Actin-filament cross-linking protein T-plastin increases Arp2/3-mediated actin-based movement

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Summary

Increasing evidence suggests that actin cross-linking or bundling proteins might not only structure the cortical actin cytoskeleton but also control actin dynamics. Here, we analyse the effects of T-plastin/T-fimbrin, a representative member of an important actin-filament cross-linking protein by combining a quantitative biomimetic motility assay with biochemical and cell-based approaches. Beads coated with the VCA domain of the Wiskott/Aldrich-syndrome protein (WASP) recruit the actin-nucleating Arp2/3 complex, polymerize actin at their surface and undergo movement when placed in cell-free extracts. T-Plastin increased the velocity of VCA beads 1.5 times, stabilized actin comets and concomitantly displaced cofilin, an actin-depolymerizing protein. T-Plastin also decreased the F-actin disassembly rate and inhibited cofilin-mediated depolymerization of actin filaments in vitro. Importantly, a bundling-incompetent variant comprising the first actin-binding domain (ABD1) had similar effects. In cells, this domain induced the formation of long actin cables to which other actin-regulating proteins were recruited. Altogether, these results favor a mechanism in which binding of ABD1 controls actin turnover independently of cross-link formation. In vivo, this activity might contribute to the assembly and maintenance of the actin cytoskeleton of plasma-membrane protrusions.

Key words: T-Fimbrin, Bundling, CH domain, Nucleation, Cofilin, ADF

Introduction

Cell morphology and motility depend on the remodeling of the cytoskeletal architecture and require locally restricted membrane protrusion and invagination, driven by cycles of actin polymerization and depolymerization (Carlier et al., 1998; Arpin et al., 2003; Pollard and Borisy, 2003). Site-restricted generation of free barbed filament ends is the first step in the assembly process of new actin structures in cells. The Arp2/3 complex plays an important role in the temporal and spatial control of actin polymerization (Welch et al., 1998). After activation by the Wiskott/Aldrich-syndrome protein (WASP), this complex nucleates a branched actin network (Amann and Pollard, 2001; Machesky and Insall, 1998) found, for example, in the lamellipodium, a broad membrane protrusion found at the leading edge of migrating cells (Svitkina and Borisy, 1999). However, in addition to dendritic actin networks, cells also harbor structures like microvilli and microspikes, which contain a bundle of parallel-aligned actin filaments (Bartles, 2000). Although less dynamic than branched actin networks, the parallel array of actin filaments of these structures continuously turns over and monomer addition to the plasma-membrane-oriented filament plus ends is required for protrusive force generation (Loomeis et al., 2003; Moosiker et al., 1982).

Several lines of evidence support a role for actin cross-linking proteins in the biogenesis and maintenance of the actin cytoskeleton of these structures. Overexpression of actin-bundling proteins in cultured cells causes the elongation of the microvillar F-actin core bundle and affects its turnover (Arpin et al., 1994; Friederich et al., 1989; Loomis et al., 2003). Recent work supports the view that F-actin cross-linking proteins might interfere with Arp2/3-complex-dependent actin assembly. For example, the actin-cross-linking protein EPLIN inhibits secondary activation of branching nucleation by the Arp2/3 complex and reduces the frequency of branches in vitro (Maul et al., 2003). Consistent with a role in reshaping dendritic networks in cells, the actin-bundling protein fascin appeared to be required for the reorganization of Arp2/3-nucleated branched networks into bundles in a cell-free assay (Vignjevic et al., 2003). Although these findings suggest that actin-cross-linking proteins might contribute to actin-based force generation by reorganizing Arp2/3-complex-dependent actin networks, direct experimental evidence for such a mechanism is so far lacking.

T-Plastin (Bretscher, 1981), also named T-fimbrin, is a representative of an important family of actin-cross-linking and -bundling proteins (Gimona et al., 2002) that organize most of the actin networks in cells. T-Plastin is associated with the actin
core bundle of microvilli, stereocilia and microspikes in epithelial cells (Bretscher and Weber, 1980; Daudet and Lebart, 2002), and was proposed to participate in the assembly of these structures (Arpin et al., 1994). In mesenchymal cells, T-plastin localizes to focal adhesions and the leading edge (Arpin et al., 1994), a broad lamellar membrane extension harboring a dynamic branched actin network (Svitkina and Borisy, 1999). How T-plastin might contribute to actin-based force generation and actin assembly is the question we address here.

Using a cell-free motility assay, biochemical and cell-based approaches, we show that T-plastin can augment actin-based force in a dose-dependent manner and stabilize actin structures, which elongate. Importantly, a bundling-incompetent T-plastin variant had similar effects, demonstrating that the activity of T-plastin is not limited to filament cross-linking. We propose a novel mechanism of action for T-plastin in which this protein might contribute to actin assembly independently of cross-link formation.

Materials and Methods

Cell culture

Vero monkey kidney cells [CCL-81; American Type cell Culture (ATCC), Rockville, MD] and human HeLa cells (ATCC CCL-2) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum at 37°C in a 10% CO2 atmosphere.

Antibodies and fluorochrome-coupled probes

The monoclonal antibody P5D4, raised against the 11-aminoo-acid C-terminus of the vesicular stomatitis virus (VSV) glycoprotein G, and the affinity-purified T-plastin-specific polyclonal antibody have been described previously (Kreis, 1986; Lapillonne et al., 2000). The specificity of the affinity-purified polyclonal anti-plastin antibody raised against full-length recombinant human L-plastin was determined by western blot. The antibody reacted with recombinant human L- and T-plastin, and recognized a single protein of 67 kDa in HeLa extracts. Monoclonal mouse IgM antibody against bovine αt-actin was purchased from Sigma and monoclonal mouse IgG anti-human-VASP antibody from BD Transduction Laboratories. Monoclonal antibody against cofilin was a kind gift from I. Yahara and K. Moriyama (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Alexa-488-coupled donkey anti-rabbit or anti-mouse IgG antibodies and Alexa-320-coupled phalloidin were purchased from Molecular Probes. Rhodamine-coupled phalloidin was purchased from Sigma. Texas-Red-coupled goat anti-rabbit IgG and Cy2-coupled goat anti-IgM/IgG was obtained from Jackson Laboratories. N-Pyrenylisocyanate was purchased from Molecular Probes.

DNA constructs and proteins

The pCB6-T-plastin and the pUHD-10-3-ABD1 (kindly supplied by P. J. Sansonetti, Institut Pasteur, Paris, France) expression constructs have been described previously (Adam et al., 1995; Arpin et al., 1994). DNA fragments encoding T-plastin or T-plastin’s first actin-binding domain (ABD1) were inserted into the BamHI-EcoRI sites of the pGEX-2T vector. Recombinant T-plastin, ABD1 and the VCA domain of WASP, N-terminally fused to glutathione-S-transferase (VCA-GST) were produced and purified as described (Arpin et al., 1994; Golsteyn et al., 1997).

Actin was prepared from rabbit skeletal muscle (Spudich and Watt, 1971) and further purified using Sephacryl S-300 (Pharmacia) in G-buffer [5 mM Tris-HCl, pH 7.7, 0.1 mM CaCl2, 0.2 mM ATP, 0.2 mM dithiothreitol (DTT), 0.01% NaN3]. It was pyrene labeled on Cys374 following Kouyama and Michashi (Kouyama and Mihashi, 1981). The human villin headpiece cDNA sequence (villin-HP), which codes for the last 67 residues (720-826) of villin (Rossen et al., 2003), was cloned in a bacterial expression system (pET11d; Novagen). The resulting recombinantly produced villin headpiece domain was purified as described (Vardar et al., 1999) and stored in 50 mM Tris-HCl, pH 7.0, 0.025 mM EDTA. Human coflinI cDNA was cloned in pKM263 (Melcher, 2000) obtained from EUROSCARF and produced as an N-terminally tagged glutathione-S-transferase (GST) fusion. The fusion protein and GST were purified using glutathione-Sepharose (Pharmacia, protocol provided by manufacturer) followed by a gel filtration in 50 mM Tris-HCl, pH 7.0, 0.025 mM EDTA.

Sedimentation of proteins with F-actin

G-Actin from rabbit muscle was polymerized by the addition of 30 mM KCl, 1 mM MgCl2, 0.2 mM DTT, 0.1 mM ATP, 10 mM imidazole/HCl, 0.1 mM CaCl2, 2 mM EGTA, 0.5 mM Tris-HCl, pH 7.3, in the absence or presence of T-plastin or ABD1 for 12 hours at 4°C under the conditions described in the figure legends. Proteins were sedimented for 30 minutes at 150,000 g in a Beckman TL-100 ultracentrifuge to assess F-actin binding (Friederich et al., 1999) or at 12,000 g for 15 minutes to test bundling activity (Glennay et al., 1981b). To assess the Ca2+ dependency of T-plastin binding to F-actin, T-plastin (0.7 μM) was polymerized with G-actin (7 μM) overnight at 4°C in the presence of increasing concentrations of free Ca2+ and centrifuged for 30 minutes at 200,000 g. Free Ca2+ concentrations was calculated using Webmax Software standard developed by Stanford University (http://www.stanford.edu/~cpatton/webmaxcS.htm). Proteins in pellets and supernatants were analysed by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) as described (Friederich et al., 1999).

Motility assay and analysis of beads by epifluorescence microscopy

Polystyrene beads of 1 μm diameter (Polysciences) were spread in ethanol on the bottom of a Petri dish and allowed to dry. SiO2 shadowing was then performed by evaporation of a 8 nm layer at 10−5 bar (Cameron et al., 1999). Beads were resuspended in borate buffer and coated with GST-VCA protein at a concentration of 0.38 mg ml−1 in PBS. Protein binding was measured by quantitative western blotting using anti-GST antibody (Santa Cruz Biotechnology).

Extracts from HeLa cells were prepared as described (Fradelizi et al., 2001) and contained a total protein concentration of 23 mg ml−1. The amounts of actin and T-plastin in extracts were measured by quantitative western blotting as described (Fradelizi et al., 1999) using anti-human-β-actin (Sigma) and anti-T-plastin (Lapillonne et al., 2000) primary antibodies, respectively. The total β-actin and T-plastin levels in the extract were 5.12 μM and 2.06 μM, respectively.

0.5 μl beads were mixed with 8 μl HeLa extract supplemented with 1 mM ATP, 30 mM creatine phosphate and 1 μM Alexa-568-labelled actin (Molecular Probes). The final mixture was observed between glass coverslips, sealed with a mixture of lanolin, paraffin and vasolin (1:1:1) by fluorescence and phase-contrast microscopy (inverted Olympus microscope, BX51, 100× objective). Images were recorded every 5 seconds for 5-20 minutes and velocities measured using Metamorph tracking software (Universal Imaging).

For immunofluorescence staining, 2 μl bead-extract mixture was sandwiched between two coverslips, spaced by paraffin strips. The chambers were opened after a 5-minute incubation at room temperature in a solution containing 0.2% Triton X-100 and 2.5 μM phalloidin in CB (10 mM morpholino ethanesulfonic acid (MES), 150 mM NaCl, 5 mM EGTA, 5 mM MgCl2, 5 mM glucose, pH 6.1). After a 1 minute incubation, samples were fixed in 2.5 μM phalloidin, 1% glutaraldehyde in CB and processed for immunofluorescence staining with the antibodies indicated in figure legends. Identical exposure
times were used to acquire images of beads stained with the same antibodies. Images of comets were recorded using a linear CCD camera (Coolsnap; Princeton Instruments) and integrated emission intensities of rhodamine and Alexa 488 were quantified in a square section of the actin tail two bead diameters behind the bead using MetaMorph software.

**F-Actin depolymerization assay**

Pyrene-labeled-F-actin filaments (4 µM, 25% pyrene labelled) were prepared by a 30 minute incubation at room temperature in F buffer (0.5 mM Tris-HCl, pH 7.7, 0.1 mM CaCl2, 30 mM KCl, 1 mM MgCl2, 2 mM EGTA, 10 mM imidazole, 0.1 mM ATP, 0.2 mM DTT) in the presence or absence of different concentration of T-plastin, ABD1 or villin HP, as described in the figure legends. Depolymerization of F-actin was induced by dilution in F buffer to a final concentration of 0.4 µM in the presence or absence of GST-cofilin at the concentration indicated in the legends of the figures. The fluorescence decrease was recorded over the course of 6 minutes by an F-4500 fluorimeter (Hitachi) (excitation at 365 nm and emission at 388 nm).

**Transfection and epifluorescence analysis of cells**

Electroporation of cells was carried out with 10 µg plasmid for 5×10^6 cells at 240 V and 900 µF. Cells were plated onto glass coverslips and analysed 48 hours after transfection. Cells were processed for immunofluorescence staining as described previously (Friederich et al., 1999). For triple staining, ABD1-vsv transfected cells were labeled with Alexa-320-conjugated phalloidin as a probe for F-actin, anti-vsv-tag antibody followed by Texas-Red-coupled anti-rabbit IgG secondary antibody, mouse anti-α-actinin primary antibody followed by anti-mouse IgM/IgG Cy2-coupled secondary antibody or mouse anti-VASP primary antibody followed by anti-mouse IgG secondary antibody. Cells were analysed by epifluorescence microscopy (Leica DMRX, HCX PL APO ×100) equipped with the appropriated excitation and emission filters. Images were acquired with a linear CCD camera (Micromax; Princeton Instruments) and Metaview software (Universal Imaging).

**Results**

We used a cell-free assay consisting of beads coated with the VCA domain of WASP and placed in HeLa-cell extracts (Fradelizi et al., 2001; Noireaux et al., 2000) to assess how T-plastin participates in actin assembly and force generation. The VCA domain specifically recruits the actin-nucleating Arp2/3 complex to the bead surface. VCA beads undergo actin-polymerization-driven motility, accompanied by the assembly of a comet-like structure composed of F-actin. This system is extremely powerful for a qualitative and quantitative evaluation of the relative contributions of cytoskeletal components to the assembly of actin structures (Fradelizi et al., 2001; Vignjevic et al., 2003) and actin-based movement (Bernheim-GrosWasser et al., 2002).

Asymmetric polystyrene beads with a 1 µm diameter were asymmetrically coated with VCA (Cameron et al., 1999). Once placed in HeLa-cell extracts, 50% of the VCA-coated beads started to move within 2 minutes, propelled by a comet tail visible by phase-contrast microscopy (Fig. 1A). HeLa-cell extracts contained 2 µM endogenous T-plastin as determined by immunoblotting with T-plastin-specific antibodies. Supplemening extracts with exogenous recombinant T-plastin in the range 0.5-2.2 µM caused a prominent densification of the actin tails (Fig. 1A) and an increase in the proportion of comet formation from 50% to 80%. Densification of actin tails occurred concomitantly with tail growth and was also observed at high T-plastin concentrations (Fig. 1A). The densification of the comets was not due to an increase of the medium viscosity, as described by Wiesner et al. (Wiesner et al., 2003). Indeed,
by Brownian-motion analysis, we found that the diffusion coefficient of 1 µm diameter bovine serum albumin (BSA) coated beads placed in extracts was not affected by the presence of T-plastin, the viscosity being $3 \times 10^{-6} \pm 1 \times 10^{-3}$ kg m$^{-1}$ second$^{-1}$ in all cases.

In the absence of exogenous T-plastin, the mean length of actin tails was 4.0±1.5 µm. Tail length was significantly increased to 7.2±2.1 µm by the addition of 1.1 µM T-plastin. The length of actin tails being defined by the balance of cycles of actin assembly and disassembly, this result suggested that T-plastin affected actin-filament turnover and, as a consequence, the rate of bead movement. To test this hypothesis, we measured bead velocity in the absence (control) or presence of exogenous T-plastin at various concentrations (Fig. 1B). VCA-coated control beads moved with a maximum velocity of 1.9 µm minute$^{-1}$, comparable to that obtained in previous studies (Bernheim-Grosmaxx et al., 2002; Wiesner et al., 2003). Plotting bead velocity as a function of T-plastin concentration yielded a bell-shaped curve. At a concentration of 0.5-1.1 µM, exogenous T-plastin increased the velocity up to 1.5 times (Fig. 1B). At higher concentrations (up to 2.3 µM), velocity decreased to a value similar to that of control beads. Such a behavior might result from changing the balances of different aspects of actin dynamics (e.g. F-actin stabilization versus turnover).

T-Plastin might regulate actin organization and dynamics by cross-linking actin filaments into tight unipolar bundles. To assess whether this activity of T-plastin contributed to the modification of comet structure and bead movement, we generated a bundling-incompetent variant comprising the N-terminal calmodulin-like Ca$^{2+}$-binding domain followed by the first actin-binding domain (Fig. 2A). This variant, named ABD1, bound F-actin like T-plastin, as shown in a high-speed F-actin cosedimentation assay (Fig. 2B, top) but did not, as expected, bundle actin filaments as shown by low-speed cosedimentation, which sedimented F-actin bundles but not simple actin filaments (Fig. 2B, bottom).

Importantly, when tested in the bead assay, similar effects on bead velocity, comet length and density were observed with ABD1 (Fig. 2C,D) as with T-plastin (Fig. 1A,B). Surprisingly, ABD1 still densified and stabilized actin comets (Fig. 2C). Furthermore, as for T-plastin, bead velocity varied following a bell-shaped curve for ABD1 concentrations in the range 0-2.2 µM (Fig. 2D). No significant difference was detected between T-plastin and ABD1 results, as evidenced by Student’s t test $P$ values of 0.052 and 0.63 at 1.1 µM and 2.2 µM, respectively. This result clearly showed that the effects of T-plastin on actin-based movement and comet organization were independent of its bundling activity. Therefore, binding of T-plastin to the sides of actin filaments must be sufficient to cause these structural and dynamic modifications.

To verify that T-plastin and ABD1 indeed acted through their association with the comet actin cytoskeleton, we examined the distribution of actin, T-plastin and ABD1 in the actin comet tails by epifluorescence microscopy. Exogenous T-plastin and ABD1 localized throughout the comet, as did endogenous T-plastin (Fig. 3A, left). To correlate actin density to amounts of T-plastin or ABD1 bound to actin comets, fluorescence signals were integrated. Fluorescence intensities were measured close to the bead surface where actin polymerization occurs. ABD1 intensity was on average twofold higher than that of exogenous T-plastin, suggesting that more of this plastin variant bound to comet tails (Fig. 3A, right). This difference might be explained by the fact that, at saturating conditions, ABD1 binds pure F-actin with a higher stoichiometry (1:1) than T-plastin (1:3) in vitro (Glenney et al., 1981b; Hanein et al., 1998). Correlated to the increase in T-plastin or ABD1.

![Fig. 2. The first actin-binding domain of T-plastin (ABD1), which binds but does not bundle actin filaments, has a similar effect on bead movement to wild-type T-plastin. (A) Protein domain scheme of T-plastin and ABD1 variant. (B) Cosedimentation of T-plastin or the ABD1 variant with F-actin in vitro. G-Actin was polymerized in the presence of T-plastin (+ T-plastin) or ABD1 (+ ABD1), or their absence (control). The molar ratio of actin:plastin or actin:ABD1 was 4:1. Proteins were centrifuged at high speed to sediment actin filaments (top) or at low speed to sediment actin bundles (bottom). Proteins in supernatants (S) and pellets (P) were separated by SDS-PAGE under reducing conditions. Protein bands were stained with Coomassie Brilliant Blue. (C) Time-lapse phase-contrast microscopy of bead movement. Beads were incubated in HeLa extracts supplemented with 1.1 µM or 2.2 µM ABD1. No depolymerization of the actin tail is observed when ABD1 is added (black arrow). (D) Bead velocity as a function of ABD1 concentration. Bead velocity shows a bell-shaped dependence on ABD1 concentration, with a maximum velocity for 1.1 µM ABD1. Each bar represents the mean±s.d. of ten measurements. An asterisk (*) indicates results that differ significantly from the result obtained with the addition of 1.1 µM ABD1 ($P<$0.0006).]
fluorescence intensity, a moderate but significant increase in rhodamine-actin fluorescence intensity was observed close to the bead surface where actin assembly occurs (Fig. 3A, right).

Because bundling seemed not to be important for the densification and elongation of comets, we looked for another mechanism by which T-plastin could affect actin dynamics. Comets in the presence of 2.2 µM T-plastin or ABD1 were on average 25% longer than those of control VCA beads moving at the same rate, indicating differences in depolymerization and directing us to ADF/cofilin as a candidate target for T-plastin action. ADF/cofilin family members control cellular actin dynamics by enhancing the off rate from the pointed end of actin filaments (Carlier et al., 1997) or by severing actin filaments (Maciver et al., 1991) and creating new barbed

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**Fig. 3.** T-Plastin and ABD1 increase in actin tails concomitant with a significant increase in actin content and partial displacement of cofilin.

(A) Quantification of actin and T-plastin or ABD1 in actin comets. Beads were incubated in HeLa-cell extracts supplemented with 1 µM Alexa-568-labeled actin in the presence of 1.1 µM T-plastin (middle) or ABD1 (bottom), or their absence (control, top); actin is shown on the left. Samples were immunostained with anti-plastin primary antibody and Alexa-488-coupled secondary antibody (right). Graphs indicate the integrated fluorescence intensities of actin and T-plastin in the comets, measured at 2 µm from the bead. Each bar represents the mean±s.d. of 25 measurements. Asterisks (***) indicate results that differ significantly from the results obtained with the control (*, P<0.0031; ***, P<0.0063).

(B) Quantification of cofilin/actin ratio in actin comets. Beads were incubated in extracts and processed as described in A, with the exception that anti-cofilin antibody was used for staining (middle). Representative fluorescence images are shown. Graphs indicate the ratios of the integrated fluorescence intensities of cofilin/actin in the comets, measured at 2 µm distance from the bead.
filaments ends (Ghosh et al., 2004). As visualized by immunofluorescence staining, cofilin distributed throughout the actin tail in control samples and in the presence of T-plastin or ABD1 (Fig. 3B, left). Measurement of ratios of actin to cofilin fluorescence intensities revealed that T-plastin and ABD1 partially displaced cofilin from comets (Fig. 3B, right). Considering that cofilin depolymerizes F-actin, its displacement might contribute to the elongation and densification of actin comets, as observed in a similar assay (Rosenblatt et al., 1997).

Taken together, these results suggested that T-plastin and ABD1 prevented actin filaments from disassembly. To examine further how T-plastin and ABD1 might stabilize F-actin, we analysed their effects on the in vitro depolymerization kinetics of pyrene-labeled F-actin diluted to a concentration below the critical concentration of the filament minus end (400 nM), in the absence or presence of 500 nM GST-cofilin (Fig. 4). As expected, fluorescence intensity decreased in function of time when pyrene-labeled F-actin was diluted in F-buffer. By contrast, no such decrease was observed when we diluted T-plastin/F-actin filaments, suggesting that T-plastin stabilized actin filaments (Fig. 4A). A similar result was observed with ABD1 (Fig. 4B), although at higher ABD1 to F-actin ratios. This suggested that binding of ABD1 stabilized actin filaments independent of cross-link formation and is in agreement with the increased length of actin comets that we described above. When F-actin filaments were diluted in F-buffer containing GST-cofilin, these filaments rapidly depolymerized (Fig. 4C). GST alone, used as control, had no effect on depolymerization kinetics (data not shown). Almost no decrease in fluorescence intensity was observed when actin co-polymerized with T-plastin (1:1) was diluted in the presence of GST-cofilin. Similarly, ABD1 also protected actin filaments against GST-cofilin-induced depolymerization, but at higher ratios of ABD1 to GST-cofilin (Fig. 4D); indeed, compared with full-length T-plastin, four times more ABD1 was needed to inhibit

**Fig. 4.** T-plastin or ABD1 stabilize actin filaments and protect them against cofilin-mediated depolymerization. G-Actin (4 µM, 25% pyrene labeled) was co-polymerized with various concentrations of T-plastin or ABD1. To induce depolymerization, T-plastin- or ABD1-decorated pyrene-labeled F-actin was diluted under the critical concentration of the filament minus end (400 nM), in the absence or presence of 500 nM GST-cofilin (Fig. 4). As expected, fluorescence intensity decreased in function of time...
depolymerization. These results confirmed the F-actin-stabilizing capacity of T-plastin and ABD1.

It is documented that physiological Ca\(^{2+}\) concentrations (1-2 µM) inhibit the F-actin binding of L-plastin (Namba et al., 1992; Pacaud and Derancourt, 1993), raising the possibility that the stabilizing effects we observed with T-plastin were also Ca\(^{2+}\)-dependent. Because cell extracts contain other Ca\(^{2+}\)-regulated proteins like gelsolin that might also contribute to actin turnover in comets (Samarin et al., 2003), we decided to examine the effect of Ca\(^{2+}\) in the F-actin depolymerization assay (Fig. 5). Importantly, when F-actin was co-polymerized with T-plastin in the presence of free Ca\(^{2+}\) concentrations ranging from 4.6 nM to 1.6 µM, almost identical depolymerization curves were obtained (Fig. 5A). Next, we verified that T-plastin indeed bound F-actin in the presence of Ca\(^{2+}\) using an F-actin co-sedimentation assay. Free Ca\(^{2+}\) concentrations up to 2.2 µM did not affect the binding of T-plastin to F-actin (Fig. 5B). Conversely, F-actin binding of the closely related L-plastin isoform was Ca\(^{2+}\)-sensitive when analysed under identical experimental conditions (data not shown), as observed previously (Namba et al., 1992; Pacaud and Derancourt, 1993). This result confirmed the observation that plastins differ in their Ca\(^{2+}\) regulation, as previously proposed (de Arruda et al., 1990; Lin et al., 1994).

To examine whether other F-actin-binding domains also stabilized and elongated actin comets, we analysed the effects of the C-terminal F-actin domain of the F-actin-bundling protein villin (Friederich et al., 1992) in our bead assay. This 67-residue-long domain, called the headpiece, binds F-actin but does not bundle filaments (Glenney et al., 1981a). In addition, it was previously shown to compete with ADF, a cofilin family member, for binding to actin filaments using a F-actin co-sedimentation assay (Pope et al., 1994). Accordingly, we found using a similar assay that also cofilin interfered with binding of villin headpiece to F-actin (data not shown). As observed with ABD1, addition of villin headpiece (1 µM) to cell extracts that do not contain villin (Fig. 6) caused an approximately doubled actin comet length (6.70±2.05 µm) compared with control beads (3.89±0.94 µm). Addition of BSA (1 µM), an unrelated protein that does not bind actin, did not induce elongation of actin comets (3.53±0.74 µm), showing that the phenotype observed with villin headpiece was specific. These results suggest that structurally distinct F-actin binding domains might share common properties, contributing to the control of actin turnover.

The bead assay enabled us to reveal an unexpected property of ABD1 in comet tail stabilization. To corroborate this result, we analysed the effects of ABD1 in the more complex environment of a living cell (Fig. 7). Fibroblast-like Vero cells were transfected with a DNA construct encoding vsv-tagged ABD1. Overexpression of ABD1 caused the formation of long, T-Plastin-dependent force generation

**Fig. 5.** Wild-type T-plastin stabilizes actin filaments in a Ca\(^{2+}\)-independent manner. (A) Depolymerization of T-plastin-decorated pyrene-labeled F-actin in the presence of increasing free Ca\(^{2+}\) concentrations. G-Actin (4 µM, 25% pyrene labeled) was co-polymerized with T-plastin (4 µM) in the presence of increasing concentrations of free Ca\(^{2+}\) (4.6 nM to 1.6 µM). F-Actin was diluted and depolymerization was measured as described in Fig. 4. Control (actin) was the depolymerization of F-actin in the absence of T-plastin. (B) Co-sedimentation of T-plastin with F-actin in the presence of increasing free Ca\(^{2+}\) concentrations. G-Actin (7 µM) was co-polymerized with T-plastin (3 µM) in the presence of increasing concentrations of free Ca\(^{2+}\). Proteins were centrifuged at high speed to sediment actin filaments. Proteins in supernatants (S) and pellets (P) were separated by SDS-PAGE under reducing conditions. Protein bands were stained with Coomassie Brilliant Blue. The migration positions of plastin and actin are indicated. Notice that, owing to the presence of Ca\(^{2+}\), more actin is present in the supernatant than observed in the experiment in Fig. 2.

**Fig. 6.** The C-terminal F-actin-binding domain of villin causes the elongation and densification of F-actin comets. Beads were incubated in HeLa-cell extracts in the presence of villin headpiece domain (VHP, 1 µM) or its absence (control). Phase-contrast images are shown.
prominent actin cables spanning the cell body and peripheral, actin-rich membrane extensions that were not observed in neighboring (Fig. 7A, 1,2,4,5). Co-staining of cells for α-actinin (Fig. 7A, 3) or VASP (Fig. 7A, 6), two proteins that contribute to the assembly and stability of actin networks, showed that they were recruited to these structures. As reported in previous studies (Ichetovkin et al., 2002), cofilin antibody yielded a diffuse staining pattern that did not co-distribute with the actin cables, even in untransfected control cells (data not shown). In agreement with a stabilizing effect, ABD1-induced cables were more resistant to latrunculin A, an F-actin depolymerizing drug that sequesters actin monomers (Spector et al., 1989), than those of neighboring untransfected cells (Fig. 7B, 1,2). These results confirmed that ABD1 reorganizes and stabilizes F-actin under cellular conditions and suggested that it might act in synergy with other actin-regulating proteins. Accordingly, measuring the α-actinin to actin ratios of fluorescence intensities in the bead assay showed that significantly more α-actinin (α-actinin/actin ratio of 1.4) was recruited to the actin comets in cell extracts supplemented with ABD1 (1 µM) compared with control to extracts (α-actinin/actin ratio of 1.09).

Discussion
We used a simplified, quantitative cell-free assay for actin-based motility to gain new insights into the mechanism by which T-plastin, a representative CH-domain actin-bundling protein, might contribute to actin-based force generation and actin assembly. In our cell-free motility assay, T-plastin enhanced comet formation, stabilized the comets and increased bead velocity. Importantly, these effects were also observed with the bundling-incompetent ABD1 variant, arguing for a mechanism independent of actin-filament bundling. Also in line with the stabilization effect, we found that T-plastin and ABD1 protected filaments from cofilin-mediated depolymerization in vitro. Taken together, these findings give, to our knowledge, the first direct evidence that T-plastin can modulate actin dynamics and generate force independent of cross-bridge formation in a WASP-Arp2/3-dependent polymerization system, a mechanism that might be applicable to other F-actin-stabilizing and/or bundling proteins, as suggested by our results with villin headpiece domain.

How do T-plastin and ABD1 stabilize actin comets and increase bead velocity? Actin-cross-linking proteins were generally thought to generate force by organizing actin filaments into bundles (Loomis et al., 2003; Vignjevic et al., 2003). Although filament cross-linking activity is obviously required for their biological function in microvillus formation (Friederich et al., 1989; Loomis et al., 2003), our results indicate that this might be only part of the activity of T-plastin. Our assays allowed us to separate the effects of F-actin bundling and binding of T-plastin. ABD1 stabilized actin comets and induced their elongation and densification, as did wild-type T-plastin. The in-vitro F-actin depolymerization assay showed that both T-plastin and ABD1 stabilized actin filaments, protecting them from depolymerization. Based on our findings, at least two mutually non-exclusive and potentially synergistic mechanisms might account for these effects. ABD1 might stabilize actin filaments by binding along the side of the actin filament (Hanein et al., 1997), thereby affecting its dynamics. Consistently, ABD1 of the closely related L-plastin isoform also inhibited F-actin depolymerization (Lebart et al., 2004). In addition, ABD1 might protect actin filaments against the depolymerizing action of cofilin, a protein that controls actin turnover (Carlier et al., 2003; Pollard and Borisy, 2003) and polymerization by generating free barbed ends (Ghosh et al., 2004). In agreement with such a mechanism, yeast plastin also

![Fig. 7. ABD1 stabilizes F-actin structures in transfected cells.](image-url)
stabilized F-actin structures by displacing cofilin (Nakano et al., 2001). Whether T-plastin and cofilin compete for binding to the same site on the actin filament or might indirectly influence their binding by inducing a conformational change in the actin subunit, as supported by structural and biochemical data (Hanein et al., 1998; McGough et al., 1997), remains to be experimentally evaluated. Our data do not exclude the possibility that T-plastin also protects actin filaments from depolymerization caused by other proteins, as suggested by the observation that plant plastin inhibits profilin-induced actin disassembly (Kovar et al., 2000).

The rate of actin-based movement is correlated with the rate of actin polymerization (Theriot et al., 1992). Consistently, we observed a small but significant increase in rhodamine-actin close to the bead surface, where actin assemblies (Bernheim-Grosawsser et al., 2002), and a 1.5-times increase in bead velocity. Notably, T-plastin or ABD1 were previously shown to bind more efficiently to F-actin when present during polymerization (Hanein et al., 1997), suggesting that these proteins might be incorporated into actin filaments as soon as they are nucleated at the bead surface. Because T-plastin does not affect the polymerization kinetics of pure actin in vitro (data not shown), it is unlikely that this protein increases the rate of actin polymerization by keeping Arp2/3-nucleated filaments uncapped or by nucleating new free barbed ends. Considering the newly discovered biochemical property of T-plastin (or ABD1), it might act through this stabilizing effect and influence the elongation phase of actin filaments nucleated by Arp2/3 or generated by cofilin-mediated severing. In line with this, a recent study using a semi-in-vitro *Listeria*-based motility assay showed that, when Arp2/3 activity is inhibited after nucleation, cross-linking proteins are absolutely required for filament elongation and bacterial movement (Briehler et al., 2004). Stabilizing the capped actin filaments that are distributed throughout the comet, T-plastin or ABD1 might consequently prevent actin depolymerization, causing the densification and lengthening of the comet.

ABD1 does not cross-link actin filaments but does induce the formation of dense actin comets in the bead assay and straight actin cables in cells. α-Actinin and VASP, proteins that control actin organization and assembly (Pavalko and Burridge, 1991; Sechi and Wehland, 2004), were recruited to these structures, suggesting that ABD1 might cooperate rather than compete with these proteins. Conceivably, cell extracts might contain additional factors acting in synergy with T-plastin, as suggested by our results, or increasing its activity, as shown in other cell-based studies (Galkin et al., 2002; Ohsawa et al., 2004). This might also explain why α-actinin, a plastin-related protein, did not affect the velocity of the *Listeria* bacteria in a cell-free motility assay based on a minimal set of purified proteins required for actin-based movement (Loisel et al., 1999).

Taken together, these findings support the view that the balance of the antagonist F-actin-stabilizing effect of T-plastin or ABD1 and the destabilizing activity of cofilin are important for the assembly and turnover of F-actin structures, as also highlighted in a study addressing the antagonist activities of cofilin and tropomyosin homologs in *Caenorhabditis elegans* (Ono and Ono, 2002). Recently, it was shown that cofilin not only depolymerizes actin but also generates free barbed ends by severing actin filaments (Ghosh et al., 2004). Although stabilization of short-lived actin filaments by T-plastin or ABD1 might increase force, large amounts of these proteins might impede actin turnover, yielding the opposite effect. This might explain why a bell-shaped curve was obtained when bead velocity was measured as a function of T-plastin or ABD1 concentrations. Consistently, other proteins that regulate actin dynamics (including VASP, profilin, or Arp2/3) yielded a dose-dependent, bell-shaped velocity curve in a similar cell-free motility assay measuring the rate of *Listeria* movement (Loisel et al., 1999).

Our observation that the C-terminal headpiece domain of villin also increased the length of actin comets suggests that the stabilizing effects observed with ABD1 might be a more general property of F-actin-binding domains. Both domains influence F-actin dynamics and interfere with binding of cofilin (Pope et al., 1994). However, the small villin domain did not prevent filament disassembly in the F-actin depolymerization assay (data not shown). Thus, this domain might stabilize actin filaments less efficiently than ABD1. The F-actin-stabilizing capacity of these actin-binding domains might depend on their contact interfaces with adjacent subunits of the actin filament (Hanein et al., 1998; Van Troyes et al., 1999). However, the precise mechanism by which these domains regulate actin dynamics remains to be elucidated.

What might be the functions of T-plastin in vivo? Our findings support the view that T-plastin contributes to protrusive force generation in two ways – by cross-link formation and by filament stabilization. This is in agreement with the observation that overexpression of these protein in cultured epithelial cells can cause the elongation of microvilli (Arpin et al., 1994). In line with a role in the biogenesis of membrane protrusions, T-plastin associates with the developing actin cytoskeleton of embryonic intestinal microvilli (Chafel et al., 1995) and stereocilia of the inner ear (Daudet and Lebart, 2002). Based on our data, T-plastin might control actin turnover and the length of the actin filaments in these structures, which is crucial for their biological function (Schneider et al., 2002). T-Plastin is associated not only with the F-actin bundle of epithelial cell microvilli but also with the leading edge of migrating fibroblasts (Arpin et al., 1994), which contains a branched network of filaments (Svitkina and Borisy, 1999) that this protein might stabilize. In line with this, yeast plastin is required for the stabilization of Arp2/3-dependent branched network of filaments in actin patches (Young et al., 2004).

Taken together, our work suggests that T-plastin causes the reorganization and elongation of an actin structure initiated by the WASP-Arp2/3 complex polymerization unit, and concomitantly increase actin-based force. This provides a framework in which we can further elucidate the function of cross-linking proteins in the morphogenesis of membrane protrusions.

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