THE LIM DOMAINS OF WLIM1 DEFINE A NEW CLASS OF ACTIN BUNDLING MODULES
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Running head: LIM domains in actin bundling
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Actin filament bundling, i.e. the formation of actin cables, is an important process that relies on proteins able to directly bind and crosslink subunits of adjacent actin filaments. Animal cysteine-rich proteins and their plant counterparts are two-LIM domain-containing proteins that were recently suggested to define a new family of actin cytoskeleton regulators involved in actin filament bundling. We here identified the LIM domains as responsible for F-actin binding and bundling activities of the tobacco WLIM1. The deletion of one of the two LIM domains reduced significantly, but did not entirely abolish, the ability of WLIM1 to bind actin filaments. Individual LIM domains were found to interact directly with actin filaments, although with a reduced affinity compared to the native protein. Variants lacking the C-terminal or the inter-LIM domain were only weakly affected in their F-actin stabilizing and bundling activities and trigger the formation of thick cables containing tightly packed actin filaments as does the native protein. In contrast, the deletion of one of the two LIM domains negatively impacted both activities and resulted in the formation of thinner and wavier cables. In conclusion, we demonstrate that the LIM domains of WLIM1 are new autonomous actin binding and bundling modules that cooperate to confer WLIM1 high actin-binding and bundling activities.

Not only is actin one of the most abundant and conserved proteins in eukaryotes, it also plays a central role in diverse physiological processes such as cell division, adhesion and motility, muscle contraction, organelle and vesicle trafficking and cytoplasmic streaming. Within the cytoplasm, monomeric globular actin (G-actin) assembles into actin filaments (F-actin) that constitute the basic functional elements of the actin cytoskeleton. Depending on cellular requirements, they elongate or depolymerize, form fine networks or assemble into more rigid cables also called actin bundles. The plasticity and dynamic nature of the actin cytoskeleton are dictated by the multiplicity of its functions, and rely on a complex regulation system orchestrated by hordes of regulators, namely the actin-binding proteins (ABPs). ABPs bind directly to G-actin and/or actin filaments to promote actin filament nucleation, polymerization/depolymerization, severing, stabilization, capping, as well as the assembly of actin networks and bundles (1, 2). In animal cells, actin bundles provide mechanical support to the cytoplasm and reinforce cellular protrusions and invaginations. Consequently they play particularly important roles during cell morphogenesis, division, adhesion, motility and signaling. In plant cells, actin bundles serve as tracks for intracellular transport and confer stability to transvacuolar strands (3, 4, 5, 6). In addition, the defects in actin cytoskeleton organization observed in plant mutants impaired in trichome morphogenesis or in root cell expansion strongly suggest that actin bundles are also required for proper cell elongation and morphogenesis (7, 8, 9).

The assembly of actin filaments into higher order F-actin structures is driven by a subset of ABPs called actin cross-linkers or...
bundlers. The latter usually display a modular organization comprising two actin-binding domains (ABDs), variable spacer domains and regulatory domains that confer sensitivity to stimuli such as modifications in the calcium concentration (10, 11, 12, 13). The length of the spacer domain that separates the ABDs is a critical determinant of the nature of higher order structures induced, i.e. tightly packed bundles or loose assemblies. Depending on the type of ABD present in their amino acid sequence, ABPs can be classified into different families. As an example, members of the spectrin family, including α-actinin, β-spectrin, utrophin, dystrophin and fimbrin, use pairs of calponin homology (CH) domains as ABD (14). In the case of fimbrin, two pairs of CH domains are arranged in tandem on the same polypeptide chain without any spacer, directing the formation of tightly packed bundles. In contrast, other cross-linkers such as α-actinin and β-spectrin require protein homodimerization to achieve a bivalent organization that supports the crosslinking of adjacent actin filaments.

In animal cells, a multitude of actin bundling proteins has been identified and characterized. During the last few years it has become clear that all bundles are not functionally equivalent and that cells utilize different combinations and sequences of actin bundling proteins to build the adapted actin structures with specific properties (10). The deciphering of the cooperative action of actin cross-linking proteins, such as alpha-actinin and fascin, has highlighted the evolutionary benefit conferred by the existence of multiple actin cross-linking proteins with overlapping but non-identical biochemical properties (15, 16). In comparison, rather little is known about the molecular mechanisms underlying actin bundle regulation in plants. However, substantial progress has been achieved through the characterization of important plant actin bundling protein families including villins (17, 18, 19), fimbrins (20, 21) and formins (22, 23). We recently suggested the existence of an additional family, namely the plant LIM-domain-containing (LIM) protein family (24, 25).

The LIM domain is a tandem zinc finger structure of approximately 55 amino acids that is found in numerous eukaryotic proteins. It functions as a modular protein-binding interface that targets proteins to subcellular locations and triggers the assembly of protein complexes. Through interaction with protein partners, LIM proteins participate in diverse biological processes including the regulation of gene expression and cytoskeleton organization. Animal proteins contain 1 to 5 LIM domains, which can be associated with other domains such as homeodomains, kinase domains or other protein-binding modules.

The tobacco WLIM1 protein contains two LIM domains as only defined domains and is structurally related to the vertebrate cysteine-rich protein (CRP) family, which comprises the three proteins CRP1, CRP2 and CRP3 (also called muscle LIM protein or MLP). WLIM1 binds directly to actin filaments with a high affinity and protects them from depolymerization (24). In addition, it triggers the formation of thick actin cables both in vitro and in vivo, suggesting that it participates to the regulation of actin bundling in plants. Like their plant counterparts, vertebrate CRPs exhibit a dual cellular localization (26). They accumulate in both the nucleus and the cytoplasm where they interact with the actin cytoskeleton. The nuclear functions of CRPs have been studied over the past two decades and it is now well established that this subset of LIM proteins are important regulators of cell differentiation and transcription (27, 28). In contrast, their actin cytoskeleton-related roles have remained obscure over a long period. CRPs were first believed to interact with actin filaments in an indirect manner through the intermediation of ABP partners such as alpha-actinin or zyxin (29, 30, 31). However, in agreement with our data on tobacco WLIM1, it has been recently demonstrated that CRP1 and CRP2 have the ability to interact with the actin filaments in a direct manner (32, 33). Importantly, CRP1 has been shown to induce actin filament bundling in vitro as well as in transformed rat embryonic fibroblasts (33). Together, these data strongly suggest that animal CRPs and plant CRP-related LIM proteins participate in the regulation of the actin cytoskeleton architecture.

Understanding the mechanism of actin filament stabilization and bundling triggered by WLIM1 and animal CRPs requires in the first instance the identification of the actin binding domains. To date, none of the ABD sequences registered in databases are present
in plant LIM or animal CRP proteins. Here, we conducted a detailed domain analysis aimed at identifying the domain(s) implicated in the actin-related activities displayed by the tobacco WLIM1 protein. Two domains were found to have autonomous actin-binding, stabilizing and, more surprisingly actin-bundling, activities: the LIM domains.

**Experimental Procedures**

*Generation of plasmid constructs*- The plasmids used for the transient transformation of BY2 cells are derived from an in-house-constructed group of plasmids (pNTL2103 and pNTL3103) produced by the assembly of the regulatory regions (35S promoter and nos terminator) and the fused coding sequences of GFP and tobacco WLIM1 in a derivate of pBluescript II KS (Stratagene). The pNTL2103 and pNTL3103 plasmids differ merely by the nature of the fusion in that the former produces the N-terminal GFP fusion and the latter the C-terminal GFP fusion. In both cases, the WLIM1-coding cDNA fragment is located between the *Nco* I and *Bam HI* restriction sites. cDNA fragments corresponding to the C-terminal domain-deleted WLIM1 variant (WLIM1ΔCt) and to the N-terminal and C-terminal single LIM domains (LIM1 and LIM2 respectively) were generated by PCR using primers containing *Nco* I and *Bam HI* restriction sites. They were inserted into the pNTL2103 and pNTL3103 plasmids from which the original full-length WLIM1 cDNA has been previously removed. The other WLIM1 variants containing an internal sequence deletion, i.e. deletion of the N-terminal LIM domain, the interLIM spacer or the C-terminal LIM domain (respectively named WLIM1ΔLIM1, WLIM1ΔIL and WLIM1ΔLIM2), have been produced using the PCR-based QuikChange method (Stratagene). All the primer sequences are available upon request. For synthesis of the 6xHis-tagged proteins, the coding sequences of WLIM1 and of the WLIM1 variants were sub-cloned into the bacterial expression vector pQE-60 (Qiagen) using the *Nco* I and *Bam HI* sites.

*BY-2 transient expression and confocal laser scanning microscopy observations*- GFP-fusion proteins were transiently expressed in BY-2 tobacco suspension cells (*Nicotiana tabacum* cv. Bright Yellow 2) maintained as described (34). Cells were subcultured every 7 days and harvested 3 days after medium renewal for biolistic bombardment. The harvested cells were filtered onto Whatman discs and placed on 0.8% agar Murashige-Skoog (MS) media plates supplemented with 0.25 M mannitol for 2-4 h. Particle preparation and biolistic assays were performed as described (35) with the following modifications: 4 mg 1.1 μm tungsten particles (Bio-Rad) were sterilized in 1 ml absolute alcohol for 20 min. Particles were then mixed with 10 μg plasmid DNA supplemented with 18% glycerol, 1.5 M CaCl₂ and 90 mM spermidine in a final volume of 180 μl. The firing distance was 11 cm and helium pressure 7 bars. After bombardment, cells were transferred to 0.8% agar MS media plates and incubated in the dark for 16 h at 28°C. BY-2 transfected cells were collected under HBO binocular microscope (excitation/emission wavelength 488/505–545 nm) 16 h post-bombardment and cultured in MS liquid media prior to confocal laser scanning microscopy (CLSM) observations. GFP fluorescence in transfected BY-2 cells was visualized by CLSM with an LSM510 Zeiss laser scanning confocal microscope (Jena, Germany) equipped with a C-Apochromat 63x, 1.2 water-immersion objective. For each construct, experiments were reproduced at least three times and 50 GFP-expressing BY-2 cells were observed in each case. Laser scanning was performed using excitation/emission wavelengths (488/505-530 nm). Image processing was carried out with LSM510 version 3.2 (Zeiss) and Photoshop 6.0 (final image assembly; Adobe Systems, San Jose, CA).

*Expression and analysis of recombinant proteins-* 6xHis-tagged proteins were expressed in M15 bacteria and purified using a Ni-NTA resin (Qiagen) following procedures described by the manufacturer. The purified proteins were concentrated in a centrifugal filter (Amicon), buffer-exchanged (100 mM NaH₂PO₄, 10 mM Tris-Cl, pH 7.0) using a 10 K molecular weight cut-off dialysis cassette (Pierce) and stored on ice. Prior to an experiment, proteins were pre-clarified at 150 000 g, checked for correct mass by SDS-PAGE analysis and their concentration was determined by Bradford assay (Bio-Rad) using BSA as standard.
High-speed and low-speed cosedimentation assays- High- and low-speed assays were respectively used to examine the actin-binding and -crosslinking properties of WLIM1 and its variants. Rabbit muscle actin (Cytoskeleton) was diluted at 2 mg/ml in A-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM CaCl2, 0.2 mM Na2ATP, 0.5 mM DTT). Polymerization was induced by the addition of actin polymerization inducer (final concentration of 2 mM MgCl2, 1 mM ATP, 50 mM KCl). Recombinant proteins were pre-clarified at 150 000 g prior to an experiment.

In high-speed experiments, F-Actin (8 μM) was incubated with different amounts of WLIM1 or WLIM1-derived peptides ranging from 0.5-48 μM (up to 72 μM for WLIM1ΔLIM1 and LIM2) for 30 min at 25°C and subsequently pelleted at 200 000 g for 45 min in an Optima TLX ultracentrifuge (Beckman) at 4°C. After the supernatant was removed, an identical volume of protein sample buffer was added to the pellet. Equal amounts of pellet and supernatant samples were analyzed by 12% SDS-PAGE and Coomassie Brilliant Blue R (Sigma) staining. The amount of protein in the pellets (actin-bound) or supernatants (unbound) was quantified using ImageJ v1.37b software (National Institutes of Health, USA). Dissociation constants (Kd) values were determined as previously described (24) by fitting the data of bound protein versus unbound protein to a hyperbolic function with Sigmaplot v10 software (Systat Software). A mean Kd (± SD) value was calculated for each protein from three independent experiments.

In low-speed cosedimentation assays, actin (8 μM) was copolymerized with different amounts of WLIM1 or WLIM1-derived peptides (1-24 μM) for 1 h at 25°C, subsequently centrifuged at 12 500 g for 30 min in a microcentrifuge at 4°C and analyzed by SDS-PAGE as previously described. After quantification, results were expressed as percentage of actin in the pellet as a function of WLIM1 or WLIM1-derived peptide concentration. The presence or absence of actin bundles was checked by direct visualization using fluorescence microscopy. An aliquot of the copolymerized actin samples was labeled with 4 μM of rhodamine-phalloidin (Sigma). 1 μl of sample was diluted in one drop of cityfluor (Agar Scientific, UK) and applied to a coverslip coated with poly-L-lysine (0.01%). Images were recorded with our Zeiss LSM510 laser scanning confocal microscope (Jena, Germany) using a pinhole set to produce optical sections approximately 2 μm thick.

F-Actin depolymerization assays- Pyrene-labeled-actin (4 μM, 25% pyrene labeled) was polymerized at room temperature by a 30 min incubation in F-buffer (0.5 mM Tris-HCl, pH 7.5, 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 absence or presence of different amounts of WLIM1 or WLIM1-derived peptides (ranging from 2 to 24 μM). To induce depolymerization, F-actin was diluted in F-buffer to a final concentration of 0.4 μM. When specified in the results section, cofolin (2 μM) was added to the dilution buffer. The fluorescence decrease was recorded over the course of 300 or 400 sec using an F-4500 fluorimeter (Hitachi; excitation at 365 nm and emission at 388 nm).

Electron microscopy- Actin (4 μM) was copolymerized with WLIM1 or WLIM1 variants as previously described for low-speed cosedimentation assays. The mixture was applied to a carbon-coated nickel grid. Actin-filament bundles were allowed to adhere to the carbon film during a few seconds and stained with 2% (w/v) uranyl acetate prior to examination with an electron microscope (Hitachi H 600).

RESULTS

Generation of GFP-fused and 6-His-tagged recombinant WLIM1 and WLIM1 variants. The tobacco WLIM1 protein is a 193 amino acid protein that comprises a short N-terminal domain (9 amino acids), two LIM domains (LIM1, LIM2, 52 amino acids each) that share about 43% identities, an interLIM spacer (IL, 48 amino acids) and a C-terminal domain (Ct, 32 amino acids). To better characterize the mechanism of action of WLIM1 and define the critical domains conferring the protein F-actin binding, stabilizing and bundling activities we generated four deletion variants (WLIM1ΔLIM1, WLIM1AIL, WLIM1ΔLIM2 and WLIM1ΔCt) as well as two single LIM domains (LIM1 and LIM2). The domain topology of WLIM1 and of the derived peptides is shown in Figure 1A. In order to study their binding properties in the cellular environment, these peptides were fused to the
fluorescent marker GFP. To further dissect the mechanism by which WLIM1 binds to, stabilizes and bundles actin filaments, recombinant proteins were expressed in *Escherichia coli* with a 6-His tag at the C-terminus, affinity-purified on a nickel-nitrilotriacetate agarose (Ni-NTA) matrix (Figure 1B) and used in a series of *in vitro* experiments.

**The in vivo WLIM1 / F-actin interaction relies on the two LIM domains of WLIM1.** The subcellular localization of GFP-fused WLIM1 deletion variants was analyzed in tobacco BY2 cells transiently transformed by biolistics. To make sure that GFP does not interfere with the proper targeting of the peptides, both N- and C-terminal fusions were constructed and tested. As the two types of fusions produced equivalent results, only those corresponding to C-terminal GFP fusions are presented here. Consistent with the pattern previously described using a stably transformed BY2 line (24), the full-length protein accumulated in both the nucleus and the cytoplasm where it predominantly associated with the actin cytoskeleton. A statistical analysis revealed that 98% of the cells exhibited this pattern whereas only 2% exhibited a diffuse fluorescent pattern in both the nucleus and the cytoplasm (Figure 2A). WLIM1∆IL-GFP and WLIM1∆Ct-GFP showed a distribution identical to that of WLIM1. Indeed, respectively 95% and 96% of the cells exhibited a sharp actin cytoskeleton labeling (Figure 2A). Such a labeling is illustrated in Figure 2B for cells expressing the WLIM1∆Ct-GFP fusion protein. In contrast, the ability of the one-LIM domain deleted variants and of the single LIM domains to interact with actin filaments in BY2 cells was dramatically impaired. Indeed, only 7%, 9%, 5%, and 3% of the cells transformed with WLIM1∆LIM1-GFP, WLIM1∆LIM2-GFP, LIM1, and LIM2 respectively displayed a clear actin cytoskeleton labeling (Figure 2A). The vast majority of the cells exhibited a diffuse fluorescent pattern or an only weak actin cytoskeleton labeling as illustrated in Figure 2B for a WLIM1∆LIM1-GFP expressing cell. Together these observations indicate that the interLIM spacer and the C-terminal domain are not essential for actin cytoskeleton targeting whereas both LIM domains are important for efficient actin-binding activity.

**The two LIM domains of WLIM1 exhibit autonomous actin-binding activity in vitro.** The actin-binding activity of WLIM1 and its deletion variants was assessed in high-speed (200 000g) cosedimentation assays. Each recombinant protein (4 µM) was incubated 1 h with prepolymerized F-actin (8 µM), the mixture was centrifuged, and the resulting pellet and supernatant fractions were analyzed by SDS-PAGE. Control experiments indicated that the recombinant proteins do not sediment when centrifuged without F-actin (data not shown). WLIM1, WLIM1∆IL and WLIM1∆Ct were found almost exclusively in the pellet fraction, indicating that these proteins interact with a high affinity with F-actin (Figure 3A). In contrast the deletion of one LIM domain impacted negatively the affinity of WLIM1 for F-actin, as indicated by the high amount of WLIM1∆LIM1 and WLIM1∆LIM2 detected in the supernatant fraction. Importantly, the interaction with actin filaments was not totally abolished, suggesting that each of the LIM domains has actin-binding activity.

To further compare the affinity for actin filaments of the WLIM1 variants, additional high-speed cosedimentation assays were performed using increasing protein concentrations. To test the hypothesis that each LIM domain possesses an intrinsic actin-binding activity, single recombinant LIM1 and LIM2 domains were included in the study. After centrifugation, the amount of protein in the supernatant and pellet fractions was quantified by densitometry and the F-actin-bound protein was plotted against unbound protein as illustrated Figure 3B for WLIM1∆Ct and WLIM1∆LIL. *K₅d* values were determined by fitting experimental data with a hyperbolic function. A mean dissociation constant (*K₅d* ± SD) value was calculated for each protein from independent experiments (Table 1). Relatively similar *K₅d* values were obtained for wild-type WLIM1, WLIM1∆IL and WLIM1∆Ct, i.e. 1.7 ± 0.2 µM, 2.6 ± 0.5 µM and 1.5 ± 0.2 µM respectively. This is consistent with the statement that the interLIM and the carboxy terminal domains are not essential for the actin-binding activity of WLIM1. In contrast, the deletion of one LIM domain significantly reduced the affinity of the protein for actin filaments. Interestingly, the deletion of LIM1 appeared more detrimental (*K₅d* = 16.6 ± 2.6 µM for WLIM1∆LIM1) than the deletion of LIM2 (*K₅d* = 8.2 ± 1.8 µM for WLIM1∆LIM2),
suggested that LIM1 has the highest affinity for actin filaments. This hypothesis was confirmed by the data obtained with single LIM domains. Indeed, mean $K_d$ values of $6.2 \pm 1.8$ µM and $16.6 \pm 1.5$ µM were respectively calculated for LIM1 and LIM2. Strikingly, mean $K_d$ values obtained for WLIM1ΔLIM1 and LIM2, which both contain the LIM2 domain as only LIM domain, do not differ significantly ($16.6 \pm 2.6$ µM and $16.6 \pm 1.5$ µM respectively). In the same way, the mean $K_d$ values obtained for WLIM1ΔLIM2 and LIM1, that both contain the LIM1 domain as only LIM domain, are similar ($8.2 \pm 1.8$ µM and $6.2 \pm 1.8$ µM respectively).

Together, these results confirm that the actin binding activity of WLIM1 is specifically attributable to its LIM domains and that each of these domains displays an autonomous ability to interact with actin filaments. However the affinity for actin filaments of single LIM domains is significantly reduced compared to the one of the wild type protein and thus the two domains cooperate to mediate full F-actin binding capacity.

**WLIM1 stabilizes actin filaments and protects them against cofilin-mediated depolymerization.** We previously reported that WLIM1 stabilizes F-actin *in vitro* as well as in tobacco BY2 cells (24). To extend these data and test whether WLIM1 could prevent actin filaments from disassembly in presence of cofilin, an F-actin severing protein that accelerates the depolymerization rate of actin filaments, we conducted new pyrene-labeled F-actin assays. After polymerization in the absence or in the presence of different amounts of WLIM1, pyrene-labeled F-actin (4 µM) was diluted to a concentration below the critical concentration of the pointed end (i.e. 0.4 µM) using a cofilin-free or a cofilin-containing (2 µM) buffer. The depolymerization kinetics were analyzed by monitoring the fluorescence intensity. In the absence of WLIM1 and cofilin, the fluorescence intensity decreased in a time-dependent manner as a result of actin filament depolymerization (Figure 4). As expected, the addition of cofilin to the dilution buffer accelerates the depolymerization rate. In contrast, when actin filaments were first copolymerized with 2 µM WLIM1 (corresponding to 0.2 µM WLIM1 in the diluted sample), the depolymerization rate was reduced whatever dilution buffer used. However, still a significant effect of cofilin could be observed. In the presence of 4 µM of WLIM1, very slow decrease of fluorescence intensity occurred and the presence of cofilin was without any significant effect. These results confirm the F-actin stabilizing activity of WLIM1 and demonstrate that WLIM1-decorated actin filaments are protected against cofilin activity.

**Actin filament stabilization is triggered by the LIM domains of WLIM1.** The relative contribution of each domain of WLIM1 to F-actin stabilization was evaluated by submitting WLIM1 variants (Figure 1) to the above-described pyrene-labeled F-actin depolymerization assay. In this set of experiments, given the lower affinity of the mutants containing only one LIM domain (see above), actin filament depolymerization was only induced by sample dilution (no cofilin). At equimolar ratio with actin (4 µM each before dilution), the deletion mutants WLIM1ΔL1 and WLIM1ΔCt showed an ability to stabilize actin filaments similar to the WT protein (Figure 5A). Indeed, fluorescence intensity remained nearly stable over time indicating that depolymerization is very low or does not occur at all. On the contrary, at the same concentrations, the peptides containing only one LIM domain, i.e. WLIM1ΔL1, WLIM1ΔL2, LIM1 and LIM2, only weakly stabilized actin filaments as shown by the rapid decrease of fluorescence intensity (Figure 5A). By increasing their concentration to 8 µM in the preincubation phase, WLIM1ΔL2 and LIM1, both containing LIM1 as only LIM domain, were found to prevent actin filaments from depolymerization (Figure 5B). In contrast, WLIM1ΔL1 and LIM2, both containing LIM2 as only LIM domain, did not stabilize actin filaments efficiently. An efficient stabilization effect could however be achieved by increasing their concentration to 18 µM before dilution (Figure 5C).

Together these data indicate that the F-actin stabilizing activity of WLIM1 is mediated by its two LIM domains which both can autonomously stabilize actin filaments *in vitro*. The observation that LIM1 is more efficient than LIM2 in stabilizing actin filaments is in agreement with the actin-binding experiments showing that LIM1 has a higher affinity for actin filaments than LIM2. In the full-length protein, both LIM domains appear to cooperate in a synergistic manner,
conferring WLIM1 a high F-actin stabilization ability.

LIM domains of WLIM1 are autonomous actin bundling elements. In view of these results, LIM domains likely also play a central role in the actin bundling activity of WLIM1. To test this hypothesis, the ability of the different WLIM1 variants to trigger the formation of actin bundles was investigated by low-speed cosedimentation assays. 8 µM F-actin was co-polymerized with increasing amounts ranging from 1 to 24 µM of each recombinant protein and subsequently submitted to a 12 500 g centrifugation, which only allows pelleting of higher order structures of cross-linked F-actin. Samples were analyzed by SDS-PAGE and the amounts of actin in the pellet and supernatant determined by densitometry. Results are expressed as the percentage of actin in the pellet fraction (Figure 6). The presence of actin bundles was always confirmed by direct visualization using a fluorescence light microscope as illustrated in Figure 6B for a number of WLIM1 variants. In agreement with our former published data, WLIM1 induced the formation of actin bundles in a concentration-dependent manner, as demonstrated by the increasing amount of actin in the pellet fraction and the long and thick actin cables observed by microscopy (Figures 6A and B respectively). Maximal bundling (about 85% of total actin is pelleted) occurred for WLIM1 concentrations higher than 4 µM. The interLIM spacer and the carboxy-terminal domain were found dispensable to the actin bundling activity since both WLIM1IL and WLIM1Ct displayed effects very similar to the wild-type protein (Figure 6A). As expected, the deletion of one of the two LIM domains reduced the ability of WLIM1 to induce actin sedimentation. As previously observed for actin binding and stabilizing activities, the deletion of LIM1 had more pronounced effects than the deletion of LIM2. Indeed, whereas WLIM1∆LIM2 exhibited an activity approximately 2-fold lower than the full-length protein, i.e. maximum of sedimentation occurred only at 8 µM WLIM1∆LIM2, WLIM1∆LIM1 was unable to sediment high amounts of actin in the range of concentrations tested (Figure 6A). By testing higher protein concentrations, we determined that 48 µM WLIM1∆LIM1 induced the sedimentation of about 85% of total actin (data not shown), indicating that the ability, although significantly reduced, to form high order F-actin structures was still preserved in WLIM1∆LIM1. The analysis of single LIM domains confirmed that each LIM domain possesses an autonomous cross-linking activity. The ability to sediment F-actin did not significantly differ between LIM1 and LIM2 except at 4 µM, a concentration at which LIM1 triggers the sedimentation of a higher amount of actin than LIM2, i.e. 45% versus 25% of total actin respectively. As could be expected, LIM1 and WLIM1∆LIM2, which contain LIM1 as only LIM domain, exhibited a similar ability to induce actin sedimentation. In contrast, the observation that the cross-linking activities of LIM2 and WLIM1∆LIM1 differed was surprising. Indeed, a lower concentration of LIM2 than of WLIM1∆LIM1 was required to achieve the maximum of sedimentation.

In conclusion, the actin bundling activity of WLIM1 is specifically attributable to its LIM domains that both can function as autonomous bundling elements in vitro. However, as already observed for actin stabilization, the N-terminal LIM domain (LIM1) was found to be more efficient, i.e. it bundles actin filaments at a lower concentration than does the C-terminal LIM domain (LIM2).

Structural organization of actin bundles as visualized by electron microscopy. The organization of actin bundles induced in the presence of WLIM1 or the different variants was examined by negative staining electron microscopy. In the control sample that contains F-actin alone (4 µM), single or intermingled curved actin filaments were observed (Figure 7A). When wild type WLIM1 (2 µM) was added to F-actin, rather straight bundles with closely aligned filaments were observed (Figure 7B). Very few free actin filaments were present in the sample. Using identical concentrations, the deletion variants lacking the C-terminal domain or the interLIM spacer generated actin bundles indistinguishable from those generated by WLIM1 (Figure 7D and F). In contrast, the bundles formed in the presence of 2 µM of one-LIM domain containing proteins, i.e. WLIM1∆LIM1, WLIM1∆LIM2, LIM1 and LIM2, presented distinct features (Figure 7C, E, G and H respectively). Indeed, bundles were thinner and, most likely as a consequence of that, had a more wavy appearance than those induced by WLIM1, WLIM1∆Ct and WLIM1∆IL. Strikingly, most
of them contained few filaments, usually two, as illustrated in Figure 7C, E, G and H. Free actin filaments were also more frequently observed than in the case of WLIM1, WLIM1ΔCt or WLIM1ΔIL, which is consistent with the weaker actin sedimentation efficiency revealed by low-speed cosedimentation experiments. Thicker actin bundles could however be induced by the one-LIM domain containing proteins but only at high protein-to-actin ratio. In addition, these bundles frequently appeared loosely packed and twisted (data not shown), indicating that they are not entirely equivalent to those formed in the presence of the full-length WLIM1.

DISCUSSION

We recently reported the characterization of a new plant actin bundling protein, namely the tobacco WLIM1 protein (24). This two LIM domain-containing protein was shown to interact directly with actin filaments and to stabilize and bundle them in vitro as well as in plant cells. The present work specifically ascribes the F-actin binding, stabilizing and bundling activities of WLIM1 to its LIM domains. Importantly, our in vitro investigations revealed that the single LIM domains can bind directly to actin filaments and stabilize these. More surprisingly, each of the single domains is able to generate bundles in an autonomous manner, suggesting a different mechanism for actin filament bundling than the canonical bundling mechanisms using two separate actin-binding units linked by a spacer or by dimerization. The lower bundling efficiency of the separate LIM domains compared to the combined domains suggests however that both LIM domains cooperate to achieve efficient bundling in vivo (see below).

The LIM domain is a tandem zinc-finger structure that functions as a protein-protein interaction module (26, 36, 37). It is common to a wide variety of eukaryotic proteins participating in diverse biological processes including regulation of gene expression, cell motility, signal transduction and cytoarchitecture (38, 39). Conserved features in the LIM sequence direct the formation of a stable structural core whereas variable features provide high-affinity binding to many structurally and functionally diverse protein partners. Several actin cytoskeleton components have been identified among these partners, including actin filaments themselves. However, this report is the first example where the LIM domains are demonstrated to be the elements directly responsible for interaction with actin filaments. Interestingly, high-speed cosedimentation assays revealed that the C-terminal LIM domain (LIM2) has a lower affinity for actin filaments than the N-terminal LIM domain (LIM1). Consistent with these data the stabilization and bundling activities caused by LIM2 were also found lower than those triggered by LIM1, suggesting sequence specific effects in the F-actin-LIM interaction. The observation that the deletion of either LIM1 or LIM2 reduced significantly the ability of the protein to bind to, stabilize and bundle actin filaments, strongly supports the fact that both LIM domains operate in concert to confer WLIM1 optimal activities. Such a synergistic cooperation between LIM domains has formerly been deduced from a domain analysis conducted on a member of the CRP family, the animal counterpart of plant LIM family members (26). Although in the case of the rat muscle LIM protein (MLP, also called CRP3), actin cytoskeleton association was attributed to the C-terminal LIM domain (LIM2), the N-terminal LIM domain (LIM1) was required for stabilization, indicating that both LIM domains are necessary for efficient actin cytoskeleton targeting in vivo. Another mapping study suggested that chicken CRP1 associates with the actin cytoskeleton via its N-terminal part which contains the LIM1 domain (30), which is the one that corresponds to the LIM domain displaying the highest F-actin affinity in tobacco WLIM1. At that time, the interaction between CRPs, including CRP1, and actin filaments was believed to be indirect, requiring intermediary proteins such as alpha-actinin or zyxin (29, 30, 31). However, it is now clearly established that CRP1 as well as other members of the CRP family such as CRP2 are autonomous F-actin binding proteins (32, 33). The present in vitro investigations provide for the first time strong arguments supporting the idea that both LIM domains participate in the F-actin binding and bundling activities displayed by a plant CRP-related protein. This perfectly correlates with in vivo data showing that the deletion of one LIM domain dramatically reduced the ability of WLIM1 to target the actin cytoskeleton in BY2 cells, whatever LIM domain deleted.
Another argument in favor of the participation of the two LIM domains of WLIM1 in actin bundling is the observation that actin bundles generated by the one-LIM domain-containing variants are not the equivalent of those induced by the full-length WLIM1. At low LIM-to-actin ratios, the bundles induced by the one-LIM domain-containing variants contain fewer filaments, usually two, than those induced by native WLIM1. In addition, the occasional thick bundles formed in presence high amounts of one-LIM domain-containing variants appear relatively loosely packed and wavy. Therefore, the deletion of one LIM domain impacts both quantitatively and qualitatively F-actin bundling, suggesting that both LIM domains of WLIM1 cooperate to crosslink F-actin in such a way as to increase the thickness and the rigidity of the bundle formed. This is consistent with the dramatic reduction of the number of actin filaments and bundles as well as with the overall increase of bundle thickness observed in WLIM1-overexpressing leaf epidermal cells (24). Supporting a biological role in actin bundle formation and maintenance, members of the plant LIM protein family, namely the pollen LIMs or PLIMs, have been reported to be expressed at particularly high levels in pollen grains (40, 41), which upon pollen tube growth develop very long actin cables orientated parallel to the tube axis (42, 43, 44).

In line with our observation that the deletion of the interLIM or C-terminal domains does not alter the actin-binding activity of WLIM1 in BY2 cells, in vitro analyses allow to conclude that WLIM1, WLIM1∆IL and WLIM1∆Ct display a very similar affinity for actin filaments. This confirms and extends earlier data indicating that the association of MLP with actin filaments is independent of the length of the interLIM spacer (26). In addition, our data suggest that the interLIM and C-terminal domains do not participate in the F-actin stabilizing and bundling activities of WLIM1. A tempting hasty conclusion would be that modifications of the interLIM spacer length could not affect actin stabilizing and bundling efficiencies. However, considering that each LIM domain functions as an autonomous in vitro actin bundling element, it could be anticipated that the length between the two LIM domains may influence the structure of the actin bundles induced by WLIM1. It is noteworthy that the length of the interLIM spacer is well conserved (~ 50 amino acid residues) among CRPs and plant LIM proteins, suggesting that it is an important requirement for their function(s). Although our electronic microscopy investigations failed to reveal any clear differences in the actin bundles generated by WLIM1 and WLIM1∆IL, it should be kept in mind that only one mutant, namely WLIM1∆IL, with one modified spacer length has been analyzed in this work. Further investigations including different interLIM spacer lengths have to be carried out to definitely state on the influence of the interLIM spacer in the bundle architecture. As an alternative, the interLIM spacer as well as the C-terminal domain may be of particular importance for WLIM1 nuclear functions (45).

Although the present data do not permit to outline definitive predictions regarding the mechanism by which WLIM1 bundles actin filaments, two putative models can be proposed (Figure 8). Each model can be subdivided into two submodels depending on whether WLIM1 dimerization occurs or not. To our knowledge, fimbrin is the only example of a ABD-containing crosslinking protein that possess two tandem repeats of ABD within a single polypeptide chain and that consequently does not require dimerization to bundle actin filaments. Although several animal LIM proteins, including CRPs, have been reported to dimerize in vitro through their LIM domains (36, 26), there is no evidence for WLIM1 dimerization from our yeast two-hybrid and GST pull-down experiments (Dieterle et al., unpublished data, 2006). In both models each LIM domain is regarded as an autonomous F-actin binding and bundling entity (requiring or not dimerization) that crosslinks two actin filaments in a parallel manner (Figure 8A). In the first model, both LIM domains from one WLIM1 molecule bind to and bundle the same filament pair so that the WLIM1 body is oriented parallel to the bundle axis in a tropomyosin-like manner (Figure 8B). In the second model, each LIM domain crosslinks distinct pairs of actin filaments. In this case, the WLIM1 body would be oriented more or less orthogonally to the bundle axis (Figure 8C). As an alternative to these two models, one can also imagine that both types of crosslink occur.
Actin regulatory proteins represent one of the best examples of the economical strategy developed by cells in which elementary modules with specific catalytic and/or recognition functions are modified and combined in a cassette-like fashion to generate the multitude of proteins required to carry out the broad array of biological functions (46). The present data indicate that the LIM domain should be added to the list of the actin-binding modules available in this toolbox of short actin-binding modules from plants, and most likely from all eukaryotes. Consistent with our finding that the LIM domains of WLIM1 display autonomous actin-binding and -bundling activities in vitro, the structural studies conducted on avian CRP1 (47) and CRP2 (48) have revealed that LIM domains can fold, and consequently function, independently from each other. Importantly, the presence of one or several LIM domains in a protein is not necessarily synonymous of actin-binding activity. Indeed, despite the overall structure conservation of the LIM domain, this latter is present in a wide variety of eukaryotic proteins that have diverse functions, including actin-unrelated ones (39). Such functional plasticity has previously been reported for other ABDs such as the calponin homology domain (14) and is also underscored by the different affinities of the two LIM domains studied here.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: CRP, cysteine-rich protein; LIM, LIM domain-containing protein

**FIGURE LEGENDS**

Fig. 1. Tobacco WLIM1 and its derived variants. (A) Domain topology of tobacco WLIM1 and the peptides used in this study. WLIM1 basically comprises a short N-terminal domain (Nt), two LIM domains (LIM1 and LIM2), an interLIM spacer (IL) and a C-terminal domain (Ct). Four deletion variants (WLIM1ΔLIM1, WLIM1ΔIL, WLIM1ΔLIM2 and WLIM1ΔCt) as well as two single LIM
domains (LIM1 and LIM2) have been generated. They were either fused to GFP to study their subcellular localization in tobacco BY2 cells or expressed in E. coli for in vitro investigations. (B) Production of wild type WLIM1 and WLIM1 variants in E. coli. About 4 μg of protein was loaded on a 12 % SDS-polyacrylamide gel and submitted to electrophoresis. Protein bands were revealed by Coomassie Blue staining. The molecular masses of standard proteins are indicated on the right.

Fig. 2. Distribution of GFP-tagged WLIM1, WLIM1 deletion variants and single LIM domains (LIM1 and LIM2) in transiently transformed tobacco BY2 cells. (A) Prevaleces of subcellular localization patterns. Only cytoplasmic patterns are considered here, i.e. diffuse cytoplasmic fluorescence or weak actin cytoskeleton labeling versus sharp actin cytoskeleton labeling. Values include all transgene-expressing cells, irrespective of the apparent expression level, which appeared unrelated to the localization of WLIM1 and WLIM1 deletion variants. For each GFP-tagged protein, more than 150 transformed cells were analyzed in at least 3 independent experiments. (B) Confocal images showing the sharp cytoskeleton labeling observed in a WLIM1ΔCt-GFP expressing cell and the weak actin cytoskeleton and diffuse labeling observed in a WLIM1ΔLIM1-GFP expressing cell. Bars: 10 μm.

Fig. 3. High-speed (200 000 g) cosedimentation assays. (A) WLIM1 or domain-deleted variants (4 μM) were incubated with polymerized F-actin (8 μM) for 30 min at room temperature. After centrifugation, the resulting pellets and supernatants were subjected to SDS-PAGE and Coomassie blue-stained. The molecular masses of standard proteins are indicated on the left. (B) Example of curves obtained for WLIM1ΔCt and WLIM1ΔLIM1 after repeating the same experiment using increasing concentrations of WLIM1 variant. After SDS-PAGE and staining, the gels were scanned and the amount of protein that was present in the pellet and the supernatant was quantified. The concentration of actin-bound protein was plotted against the concentration of free WLIM1 variant. Such experimental data were fitted with a hyperbolic function to determine $K_d$ values (see Tab. 1).

Fig. 4. WLIM1 stabilizes actin filaments and protects them from cofilin-mediated depolymerization. G-actin (4 μM, 25% pyrene labeled) was copolymerized with 2 or 4 μM of full-length WLIM1. Depolymerization was induced by the dilution of the incubated mixture 10-fold to an actin concentration below the critical concentration of the minus filament end (i.e., 0.4 μM). Depolymerization was done either in a cofilin-free or a cofilin-containing (2 μM) buffer (white and gray symbols respectively). Fluorescence intensity at the start was set at 100 and recorded over time. Control is depolymerization of F-actin in the absence of WLIM1.

Fig. 5. Actin filament stabilizing activity of WLIM1 and WLIM1 variants. G-actin (4 μM, 25% pyrene labeled) was copolymerized with 4 μM (A) or 8 μM (B) of WLIM1 or WLIM1 variant. Depolymerization was induced by dilution of pyrene-labeled actin filaments below the critical concentration of the minus end (i.e., 0.4 μM). The fluorescence intensity was set at 100 and recorded over the time. Higher concentrations, i.e. 12 and 18 μM, of LIM2 and WLIM1ΔLIM1 were tested in (C).

Fig. 6. (A) Low-speed (12 500 g) cosedimentation assays. Actin (8 μM) was copolymerized in the presence of increasing amounts of WLIM1 or WLIM1 variants ranging from 1 to 24 μM. After centrifugation, the resulting pellets and supernatants were subjected to SDS-PAGE and Coomassie blue-stained. The gels were scanned and the amounts of actin present in the pellet and in the supernatant were quantified. Results are expressed as the percentage of actin that sediments as a function of the concentration of WLIM1 or WLIM1 variant. Control experiments indicate that 15 ± 0.9% of actin sediment in the absence of LIM protein. Values represent the mean of at least three independent experiments. Bars represent the standard deviation. (B) Direct visualization of actin filaments alone (left panel) and actin bundles induced in the presence of 4 μM WLIM1ΔLIM2 (middle panel) and 4 μM WLIM1 (right panel). Bars: 100 μm.

Fig. 7. Electron micrographs of negatively stained preparations containing 4 μM F-actin and 2 μM purified recombinant WLIM1 or WLIM1 variants. (A) F-actin alone. (B-I) Details of actin
Fig. 8. Putative models of actin bundling by WLIM1. (A) Single LIM domains crosslink two actin filaments in a parallel manner. (B) First model: both LIM domains of WLIM1 crosslink the same filament pair. (C) Second model: each LIM domain of WLIM1 crosslinks distinct pairs of actin filaments. In the schemes on the left, dimerization was believed not to occur whereas the schemes on the right illustrate the situation upon LIM domain dimerization. Black circles: LIM domains; black bars: interLIM spacers; grey diamonds: actin monomers.

Table 1. Affinities of the full-length WLIM1 and WLIM1 variants for actin filaments. Mean $K_d$ values (± SD) were calculated from three independent high-speed cosedimentation experiments after fitting the data (bound protein plotted against free protein, see Fig. 3) with a hyperbolic function.
Figure 1
### Figure 2

#### Table A

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#### Figure B

![Immunofluorescence images](images)

**WLIM1ΔCt-GFP**

**WLIM1ΔLIM1-GFP**

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Figure 3
Figure 4
Figure 5
Figure 6

A

B

F-actin

+ 4 µM WLIM1ΔLIM2

+ 4 µM WLIM1

Figure 6
Figure 8
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<th>Protein</th>
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<tr>
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Table 1
The lim domains of WLIM1 define a new class of actin bundling modules
Clément Thomas, Flora Moreau, Monika Dieterle, Céline Hoffmann, Sabrina Gatti, Christina Hofmann, Marleen Van Troy, Christophe Ampe and Andre Steinmetz

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