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Accumulation of profilin II at the surface of *Listeria* is concomitant with the onset of motility and correlates with bacterial speed

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SUMMARY

The spatial and temporal activity of the actin cytoskeleton is precisely regulated during cell motility by several microfilament-associated proteins of which profilin plays an essential role. We have analysed the distribution of green fluorescent protein (GFP)-tagged profilins in cultured and in *Listeria*-infected cells. Among the different GFP-profilin fusion proteins studied, only the construct in which the GFP moiety was fused to the carboxy terminus of profilin II (profilin II-GFP) was recruited by intracellular *Listeria*. The in vitro ligand-binding properties of this construct, e.g. the binding to monomeric actin, poly-L-proline and phosphatidylinositol 4,5-bisphosphate (PIP2), were unaffected by GFP. Profilin II-GFP co-localised with vinculin and Mena to the focal adhesions in REF-52 fibroblasts and was distributed as a thin line at the front of protruding lamellipodia in B16-F1 mouse melanoma cells. In *Listeria*-infected cells, profilin II-GFP was recruited, in an asymmetric fashion, to the surface of *Listeria* at the onset of motility whereas it was not detectable on non-motile bacteria. In contrast to the vasodilator-stimulated phosphoprotein (VASP), profilin II-GFP localised at the bacterial surface only on motile *Listeria*. Moreover, the fluorescence intensity of profilin II-GFP directly correlated with the speed of the bacteria. Thus, the use of GFP-tagged profilin II provides new insights into the role of profilins in cellular motility.

Key words: Profilin, *Listeria monocytogenes*, Focal contact, Actin dynamics, Ena/VASP protein

INTRODUCTION

The actin cytoskeleton is involved in the control of cell shape, polarity and motility in response to extra- and intracellular signals. Actin polymerisation itself is generally thought to be the driving force of cell motility, but many microfilament-associated proteins regulate polymerisation, supraorganisation and contraction of actin cytoskeletal structures (Small et al., 1999). *Listeria monocytogenes* and other bacterial and viral pathogens subvert the actin-based machinery and exploit the polymerisation of actin for intracellular movement and cell to cell spreading (Tilney and Portnoy, 1989; see Higley and Way, 1997; Dramsi and Cossart, 1998). This pathogen-controlled exploitation of the motile machinery of the host cell parallels in many aspects the events taking place at the plasma membrane during lamellipodia extension (see Higley and Way, 1997; Dramsi and Cossart, 1998), but the *Listeria* and *Shigella* systems seem to be restricted to fewer components (Loisel et al., 1999) and lack signal-mediated regulation (Ebel et al., 1999; Mounier et al., 1999).

Among the proteins involved in the regulation of the actin cytoskeleton dynamics, profilin was one of the first actin monomer sequestering proteins to be described (Carlsson et al., 1977). In mammals, two isoforms of profilin, profilin I and profilin II, have been identified. Profilin I is ubiquitously expressed and abundant, whereas profilin II is predominantly expressed in neuronal cells (Honore et al., 1993; Lambrechts et al., 1995, 1997; Witke et al., 1998; Giesemann et al., 1999). In vitro, profilins can either promote or inhibit actin polymerisation. They can act as actin monomer sequestering proteins causing actin depolymerisation or inhibition of actin assembly into filaments. On the other hand, profilins can promote actin polymerisation, which depends on the presence of other actin-binding proteins such as thymosin β4 and on the state of the barbed actin filament ends (Pantaloni and Carlier, 1993). In addition, they can synergise with the actin depolymerising factor (ADF)/cofilin to increase the actin filament turnover (Didry et al., 1998). Profilins also enhance the exchange rate of ADP to ATP after binding to monomeric actin (Goldschmidt-Clermont et al., 1991).

In addition to monomeric actin, profilins bind to PIP2 and poly-L-proline. The binding of profilin-actin complexes to PIP2 dissociates profilin from monomeric actin. Hence, the interaction between profilin and PIP2 is thought to be one of the strategies the cells use to link the actin cytoskeleton to external signals (Lassing and Lindberg, 1985, 1988;

Goldschmidt-Clermont et al., 1991; Machesky and Pollard, 1993). Several proteins that harbour poly-L-proline stretches are profilin ligands and some of these are associated with the actin cytoskeleton such as VASP (Reinhard et al., 1995; Kang et al., 1997), Mena (Gertler et al., 1996), p140mDia (Watanabe et al., 1997), and N-WASP (Suetsugu et al., 1998).

Profilins play an essential role in re-modelling the actin cytoskeleton. They have been localised in highly dynamic regions of cultured cells (Buß et al., 1992; Machesky et al., 1994; Neely and Macaluso, 1997). In addition, profilins are thought to stabilise specific dynamic F-actin structures (Finkel et al., 1994; Rothkegel et al., 1996). *Dictyostelium discoideum* mutants lacking both profilin isoforms identified in these cells show increased actin filament levels and defects in motility and cytokinesis (Haugwitz et al., 1994), whereas profilin I knock-out mice die at a very early stage of embryonic development (Witke et al., 1993). Moreover, *Drosophila* mutants lacking *chickadee*, a homologue of profilin, show defects such as deficient oocyte formation, abnormal mitosis and impaired cell migration (Cooley et al., 1992; Verheyen and Cooley, 1994). Finally, profilins are also involved in the actin-based motility of *Listeria* (Theriot et al., 1994; Laurent et al., 1999), although their precise function in this process is still unclear (Theriot et al., 1994; Marchand et al., 1995; Sanger et al., 1995).

Only limited information is available about the cellular localisation of profilins (Hartwig et al., 1989; Buß et al., 1992; Machesky et al., 1994; Mayboroda et al., 1997; Neely and Macaluso, 1997) especially with respect to their dynamics in living cells (Tarachandani and Wang, 1996). Due to the different affinities for their ligands (Gieselmann et al., 1995; Lambrechts et al., 1995, 1997; Jonckheere et al., 1999), the two profilin isoforms may have different physiological functions and/or be localised to different subcellular sites. Thus, the analysis of the distribution of profilins in living cells is of particular importance.

In this study, we analysed the dynamics of GFP-tagged profilin II during cell spreading and in *Listeria*-infected cells. Our results reveal that profilin II-GFP localises to focal contacts and is distributed at the front of protruding lamellipodia. Profilin II-GFP is also recruited to the bacterial surface at the onset of *Listeria* motility. Moreover, the fluorescence intensity of profilin II-GFP at the bacterial surface highly correlates with the bacterial speed. Our data demonstrate that profilin II, presumably upon association with Ena/VASP proteins, can modulate the dynamics of the actin cytoskeleton.

MATERIALS AND METHODS

Bacterial culture

The wild-type weakly haemolytic *Listeria monocytogenes* strain EGD (serotype 1/2) and its isogenic *Listeria* mutant (ΔActA5) that expresses a mutated form of ActA lacking the proline-rich region (Domann et al., 1992; Niebuhr et al., 1997) were grown in brain heart infusion broth (BHI; Difco Laboratories) at 37°C with agitation.

Cell culture and infection

Rat embryo fibroblasts (REF-52), mouse melanoma cells B16-F1 (ATCC; CRL-6323), human fibroblasts WI 38 (ATCC; CCL 75), Ntera-2 (ATCC; CRL-1973), PtK_2 (ATCC, CCL 56) and HeLa (ATCC, CCL 2) were obtained from the American Type Culture Collection (Rockville, MD, USA). Cell culture and infection of PtK_2 and HeLa cells with *Listeria monocytogenes* were performed according to the method of Sechi et al. (1997). All media and supplements were obtained from Gibco BRL. Human fibroblasts were grown in MEM supplemented with 10% FCS, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate. REF-52 cells were grown in DMEM supplemented with 10% FCS and 2 mM L-glutamine. B16-F1 and Ntera-2 cells were grown in the same medium supplemented with 4.5 g/l glucose. Both cell lines were grown at 37°C in the presence of 7% CO_2. Cells were transiently transfected using FuGENE 6 (Boehringer Mannheim) as recommended by the supplier. Sixteen hours after starting the transfection, the cells were infected with *Listeria monocytogenes*.

Cloning of GFP-tagged profilins

To obtain the cDNAs of human profilin I and profilin II (available in the National Center for Biotechnology Information data base under the accession numbers J03191 and L10678, respectively) a reverse transcription-polymerase chain reaction (RT-PCR) was done using total mRNA preparations of human fibroblasts and Ntera-2 cells, respectively, according to standard protocols. A two-step cloning strategy was then used to prepare GFP-tagged profilins. First, the cDNAs for human profilin I and profilin II were amplified using the following primers: profilin I: forward primer (CCTTCCCGAATTC-AGCGCCATGG), reverse primer for cloning into pEGFP-N1 (GGAAGGGGAATTCGAGGTCCGTAC) and reverse primer for cloning into pEGFP-C2 (GGAAGGGGAATTCGAGGTCAGTAC); profilin II: forward primer (ACCATGGCCGGTTGG), reverse primer for cloning into pEGFP-N1 (CTAACACATCAGACCTCCTCAGG) and reverse primer for cloning into pEGFP-C2 (CTTACACATCAG-ACCTCCTCAGG). The amplified profilin I and profilin II cDNAs were cloned into pCR2.1-TOPO. Second, profilin I and profilin II fragments were excised with *Eco*RI and cloned into the same restriction sites of the pEGFP-N1 or pEGFP-C2 vectors (Clontech Laboratories, Inc.). Using this cloning strategy the following hydrophilic linkers were introduced between the profilins and the GFP: profilin I-GFP and profilin II-GFP, GPRILQSTVPRARD-PPVAT and LEGRILQSTVPRARDPPVAT, respectively; GFP-profilin I and GFP-profilin II, SGRTQISSSSFEFSA and SGRT-QISSSSFEFALT, respectively. The nucleotide sequences of all constructs were verified by DNA-sequencing.

Expression and purification of profilin II and GFP-tagged profilins

The cDNA for untagged profilin II was initially cloned into the *E. coli* expression vector pGEX-2T (Pharmacia) as follows. First, the cDNA was amplified using the following primers: forward (CCCTTGGG-CGGATCCATGGCCG) and reverse (GGTAGATGGGGGGATCC-TGCTTACAC). Thereafter, the profilin II cDNA was excised with *Bam*HI and cloned into the same restriction site of pGEX-2T. Due to the low expression level of profilin II in *E. coli* BL21, we cloned profilin II into the *E. coli* expression pET28 vector (Novagen) using *Nco*I and *Eco*RI. GFP-tagged profilins were cloned into the pET28 vector. The profilin-GFP plasmids were incompletely digested using *Nco*I and *Not*I. Thereafter, profilin-GFP fragments were cloned into the same restriction sites of the pET28 vector. Human profilin II and the corresponding GFP-tagged profilins were expressed in *E. coli* BL21. Four hours after induction with IPTG, bacteria were harvested by centrifugation and lysed with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 1.5% Triton X-100, 0.1 mg/ml lysozyme and protease inhibitors. After removing cellular debris, the supernatant was applied to a poly-L-proline affinity column (Tanaka and Shibata, 1985; Lindberg et al., 1988; Kaiser et al., 1989). After a wash with 20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 2 M urea, both proteins were eluted with 8 M urea. Profilin-containing fractions were identified by SDS-PAGE, pooled and dialyzed against 0.01 M Tris-

HCl, pH 7.6, 0.2 mM $CaCl_2$ and 0.5 mM DTE. The purity of the protein preparations was higher than 95%, as judged by Coomassie Blue staining. Protein concentration was quantified by Bradford assay, using bovine serum albumin as standard. Rabbit skeletal muscle actin was prepared from acetone powder exactly as described by Giehl et al. (1994). Actin concentration was determined according to the method of Houk and Ue (1974).

Evaluation of the ligand-binding properties of profilin II-GFP

The influence of the GFP-tag on the ability of profilin II to bind monomeric actin, VASP and PIP2 was evaluated as follows. The affinity of profilin for monomeric actin was determined under polymerising conditions by measuring the shift in the critical concentration of actin at steady state. Actin polymerisation was measured by fluorimetry using 10% pyrene-labelled actin and 90% unlabelled actin (Kouyama and Mihashi, 1981). Briefly, increasing concentrations of monomeric actin in 0.01 M Tris-HCl, pH 8.0, 0.15 mM $CaCl_2$, 1.5 mM DTE, 1 mM ATP (G-buffer) were incubated with constant concentrations of profilin II or the corresponding GFP-tagged protein. Polymerisation of actin was started by adding 0.05 M KCl to the reaction mixture, and allowed to continue at room temperature for at least 12 hours. Fluorescence intensities were measured with an LS50B fluorimeter (Perkin-Elmer) using excitation at 366 nm and emission at 384 nm. The binding of profilin II and profilin II-GFP to PIP2 was determined by ELISA. PIP2 (Boehringer Mannheim) micelles were prepared as described by Hüttelmaier et al. (1998). Microlon ELISA plates were coated with 50 pmol of profilin II or profilin II-GFP per well and blocked with 1% bovine serum albumin. After adding increasing amounts of PIP2 micelles, ELISA plates were incubated for 2 hours at room temperature. Bound PIP2 micelles were detected using a specific antibody (DRG International) followed by a peroxidase-conjugated secondary antibody. A colourimetric reaction was developed and the optical density was measured at 410 nm using an ELISA reader. To compensate for optical density fluctuations, which are mainly due to small temperature variations, we normalised the values measured at each point using the highest optical density value as reference. The value of each data point is reported as the mean of three independent experiments ± standard deviation. The interaction between VASP and profilins was evaluated using a similar strategy. Coating and saturation were carried out as described for the PIP2 binding assay. Thereafter, increasing amounts of recombinant murine VASP tagged with the bipro epitope (Rüdiger et al., 1997; Hüttelmaier et al., 1999) were added to the ELISA plates and allowed to bind to profilins. Bound VASP was detected with a specific monoclonal antibody (4A6) to the bipro-tag that does not crossreact with mammalian profilins (Wiedemann et al., 1996), followed by a peroxidase-conjugated antibody. Colourimetric reaction, optical density measurements and data handling were carried out as for the PIP2 binding assay.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde in cytoskeleton buffer (CB; 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM glucose, 5 mM $MgCl_2$, pH 6.1) for 20 minutes at room temperature (RT) and then extracted with 0.1% Triton X-100 in CB for 1 minute at RT. VASP was labelled with the monoclonal antibody 273D4 (Abel et al., 1996) followed by AMCA-conjugated goat anti-mouse IgG (Dianova). Vinculin and Mena were labelled with the monoclonal antibodies hVIN-1 (Sigma) and 49C2B12, respectively, followed by Texas Red-conjugated goat anti-mouse IgG (Dianova). The actin cytoskeleton was labelled with Texas-Red phalloidin (Molecular Probes). Coverslips were mounted in Prolong (Molecular Probes).

Fluorescence video microscopy

For fluorescence video microscopy, cells were plated onto 40 mm round coverslips. Three hours after beginning the infection, coverslips carrying infected cells were mounted in a Focht Chamber System (FCS2, Bioptechs). An objective heater (Bioptechs) was used to eliminate the temperature gradient between chamber and objective. The cells were observed by phase contrast or epifluorescence with an Axiovert 135 TV microscope (Zeiss) equipped with a Plan-Apochromat ×100/1.40 NA oil immersion objective in combination with ×1.6 or ×2.5 optovar optics. Images were recorded with a cooled, back-illuminated CCD camera (TE/CCD-1000 TKB, Princeton Instruments Inc.) driven by IPLab Spectrum software (Scanalytics Inc.). Digital handling of the images was done using IPLab Spectrum, Scion Image 1.62 (Scion) and Adobe Photoshop 5.0 (Adobe Systems, Inc.).

Analysis of profilin II-GFP fluorescence intensity versus bacterial speed

For profilin II-GFP fluorescence intensity measurements, a circular region of interest (ROI; 0.4 μm in diameter) was centred on the bacterial pole adjacent to the actin comet tail. Peak fluorescence

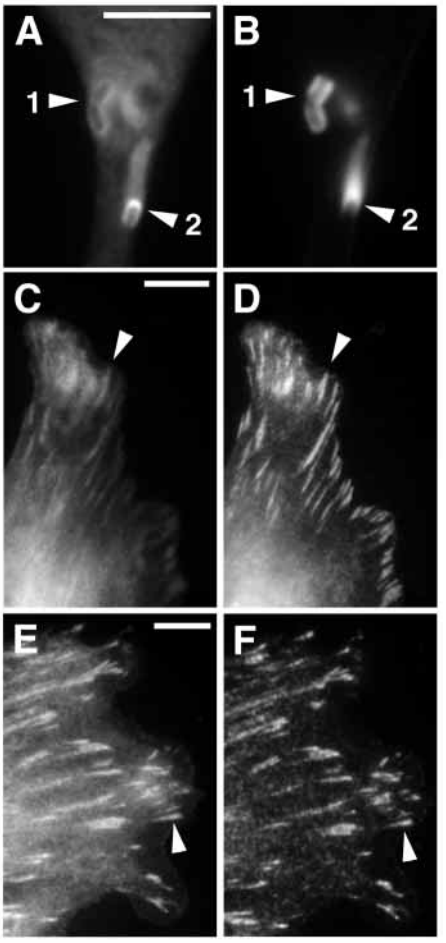

Fig. 1. Localisation of profilin II-GFP in *Listeria*-infected PtK_2 cells and in REF-52 cells. (A,B) PtK_2 cells expressing profilin II-GFP were infected with *Listeria monocytogenes* wild type, fixed and stained with Texas-Red phalloidin. (A and B) Profilin II-GFP and Texas-Red phalloidin, respectively. Profilin II-GFP is not detectable on the stationary bacterium (arrowheads 1 in A and B) whereas it localises to the pole adjacent to the actin tail of the motile bacterium (arrowheads 2 in A and B). (C,F) REF-52 fibroblasts were transfected with profilin II-GFP, fixed and stained with vinculin or Mena antibodies. Profilin II-GFP (C and E) co-localises with vinculin (D) or Mena (F) to focal contacts (arrowheads). Bars: 5 μm (A,B); 10 μm (C,F).

intensities in the ROI were considered as indicators of the relative amount of profilin II-GFP localised to the bacterial pole. Similar results were achieved when the average of the pixel intensities in the ROI was recorded instead. The same results were obtained using conventional imaging or digital deconvolution methods. To smooth out sudden speed oscillations, the instantaneous speed was calculated according to the central difference method (Maron, 1982). The highest peak intensity measured was set to 100% and all other values were calculated using this as a reference. The correlation between the profilin II-GFP fluorescence intensity and the instantaneous speed was calculated with Excel 5.0 (Microsoft Corporation).

RESULTS

Cloning of GFP-tagged human profilin I and II

Linking GFP to profilins might affect their binding activity because both the carboxy- and amino-termini of profilin are involved in the interaction with its ligands (Raghunathan et al., 1992; Bjorkegren et al., 1993; Mahoney et al., 1997; Chaudhary et al., 1998). Moreover, because profilins are considerably smaller than GFP (12-15 kDa versus 27-30 kDa, respectively), we reasoned that the binding of profilin to monomeric actin, PIP2, poly-L-proline and proteins harbouring proline-rich sequences might be affected by the presence of GFP. We constructed GFP-tagged profilin I and profilin II differing in the position of GFP relative to profilin, i.e. GFP was fused either to the carboxy- or amino terminus of profilins. Furthermore, to minimise a potential steric hindrance by GFP, all the constructs carried hydrophilic linkers between the profilin and the GFP moieties (see Materials and Methods).

GFP does not interfere with the binding properties of profilin II

We considered recruitment by intracellular *Listeria* to be a major criterium for selecting a functional construct. Only the construct in which the GFP moiety was carboxy-terminal to profilin II was recruited by *Listeria* (Fig. 1A,B). Because GFP-profilin I constructs neither associated with intracellular *Listeria* nor bound to poly-L-proline columns (as determined for profilin I-GFP, data not shown), we used profilin II-GFP for further analysis. To analyse the functionality of the binding of the profilin II-GFP fusion protein to monomeric actin, poly-L-proline ligands and PIP2, profilin II and its GFP-tagged version were expressed in *E. coli* and purified on poly-L-proline

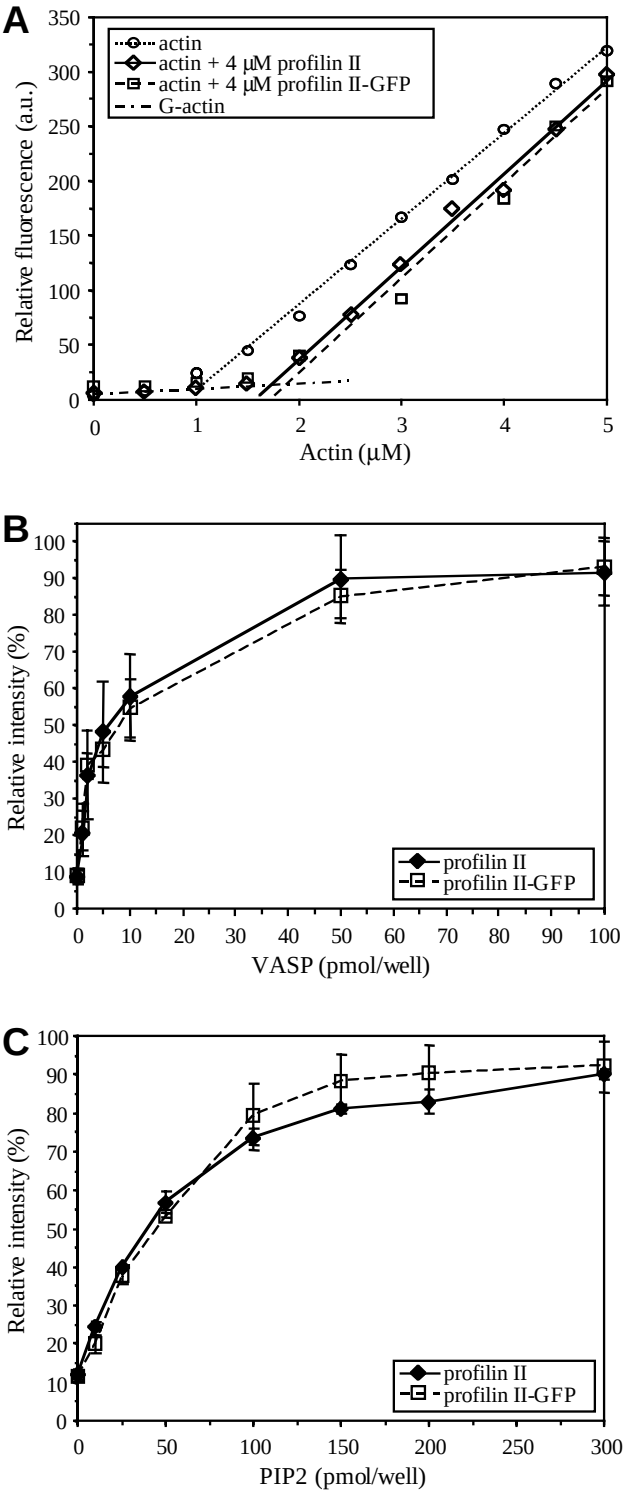

Fig. 2. GFP does not interfere with the ligand-binding properties of profilin II. (A) Affinity of profilin II and profilin II-GFP for monomeric actin. The affinity of profilin for actin was determined under polymerising conditions by measuring the shift in the critical concentration of actin at steady state. A constant amount (4 μM) of profilin II or profilin II-GFP was added to increasing concentrations of monomeric actin containing 10% pyrene-labelled actin. After inducing actin polymerisation, increase of F-actin content was monitored as increase of fluorescence intensity and plotted versus actin concentration. Lines represent regression plots for monomeric actin (unpolymerised actin), F-actin alone (open circles), F-actin plus profilin II (open rhombs) and F-actin plus profilin II-GFP (open squares). Critical concentrations can be deduced from the intercept between the monomeric actin regression line, representing the background pyrenyl-actin fluorescence, with the regression lines of F-actin, F-actin plus profilin II, or F-actin plus profilin II-GFP. (B) Binding of profilin II and profilin II-GFP to VASP. A constant amount of profilin II or profilin II-GFP was adsorbed on ELISA plates followed by incubation with increasing concentrations of purified bipro-tagged VASP. Bound VASP was detected with a specific antibody to the tag. (C) Binding of profilin II and profilin II-GFP to PIP2. A constant amount of profilin II or profilin II-GFP was adsorbed on ELISA plates. Increasing concentrations of PIP2 were added to the plates and the bound PIP2 was detected using a specific monoclonal antibody. Error bars (in B and C) represent one standard deviation above and below each data point.

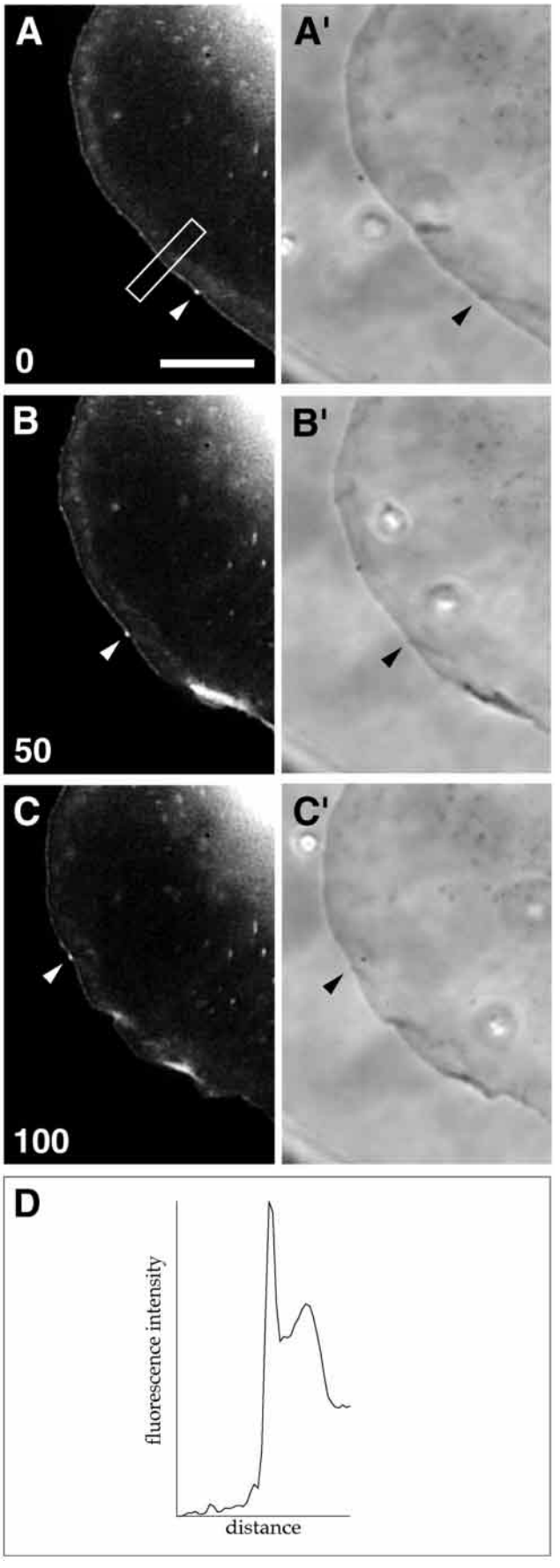

Fig. 3. Dynamics of profilin II-GFP in B16-F1 cells. (A,C) Video microscopy frames showing the distribution of profilin II-GFP during the spreading of a B16-F1 cell and (A′,C′) corresponding phase contrast images. Profilin II-GFP predominately localises as a thin line at the front of a spreading lamellipodium. Point-like structures showing lateral movement could be observed (arrowhead A,C′). (D) Plot of the intensity of profilin II-GFP across the lamellipodium (boxed area in A). Elapsed time (in seconds) is indicated in the lower left corner of A,C. Bar, 10 μm. A QuickTime movie of this experiment is available at http://www.biologists.com/JCS/movies.

columns. Elution of both profilin II and profilin II-GFP was performed under the same conditions: after stringent washing with 2 M urea, both proteins were eluted with 8 M urea, indicating that the affinity of profilin II for poly-L-proline was not affected by GFP. The affinities of both profilin II and

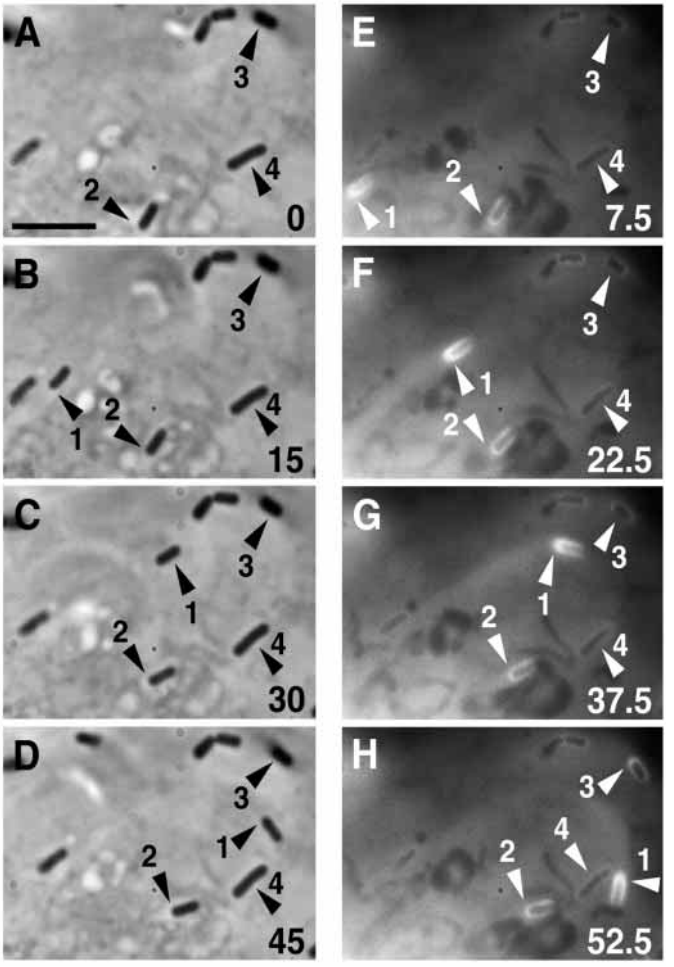

Fig. 4. Profilin II-GFP associates with *Listeria* at the onset of motility. PtK_2 cells expressing GFP-tagged profilin II were infected with *Listeria monocytogenes* wild type and observed by time-lapse videomicroscopy. (A,D) Phase contrast images, whereas E and H show the corresponding profilin II-GFP images. Profilin II-GFP localises to motile bacteria (arrowheads 1 and 2) but it is not detectable on stationary ones (arrowheads 3 and 4). Accumulation of profilin II-GFP at one bacterial pole (arrowhead 3 in H) can be observed corresponding with the onset of *Listeria* movement. Number on the bottom right corner of each panel shows the elapsed time in seconds. Note that due to the long read-out time, the fluorescence images are 7.5 seconds ahead of the corresponding phase contrast images. Bar, 5 μm. A QuickTime movie of this experiment is available at http://www.biologists.com/JCS/movies.

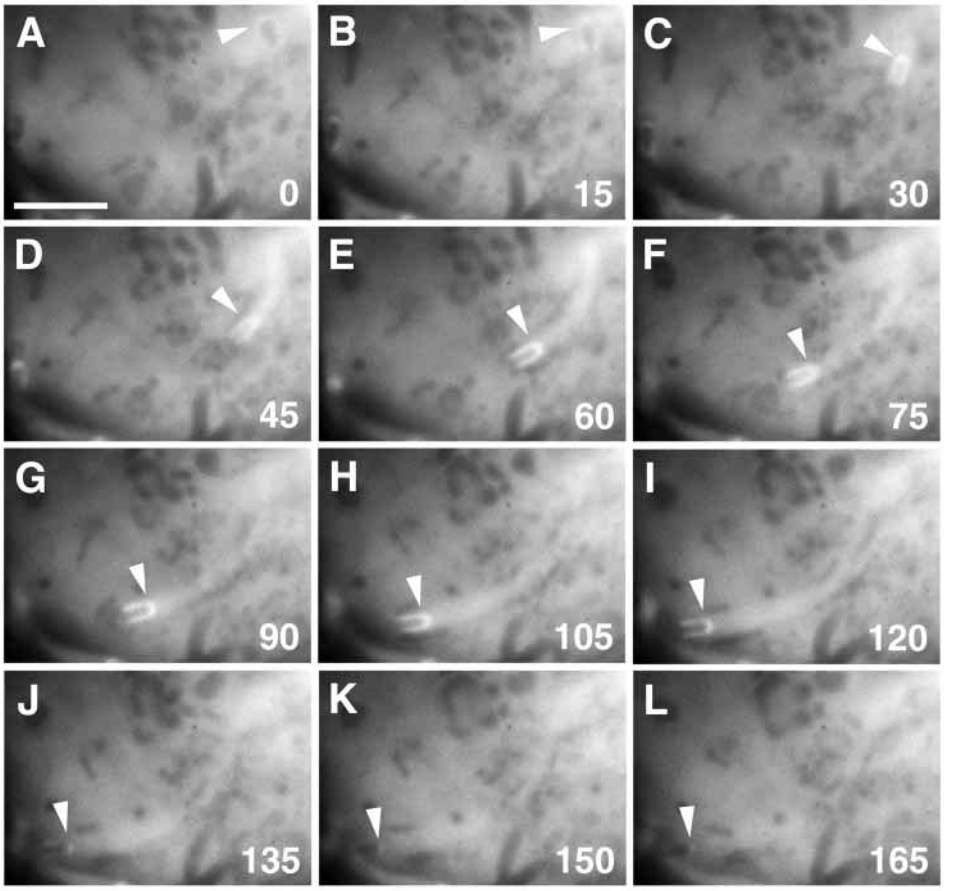

Fig. 5. Correlation between accumulation of profilin II-GFP and bacterial movement. PtK_2 cells were transfected with profilin II-GFP and then infected with *Listeria monocytogenes*. Progressive accumulation of profilin II-GFP at the rear pole of a bacterium can be observed in correspondence of onset of actin-driven *Listeria* motility (arrowhead C,G). Conversely, a decrease of profilin II-GFP associated with the same bacterium corresponded to the arrest of the bacterial motility (arrowhead H,L). Number on the bottom right corner of each panel represents the elapsed time in seconds. Bar, 5 μm. A QuickTime movie of this experiment is available at http://www.biologists.com/JCS/movies.

profilin II-GFP for actin were measured under polymerising conditions by calculating the shift in the critical concentration of actin at steady state. For this purpose, a constant amount (4 μM) of profilin II or profilin II-GFP was added to increasing concentrations of monomeric actin containing 10% pyrene-labelled monomeric actin. After inducing actin polymerisation, increase of F-actin content was monitored as increase of fluorescence intensity and plotted versus actin concentration. As judged from their influence on actin polymerisation, profilin II and profilin II-GFP had comparable affinities for monomeric actin (Fig. 2A). Thus, GFP did not interfere with the binding of profilin II to actin.

The binding of profilin II and profilin II-GFP to VASP, a representative ligand for the poly-L-proline binding site, was analysed as follows. A constant amount of profilin II or its tagged variant was immobilised on ELISA plates. Increasing amounts of recombinant murine VASP tagged with a specific sequence derived from birch profilin (bipro tag; Rüdiger et al., 1997) were then added to the immobilised profilin II. Bound VASP was subsequently detected using a monoclonal antibody (4A6) that exclusively reacts with the bipro tag. As shown in Fig. 2B, comparable amounts of VASP bound to profilin II or profilin II-GFP, indicating that the binding of profilin II to

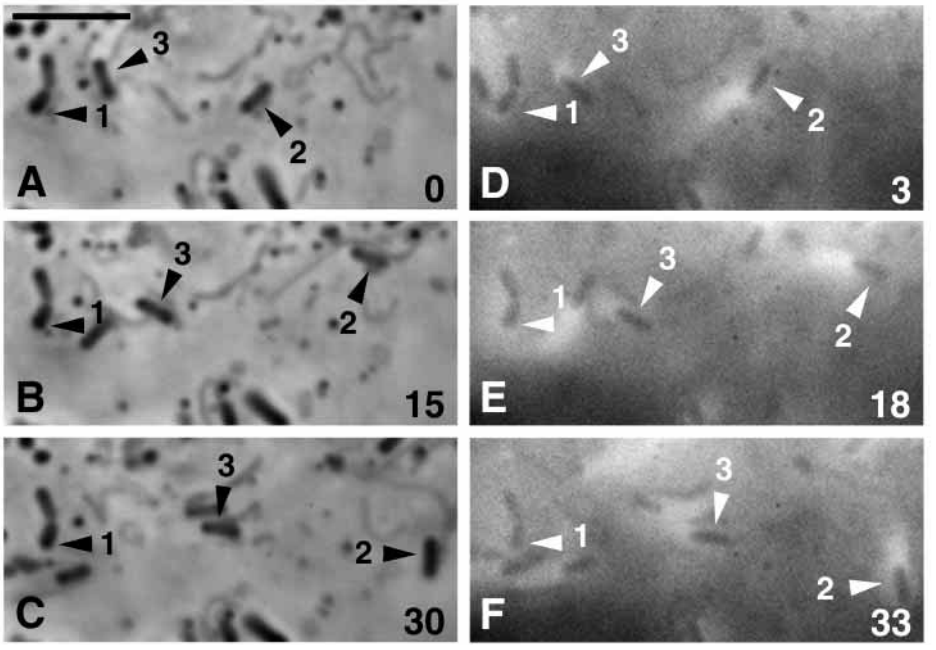

Fig. 6. Profilin II-GFP does not associate with *Listeria monocytogenes* ΔActA5. PtK_2 cells transfected with profilin II-GFP were infected with a *Listeria* mutant which expressed a mutated form of the ActA protein lacking the central proline-rich region. (A,C) Phase contrast and (D,F) corresponding fluorescence micrographs of a video sequence. In contrast with the situation typical of the wild type *Listeria*, profilin II-GFP does not associate either with motile bacteria (arrowheads 2 and 3) or with stationary ones (arrowhead 1). Elapsed time (in seconds) is shown at the bottom right corner of each panel. Note that due to the long read-out time, the fluorescence images are 3 seconds ahead of the corresponding phase contrast images. Bar, 5 μm. A QuickTime movie of this experiment is available at http://www.biologists.com/JCS/movies.

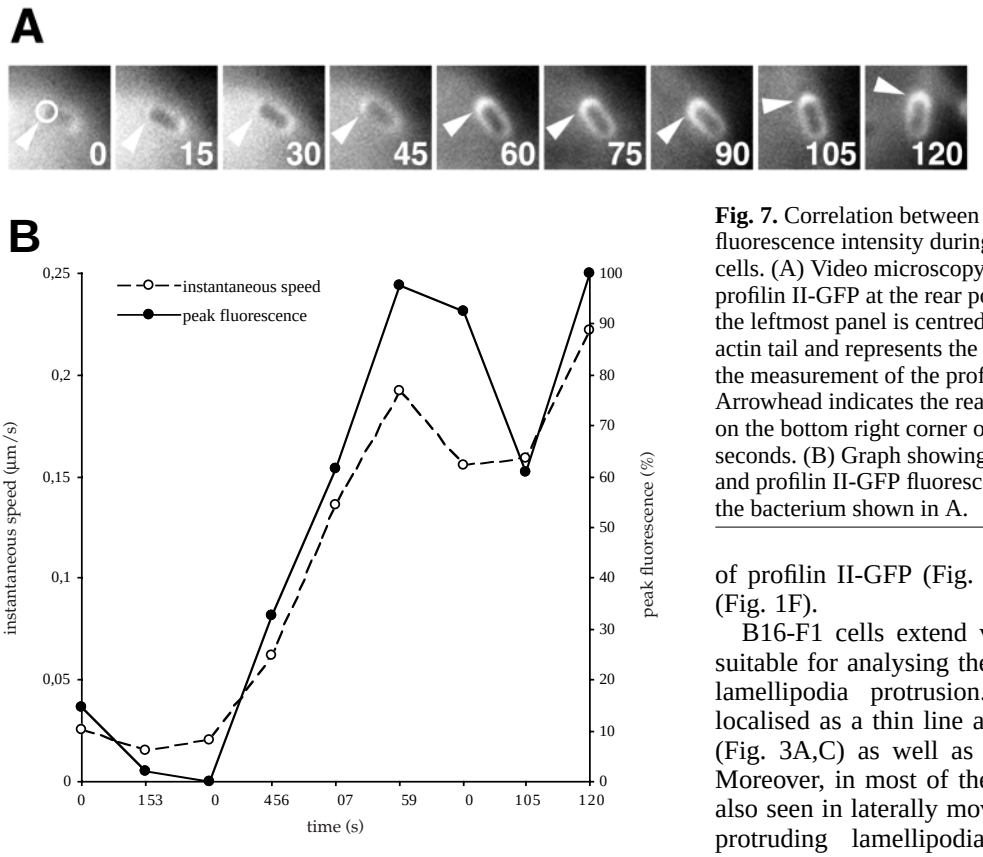

Fig. 7. Correlation between bacterial speed and profilin II-GFP fluorescence intensity during the onset of *Listeria* motility in PtK_2 cells. (A) Video microscopy frames showing the accumulation of profilin II-GFP at the rear pole of a motile bacterium. The circle in the leftmost panel is centred on the bacterial pole adjacent to the actin tail and represents the region of interest used as reference for the measurement of the profilin II-GFP fluorescence intensity. Arrowhead indicates the rear pole of the motile bacterium. Number on the bottom right corner of each panel indicates the elapsed time in seconds. (B) Graph showing the correlation between bacterial speed and profilin II-GFP fluorescence intensity at different time points for the bacterium shown in A.

proline-rich ligands was not significantly affected by GFP. This is in agreement with the comparable affinities of profilin II and profilin II-GFP for poly-L-proline columns (see above).

A similar strategy was used to study the interaction of profilin II or profilin II-GFP with PIP2. In this case, profilin II or profilin II-GFP immobilised on ELISA plates were incubated with PIP2 followed by a specific antibody to detect bound PIP2. As expected, profilin II-GFP retained its ability to bind to PIP2 (Fig. 2C).

Profilin II-GFP localises to focal adhesions and to the front of spreading lamellipodia

To gain more insight into the physiological function(s) of profilins, it is necessary to know their localisation and dynamics in living cells. Because the organisation of the actin cytoskeleton can be altered by the overexpression of profilin (Finkel et al., 1994; Rothkegel et al., 1996), for all experiments in this study we selected cells which expressed levels of profilin II-GFP suitable for being detected with a low-light-intensity CCD camera and which displayed the expected organisation of the actin cytoskeleton. Upon expression in REF-52 cells, profilin II-GFP was detected in elongated bright structures resembling typical focal contacts (Fig. 1C,E). Immunolabelling profilin II-GFP-expressing cells with antibodies to bona fide focal contacts components confirmed this view. Profilin II-GFP co-localised at these sites with vinculin (Fig. 1C,D). Ena/VASP proteins also associate with focal contacts (Reinhard et al., 1992) and are, in addition, ligands for profilin (Reinhard et al., 1995; Gertler et al., 1996). Thus, we analysed the distribution of Mena in REF-52 cells transfected with profilin II-GFP. As expected, the distribution of profilin II-GFP (Fig. 1E) perfectly matched that of Mena (Fig. 1F).

B16-F1 cells extend very thin lamellipodia making them suitable for analysing the dynamics of profilin II-GFP during lamellipodia protrusion. In these cells, profilin II-GFP localised as a thin line at the front of spreading lamellipodia (Fig. 3A,C) as well as to focal contacts (data not shown). Moreover, in most of the cells analysed, profilin II-GFP was also seen in laterally moving structures at the leading edge of protruding lamellipodia (Fig. 3A,C). As seen in the corresponding phase contrast images (Fig. 3A′,C′), these structures represent filopodia. Similar structures were also described by Rottner et al. (1999) using GFP-VASP, who verified their nature by whole-mount electron microscopy. In addition, scans of the profilin II-GFP intensity across spreading lamellipodia revealed the presence of two peaks (Fig. 3D), closely resembling the distribution of GFP-VASP in the same cell line (Rottner et al., 1999).

Profilin II-GFP associates with motile *Listeria* but not with stationary bacteria

As shown in Fig. 1A,B, profilin II-GFP accumulated at the bacterial pole adjacent to the actin tails but not around stationary bacteria. Therefore, using high resolution time-lapse imaging we sought to analyse in detail the dynamics of profilin II-GFP associated with *Listeria*. In infected cells, profilin II-GFP localised at the bacterial surface asymmetrically, being more concentrated at the interface between bacteria and actin tails (Fig. 4E,H; arrowhead 1). This asymmetric distribution was also retained in *Listeria*-induced pseudopodia-like extensions (see Fig. 9B, arrow). In contrast, profilin II-GFP was not detectable on non-motile *Listeria* (Fig. 4E,G; arrowhead 3). The speed of intracellular *Listeria* is highly variable, and thus it is common to find bacteria moving at different rates. Remarkably, the intensities of profilin II-GFP fluorescence varied among the motile bacteria analysed (Fig. 4E,H; arrowheads 1 and 2) and seemed to be related to their speed. In addition, the recruitment of profilin II-GFP to the bacterial surface was invariably associated with the onset of movement (Fig. 4E,G, arrowhead 3 and Fig. 5A,D). Conversely, the arrest of *Listeria* movement always resulted in a drastic decrease in profilin II-

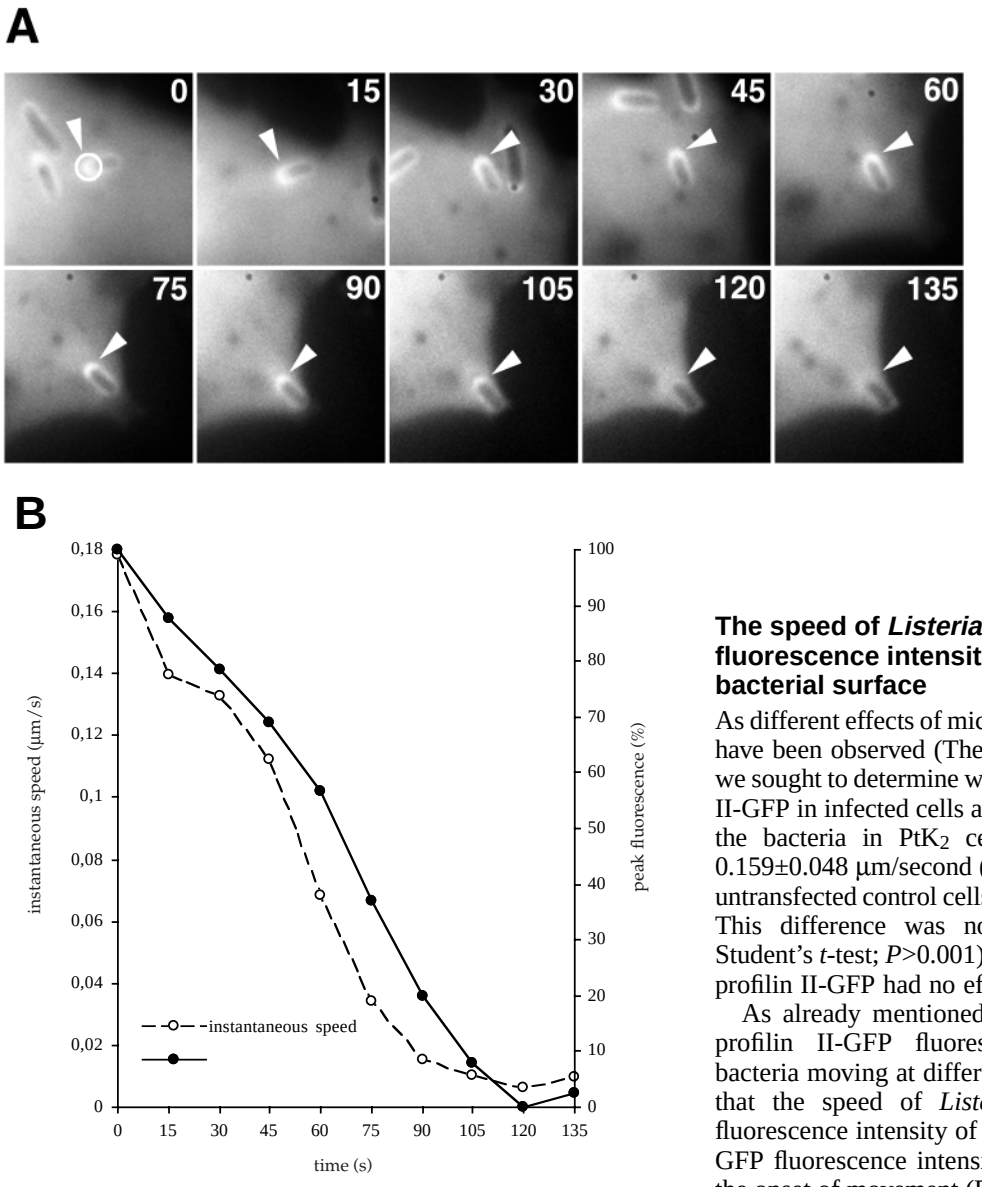

Fig. 8. Correlation between bacterial speed and profilin II-GFP fluorescence intensity during the arrest of *Listeria* motility in PtK_2 cells. (A) Video microscopy frames showing the progressive decrease of profilin II-GFP fluorescence at the rear pole of a motile bacterium. The circle in the upper left panel is centred on the bacterial pole adjacent to the actin tail and represents the region of interest used as reference for the measurement of the profilin II-GFP fluorescence intensity. Arrowhead indicates the rear pole of the motile bacterium. Number on the upper right corner of each panel indicates the elapsed time in seconds. (B) Graph showing the correlation between bacterial speed and profilin II-GFP fluorescence intensity at different time points for the bacterium shown in A.

GFP fluorescence intensity at the bacterial surface (Fig. 5A,L; arrowhead).

Ena/VASP proteins, which are profilin ligands (Reinhard et al., 1995; Gertler et al., 1996) and actin-binding proteins (Laurent et al., 1999; Hüttelmaier et al., 1999; Bachmann et al., 1999), are also recruited by *Listeria* (Chakraborty et al., 1995; Gertler et al., 1996) and are distributed around the bacterial surface in an asymmetric fashion similar to that of profilin II-GFP. To test whether the localisation of profilin II-GFP depended on Ena/VASP proteins recruited by the listerial ActA protein, we infected profilin II-GFP-transfected cells with an isogenic *Listeria monocytogenes* strain expressing a mutated form of ActA which lacks the central proline-rich regions. This mutant does not recruit Ena/VASP proteins and moves at a reduced speed (Niebuhr et al., 1997). As expected, profilin II-GFP was not found associated with these bacteria regardless of their motility state (Fig. 6; arrowheads 1-3).

The speed of *Listeria* is proportional to the fluorescence intensity of profilin II-GFP at the bacterial surface

As different effects of microinjected profilin on *Listeria* motility have been observed (Theriot et al., 1994; Sanger et al., 1995), we sought to determine whether or not the expression of profilin II-GFP in infected cells affected *Listeria* motility. The speed of the bacteria in PtK_2 cells expressing profilin II-GFP was 0.159±0.048 μm/second (n=23), whereas the bacterial speed in untransfected control cells was 0.146±0.036 μm/second (n=23). This difference was not statistically significant (unpaired Student's t-test; P>0.001). Thus, the expression of low levels of profilin II-GFP had no effect on *Listeria* speed.

As already mentioned, we consistently observed different profilin II-GFP fluorescence intensities associated with bacteria moving at different speeds. Therefore, we anticipated that the speed of *Listeria* might be correlated with the fluorescence intensity of profilin II-GFP. Typically, profilin II-GFP fluorescence intensity increased at one bacterial pole at the onset of movement (Fig. 7A,B) and always correlated with an increase of *Listeria* speed (Fig. 7B). Conversely, when bacteria slowed down profilin II-GFP fluorescence intensity decreased (Fig. 8A,B). The corresponding correlation coefficients measured for bacteria with increasing or decreasing speed were 0.971 and 0.977, respectively.

VASP and profilin II-GFP co-localise only at the surface of motile *Listeria monocytogenes*

Since the association of profilin II-GFP with *Listeria* obviously depends on the motility state of the bacteria, we sought to determine whether the localisation of VASP around *Listeria* matched that of profilin II-GFP. For this purpose, motile and stationary bacteria were observed by time-lapse video microscopy (Fig. 9A,D). This approach was necessary to precisely identify stationary and motile bacteria at the time of fixation (Fig. 9E). Fixed cells were labelled with a specific antibody against VASP (Fig. 9F,G). As expected, profilin II-GFP was only detectable on motile bacteria (Fig. 9F; arrowheads 2 and 4). In contrast, VASP was associated with both stationary and motile bacteria and co-localised with

profilin II-GFP only on motile *Listeria* (Fig. 9G). In this case, the distribution of VASP and profilin II-GFP was asymmetric and coincident at the bacterial surface (Fig. 9F,G).

DISCUSSION

In this study we have demonstrated that profilin can be tagged to GFP thus enabling the analysis of profilin dynamics in living cells. Because GFP is larger than profilin and both the carboxy- and amino-termini of profilin are involved in ligand binding, we characterised the binding properties of this fusion protein in detail and found that profilin II-GFP not only retained the ability to interact with its ligands in vitro, but also revealed affinities similar to those of untagged profilin II. Moreover, profilin II-GFP was targeted to both the surface of intracellular *Listeria* and dynamic cytoskeletal structures. Thus, our profilin II-GFP fusion protein is a reliable tool for analysing the dynamics of profilin in living cells.

Despite the fact that the same cloning strategy was used to clone all GFP-profilins, the construct in which GFP was fused to the amino terminus of profilin II and both GFP-profilin I fusion proteins failed to localise to intracellular *Listeria* and dynamic cytoskeletal structures. Furthermore, profilin I-GFP did not bind to poly-L-proline columns. Steric hindrance caused by the GFP moiety might interfere with the binding to poly-L-proline stretches, thereby inhibiting the targeting of the fusion proteins to cellular ligands such as Ena/VASP proteins in transfected cells. In addition, minor structural differences between profilin I and profilin II (Lambrechts et al., 1997) might explain why both GFP-profilin I fusion proteins did not work. In this context, fusion of GFP either to the amino- or carboxy-terminal end of human profilin I might interfere with the folding of this profilin isoform and further decrease its affinity for, or even abolish the binding to, poly-L-proline.

One might argue that the expression of profilin II in epithelial cells such as PtK_2 and HeLa is not physiologic since this profilin isoform is thought to be restricted mainly to neuronal cells (Witke et al., 1998; Giesemann et al., 1999). As a result, expression of profilin II-GFP in PtK_2 and HeLa cells might interfere with the dynamics of the actin cytoskeleton.

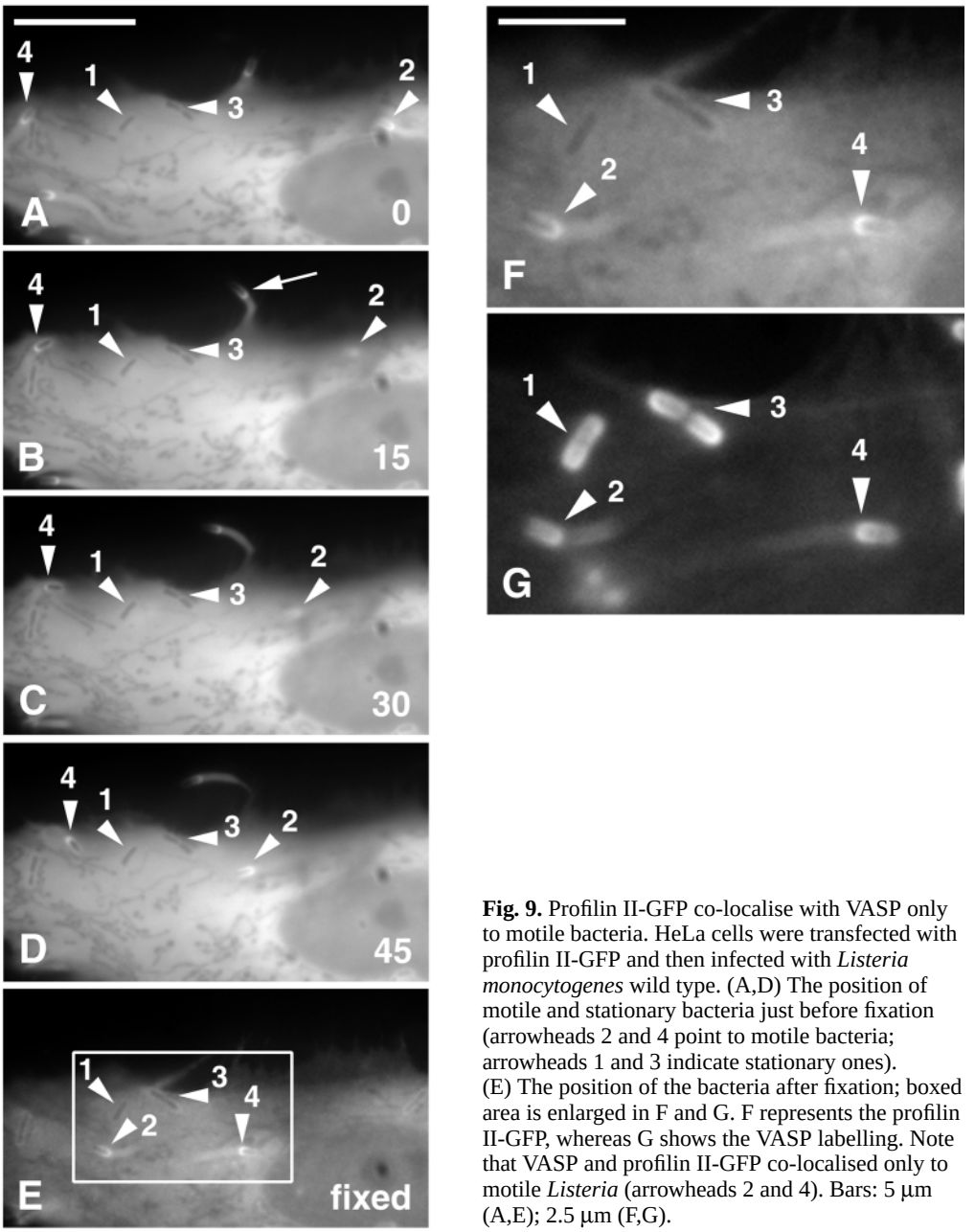

Fig. 9. Profilin II-GFP co-localise with VASP only to motile bacteria. HeLa cells were transfected with profilin II-GFP and then infected with *Listeria monocytogenes* wild type. (A,D) The position of motile and stationary bacteria just before fixation (arrowheads 2 and 4 point to motile bacteria; arrowheads 1 and 3 indicate stationary ones). (E) The position of the bacteria after fixation; boxed area is enlarged in F and G. F represents the profilin II-GFP, whereas G shows the VASP labelling. Note that VASP and profilin II-GFP co-localised only to motile *Listeria* (arrowheads 2 and 4). Bars: 5 μm (A,E); 2.5 μm (F,G).

Nevertheless, we are confident that profilin II-GFP is a reliable probe for examining the physiological role of profilins in the dynamics of the actin cytoskeleton for the following reasons. First, we only analysed cells expressing low levels of the fusion protein that displayed a morphology and actin cytoskeleton architecture indistinguishable from untransfected control cells suggesting, that under these conditions profilin II-GFP did not interfere with the actin cytoskeleton function. Second, this fusion protein displayed the expected intracellular distribution. Third, the speed of intracellular *Listeria* was not altered by the expression of profilin II-GFP. Hence, we assume that profilin II-GFP may effectively compete with endogenous profilin I for binding to proteins harbouring proline-rich stretches, possibly due to the higher affinity for poly-L-proline and Ena/VASP proteins as has been reported for bovine profilin II (Lambrechts et al., 1995, 1997; Jonckheere et al., 1999).

The exact functions of profilins and the regulation of their activities in living cells are poorly understood in spite of extensive investigations (for references see Carlier and Pantaloni, 1997; Schlüter et al., 1997). Nevertheless, studies on the *Listeria* model system have provided detailed insights into the role of profilins in actin-based motility (Theriot et al., 1994; Smith et al., 1996; Laurent et al., 1999; Loisel et al., 1999). Profilins not only contribute to the efficiency of *Listeria* motility in cell extracts by increasing the bacterial speed, but also work in cooperation with the Ena/VASP proteins to significantly support the actin-based motility of *Listeria* (Smith et al., 1996; Niebuhr et al., 1997; Laurent et al., 1999). This view is further supported by genetic studies indicating that Mena and profilin function in concert during the actin-driven process of neurulation (Lanier et al., 1999). Thus, our results showing that profilin II-GFP is associated with motile *Listeria*, but not with a slow-moving *Listeria* mutant which does not recruit Ena/VASP proteins, are consistent with these earlier findings. Moreover, the speed of intracellular *Listeria* was found to be directly proportional to the fluorescence intensity of profilin II-GFP at the bacterial surface. This is also consistent with earlier observations showing that profilins stimulate the growth of actin filaments (Pantaloni and Carlier, 1993) and that the speed of *Listeria* is proportional to the rate of actin polymerisation (Theriot et al., 1992).

Profilin II-GFP also co-localised with Ena/VASP proteins at sites of actin rearrangement such as focal adhesions in REF-52 and B16-F1 cells. In particular, the distribution and dynamics of profilin II-GFP in spreading lamellipodia of B16-F1 strikingly resembled that recently described for GFP-VASP in the same cell line (Rottner et al., 1999). Thus, our observations indicate that Ena/VASP proteins are the preferred targets for profilin II and suggest that the interaction between Ena/VASP proteins and profilins plays a pivotal role in the regulation of actin filament dynamics. This view is supported by microinjection experiments which showed that the proline-rich sequence of VASP interferes with intracellular motility of *Shigella* and *Listeria* (Zeile et al., 1996; Kang et al., 1997).

Because the co-localisation of profilin II-GFP and VASP at the bacterial surface depends on the motility state of *Listeria* (see Fig. 9), we speculate that the interaction between these proteins is regulated. To explain the behaviour of profilin II-GFP and VASP during the different stages of *Listeria* motility, we propose that the binding of profilin II to VASP depends on the activation state of VASP. In the activated form, VASP would recruit profilin II or profilin II-actin monomer complexes which in turn would provide monomeric actin to support actin filament elongation and *Listeria* motility. Conversely, deactivated VASP would not recruit profilin II and as a consequence would impair *Listeria* motility. The possibility that VASP needs to be activated to interact with profilin II is supported by the observation of Laurent et al. (1999) indicating that phosphorylation of serine 157 of VASP affects its interaction with actin filaments. This phosphorylation of VASP may also affect its binding to profilin II because serine 157, a phosphorylation site that is highly conserved among the Ena/VASP proteins (Gertler et al., 1996), is adjacent to the profilin-binding site of VASP. However, we cannot rule out the possibility that other modifications of VASP, modifications of profilin, or additional cellular factors might be involved in this process.

It is widely assumed that Ena/VASP proteins recruit profilin-actin complexes, thus providing high levels of monomeric actin at the bacterial surface. In this context, it has been proposed that profilin II dimerises upon binding to a peptide comprising the proline-rich regions of VASP, leading to an efficient release of monomeric actin and an enhancement of the nucleation and elongation of actin filaments (Jonckheere et al., 1999). Whether or not the release of actin monomers also results in the release of profilin II remains to be clarified.

In addition to the Ena/VASP proteins, several other proteins have been suggested to be involved in actin-based motility. These include WASP/N-WASP (see Bi and Zigmond, 1999), proteins of the formin homology family (see Wasserman, 1998), and the Arp2/3 complex (see Machesky and Gould, 1999). Several of these are also profilin ligands. Thus, the interactions of these proteins with profilins might also be crucial for the regulation of actin cytoskeleton dynamics.

In summary, we have demonstrated that the use of GFP-tagged profilins represents a powerful experimental approach to study the dynamics of profilins in living cells. The construction of mutated GFP-tagged profilins, which are defective in binding to one or more ligands, may be useful for gaining new insights not only into the physiological functions of the different profilin isoforms, but also into the spatial and temporal remodelling of the actin cytoskeleton.

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