho/mDia pathway in cultured cerebellar granule neurons. *J. Cell Biol.* **161**, 381-391.

Carlsson, L., Nystrom, L. E., Sundkvist, I., Markey, F. and Lindberg, U. (1977). Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* **115**, 465-483.

Da Silva, J. S. and Dotti, C. G. (2002). Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. *Nat. Rev. Neurosci.* **3**, 694-704.

Da Silva, J. S., Medina, M., Zuliani, C., Di Nardo, A., Witke, W. and Dotti, C. G. (2003). RhoA/ROCK regulation of neuritogenesis via profilin IIa-mediated control of actin stability. *J. Cell Biol.* **162**, 1267-1279.

Faivre-Sarrailh, C., Lena, J. Y., Had, L., Vignes, M. and Lindberg, U. (1993). Location of profilin at presynaptic sites in the cerebellar cortex; implication for the regulation

of the actin-polymerization state during axonal elongation and synaptogenesis. *J. Neurocytol.* **22**, 1060-1072.

Goldschmidt-Clermont, P. J., Machesky, L. M., Baldassare, J. J. and Pollard, T. D. (1990). The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. *Science* **247**, 1575-1578.

Goldschmidt-Clermont, P. J., Furman, M. I., Wachsstock, D., Safer, D., Nachmias, V. T. and Pollard, T. D. (1992). The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. *Mol. Biol. Cell* **3**, 1015-1024.

Greene, L. A. and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424-2428.

Holt, M. R. and Koffer, A. (2001). Cell motility: proline-rich proteins promote protrusions. *Trends Cell Biol.* **11**, 38-46.

Ishizaki, T., Uehata, M., Tamechika, I., Keel, J., Nonomura, K., Maekawa, M. and Narumiya, S. (2000). Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol. Pharmacol.* **57**, 976-983.

Jalink, K., van Corven, E. J., Hengeveld, T., Morii, N., Narumiya, S. and Moolenaar, W. H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J. Cell Biol.* **126**, 801-810.

Kang, F., Purich, D. L. and Southwick, F. S. (1999). Profilin promotes barbed-end actin filament assembly without lowering the critical concentration. *J. Biol. Chem.* **274**, 36963-36972.

Katoh, H., Aoki, J., Ichikawa, A. and Negishi, M. (1998). p160 RhoA-binding kinase ROKalpha induces neurite retraction. *J. Biol. Chem.* **273**, 2489-2492.

Kim, U. H., Fink, D., Jr, Kim, H. S., Park, D. J., Contreras, M. L., Guroff, G. and Rhee, S. G. (1991). Nerve growth factor stimulates phosphorylation of phospholipase C-gamma in PC12 cells. *J. Biol. Chem.* **266**, 1359-1362.

Kneussel, M. and Betz, H. (2000). Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: the membrane activation model. *Trends Neurosci.* **23**, 429-435.

Kozma, R., Sarner, S., Ahmed, S. and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol. Cell. Biol.* **17**, 1201-1211.

Lambrechts, A., Verschelde, J. L., Jonckheere, V., Goethals, M., Vandekerckhove, J. and Ampe, C. (1997). The mammalian profilin isoforms display complementary affinities for PIP2 and proline-rich sequences. *EMBO J.* **16**, 484-494.

Lambrechts, A., Braun, A., Jonckheere, V., Aszodi, A., Lanier, L. M., Robbens, J., Van Colen, I., Vandekerckhove, J., Fassler, R. and Ampe, C. (2000a). Profilin II is alternatively spliced, resulting in profilin isoforms that are differentially expressed and have distinct biochemical properties. *Mol. Cell. Biol.* **20**, 8209-8219.

Lambrechts, A., Kwiatkowski, A. V., Lanier, L. M., Bear, J. E., Vandekerckhove, J., Ampe, C. and Gertler, F. B. (2000b). cAMP-dependent protein kinase phosphorylation of EVL, a Mena/VASP relative, regulates its interaction with actin and SH3 domains. *J. Biol. Chem.* **275**, 36143-36151.

Lambrechts, A., Jonckheere, V., Dewitte, D., Vandekerckhove, J. and Ampe, C. (2002). Mutational analysis of human profilin I reveals a second PI(4,5)-P2 binding site neighbouring the poly(L-proline) binding site. *BMC Biochem.* **3**, 12.

Lanier, L. M., Gates, M. A., Witke, W., Menzies, A. S., Wehman, A. M., Macklis, J. D., Kwiatkowski, D., Soriano, P. and Gertler, F. B. (1999). Mena is required for neurulation and commissure formation. *Neuron* **22**, 313-325.

Lassing, I. and Lindberg, U. (1985). Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature* **314**, 472-474.

Laurent, V., Loisel, T. P., Harbeck, B., Wehman, A., Grobe, L., Jockusch, B. M., Wehland, J., Gertler, F. B. and Carlier, M. F. (1999). Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes. *J. Cell Biol.* **144**, 1245-1258.

Lebrand, C., Dent, E. W., Strasser, G. A., Lanier, L. M., Krause, M., Svitkina, T. M., Borisy, G. G. and Gertler, F. B. (2004). Critical role of Ena/VASP proteins for filopodia formation in neurons and in function downstream of netrin-1. *Neuron* **42**, 37-49.

Moseley, J. B., Sagot, I., Manning, A. L., Xu, Y., Eck, M. J., Pellman, D. and Goode, B. L. (2004). A conserved mechanism for Bni1- and mDia1-induced actin assembly and dual regulation of Bni1 by Bud6 and profilin. *Mol. Biol. Cell* **15**, 896-907.

Neuhoff, H., Sassoe-Pognetto, M., Panzanelli, P., Maas, C., Witke, W. and Kneussel, M. (2005). The actin-binding protein profilin I is localized at synaptic sites in an activity-regulated manner. *Eur. J. Neurosci.* **21**, 15-25.

Nikolic, M. (2002). The role of Rho GTPases and associated kinases in regulating neurite outgrowth. *Int. J. Biochem. Cell Biol.* **34**, 731-745.

Pantaloni, D. and Carlier, M. F. (1993). How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell* **75**, 1007-1014.

Qi, Y., Wang, J. K., McMillian, M. and Chikaraishi, D. M. (1997). Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. *J. Neurosci.* **17**, 1217-1225.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P. and Meyer, T. (2000). Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* **100**, 221-228.

Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B. M. and Walter, U. (1995). The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. *EMBO J.* **14**, 1583-1589.

Sebok, A., Nusser, N., Debreceni, B., Guo, Z., Santos, M. F., Szeberenyi, J. and Tigyi, G. (1999). Different roles for RhoA during neurite initiation, elongation, and regeneration in PC12 cells. *J. Neurochem.* **73**, 949-960.

Selden, L. A., Kinosian, H. J., Estes, J. E. and Gershman, L. C. (1999). Impact of profilin on actin-bound nucleotide exchange and actin polymerization dynamics. *Biochemistry* **38**, 2769-2778.

Sharma, A., Lambrechts, A., Hao, L., Le, T., Sewry, C., Ampe, C., Burghes, A. and Morris, G. (2005). A role for complexes of survival of motor neurons (SMN) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured nerve cells. *Exp. Cell Res.* **309**, 185-197.

Suetsugu, S., Miki, H. and Takenawa, T. (1998). The essential role of profilin in the assembly of actin for microspike formation. *EMBO J.* **17**, 6516-6526.

Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M. and Narumiya, S. (1997). p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* **16**, 3044-3056.

Witke, W. (2004). The role of profilin complexes in cell motility and other cellular processes. *Trends Cell Biol.* **14**, 461-469.

Yamazaki, M., Miyazaki, H., Watanabe, H., Sasaki, T., Maehama, T., Frohman, M. A. and Kanaho, Y. (2002). Phosphatidylinositol 4-phosphate 5-kinase is essential for ROCK-mediated neurite remodeling. *J. Biol. Chem.* **277**, 17226-17230.

Yang, C., Huang, M., DeBiasio, J., Pring, M., Joyce, M., Miki, H., Takenawa, T. and Zigmond, S. H. (2000). Profilin enhances Cdc42-induced nucleation of actin polymerization. *J. Cell Biol.* **150**, 1001-1012.