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Structural characteristics affecting functions of two actin regulating proteins

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ABSTRACT

Structural biology is a branch of science that concentrates on the relationship between the structure and function of biological macromolecules. The prevalence of a large number of three dimensional structures offers effective tools for bio-scientists to understand the living world. Actin is the most abundant cellular protein and one of its main functions is to produce movement in living cells. Actin forms filaments that are dynamic and which are regulated by a number of different proteins. A class of these regulatory proteins contains actin depolymerizing factor homology (ADF-H) domains. These directly interact with actin through their ADF-H domains. Although ADF-H domains possess very similar three dimensional structures to one another, they vary in their functional properties. One example of this is the ability to bind to actin monomers or filaments. During the work for this thesis two structures of ADF-H domains were solved by nuclear magnetic resonance spectroscopy (NMR). The elucidated structures help us understand the binding specificities of the ADF-H family members.

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I) Hellman, M., Paavilainen, V., Annila, A., Lappalainen, P. and Permi, P. (2004) (1)H, (13)C and (15)N resonance assignments of coactosin, a cytoskeletal regulatory protein. *J. Biomol. NMR*., **30**, 365-366.

II) Hellman, M., Paavilainen,V. O., Naumanen, P., Lappalainen, P., Annila, A. and Permi, P. (2004) Solution structure of coactosin reveals structural homology to ADF/cofilin family proteins. *FEBS Letters*., **576**, 91-96.

III) Hellman, M., Paavilainen, V. O., Annila, A., Lappalainen, P. and Permi, P. (2006) NMR assignment of the C-terminal ADF-H domain of an actin monomer binding protein, twinfilin. *J. Biomol. NMR*., **36**, 66.

IV) [1]Paavilainen, V. O., [1]Hellman, M., Helfer, E., Bovellan, M., Annila, A., Carlier, M-F., Permi, P. and Lappalainen, P. (2007) Structural basis and evolutionary origin of actin filament capping by twinfilin. *Proc. Natl. Acad. Sci. USA*., **104**, 3113-3118.

V) Mattinen, M-L., Hellman, M., Permi, P., Autio, K., Kalkkinen, N. and Buchert, J. (2006) Effect of protein structure on laccase-catalyzed protein oligomerization. *J. Agric. Food Chem.*, **54**, 8883-8890.

[1]) These authors contributed equally to the work.

ABBREVIATIONS

5-LO	5-Lipoxygenase
A	Acidic region
Abp1	Actin binding protein 1
ADF	Actin depolymerizing factor
ADF-H	Actin depolymerizing factor –homology
Aip1	Actin-interacting protein 1
Arp	Actin related protein
C	Connector region
CLP	Coactosin-like protein
FAF	Familial amyloidosis Finnish type
FH	Forming homology
FID	Free induction decay
FT	Fourier transformation
F-actin	Filamentous actin
G1-G6	Gelsolin domains 1-6
G-actin	Monomeric actin
MW	Molecular weight
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NPF	Nucleation promoting factor proteins
Pi	Inorganic phosphate
PP	Protein phosphatases
RDC	Residual dipolar coupling
PDB	Protein data bank
RMSD	Root mean square deviation
TwfN	N-terminal domain of twinfilin
TwfC	C-terminal domain of twinfilin
WH	Wiskott-Aldrich syndrome protein homology domain

1. INTRODUCTION

Proteins maintain vital processes in living organisms. They catalyze chemical reactions, carry and store other molecules in addition to participating in targeting compounds at appropriate locations. Some proteins impart shape and rigidity to organs and cells, whereas others are responsible for movements and plasticity. Protein dysfunctions are involved in several serious diseases that have a genetic aetiology. Mutations of the genes, whether innate, hereditary or age related, can cause severe abnormal protein folding and/or other protein dysfunctions (Mandemakers et al., 2007 and Kärkkäinen and Peuhkurinen, 2007). For example, accumulation of protein aggregates due to misfolding is the basis of many neurolopathological symptoms and signs in such conditions as Alzheimer's disease (Chiti and Dobson, 2006, Outeiro and Tetzlaff, 2007).

The structure of a protein is assembled from building blocks of ordered combinations of 20 different amino acids joined together to form heteropolymers. The specific sequential order of amino acids provides the primary structure of the protein and it is encoded by the nucleotide sequence of its gene. The primary structure gives rise to the secondary structure in which the protein takes up either spiral α-helices or flat β-sheets configurations or both. The primary and the secondary structures cannot give a protein its characteristic properties by themselves. However, the primary structure carries the information for a protein's three dimensional tertiary structure. It is the latter that actually determines the function of the protein. When a protein folds and takes up its three dimensional configuration or tertiary structure, some of its amino acids are packed into the core of the protein. In contrast other amino acids remain on the surface of the structure and determine the physico-chemical properties of the protein. These physico-chemical properties include electrical charge and hydrophobicity (Chothia, 1984 and Chothia and Finkelstein, 1990). Some amino acids form cavities and pores such as those found in enzymes. Special features on a protein's surface determine its interaction sites with small molecules or with other proteins. These features are often chemically complementary and also take up complementary shapes to the molecules with which they interact (the specific binding partner). The highly specific interaction sites of the protein are usually rigid, which allows the precise matching to a ligand by the exactness of fit. On the other hand, proteins with unspecific binding abilities usually have more flexible binding sites (Tsai, et al., 1999, Yuan et al., 2003, Karplus and Kuriyan, 2005, Bhalla et al., 2006, Gunasekaran and Nussinov, 2007).

Innovations in gene technology methods, particularly the production of the recombinant proteins, have revolutionized protein research. Large numbers of proteins have been made more easily available. Genetic manipulations of a gene sequence such as point mutations, are widely used for understanding the biochemical/biological functional features and mechainsims of proteins. Furthermore, as a result of these novel methodologies in protein production, data on protein structures have increased enormously and their specifications can be found in the Protein Data Bank (PDB); http://www.rcsb.org. Large worldwide structural

genomic projects have elucidated thousands of new structures, which have been deposited in the PDB. These include proteins that are already biochemically characterized and also structures of proteins with as yet unknown functions. However, prediction of protein's function from its three dimensional structure is not straightforward, because similar functions can be carried by proteins with different fold topology features. On the other hand, different functions can be carried out by proteins that have very similar fold features to each other (Todd et al., 1999, reviewed in Whisstock and Lesk, 2003). Further knowledge and experience need to be acquired from related proteins with known structures and functions in order to address the underlying questions of the structure/function relationship.

Currently there are two methods available for solving protein structures at atomic resolution, X-ray crystallography and multidimensional nuclear magnetic resonance spectroscopy (NMR). The more widely used X-ray crystallography is based on X-ray radiations and their subsequent diffraction from protein samples. X-rays are scattered primarily by the electrons of the target atoms of the protein hence their diffraction is proportional to the protein's respective constituent target atom's atomic number, i.e. electron densities (Drenth, 1999). Throughout the X-ray and its subsequent data collection procedure, the protein sample is in the crystalline state.

The NMR spectroscopy of proteins is based on the magnetic properties of the nuclei of a molecule's constituent atoms (Abragam, 1961, Wüthrich, 1986). The spectrometer is a large very powerful superconductive magnet, in whose magnetic field the spins of the nuclei of the molecules (in this case proteins) orientate themselves parallel to those of the magnetic field. These orientations are then disturbed by a radio frequency pulse. Subsequent recovery of the spin after the radio frequency pulse ends can be measured as free induction decay (FID), which is further transformed into frequency domain by Fourier transformation (FT). The manipulation of the magnetization carried by the nuclei, is achieved by a set of radio frequency pulses, given at particular frequencies at certain time intervals. The theory behind NMR spectroscopy is based on quantum mechanics (Abragam, 1961, Ernst et al., 1986). During NMR data acquisition, the protein sample can be in solution.

The structure determination by NMR is currently routine for proteins with a molecular weight less than 25 kDa. However, studies of larger proteins are possible by applying modern labeling techniques in combination with improved instrumentation and NMR methodologies (Kay and Gardner, 1997, Pervushin et al., 1997, Goto and Kay, 2000, Tugarinov et al., 2004, Ozawa et al., 2005, Kovacs et al., 2005, Kainosho et al., 2006). These have enabled NMR studies of large proteins and complexes (Salzmann et al., 2000, Fiaux et al., 2002, Tugarinov et al., 2002, Jain et al., 2004). The determination of protein structure by NMR is currently no more time-consuming than that of X-ray diffraction, contrary to was believed earlier. The resonance assignment and the structure calculation with an automated backbone assignment and structure calculation programs have sped up the structure determination of a protein to an

acceptable level (Nilges, 1995, Bartels et al., 1997, Zimmerman et al., 1997, Güntert, 2003, Jung and Zweckstetter, 2004).

Requirements for sample used for the protein structure determination by both X-ray and NMR share similar characteristics. Samples have to be: chemically pure, i.e. free from contaminants, homogeneous, i.e. free from unspecific multimerization, stable during data collection, available in large quantities, and soluble at high concentrations. If the protein fulfills the above mentioned requirements, the structure can usually be determined by either method. However, if the protein contains highly mobile parts, crystallization may fail. On the other hand, for larger systems X-ray is often an easier and cheaper way to determine a three dimensional structure, as protein enrichment with ^{15}N and ^{13}C isotopes is not required. X-ray and NMR are not competitive methods, and should therefore be used to complement each other.

In the present work, the structural information of two actin depolymerizing factor homology (ADF-H) domains, the mouse coactosin-like protein (CLP) and the C-terminal domain of twinfilin, have been acquired by NMR spectroscopy. Structural data are compared to other three dimensional structures of ADF-H domains and related to biochemical properties of the protein family. As there are several three dimensional structures already available from different ADF-H domains, they offer an attractive opportunity to investigate how minor structural differences influence a protein's function –the essence of structural biology.

2. BIOLOGY OF ACTIN

Cells have two actin-based mechanisms to generate movement (Mitchison and Cramer, 1996). One is based on motor proteins sliding past filamentous tracks. In the striated muscle cells, myosins are the 'motors' and actin filaments are the 'tracks'. Both are referred to as thick and thin filaments, respectively. They join together to form sarcomeres, which are the smallest contraction units of the muscle. The other mechanism that produces movement is based on assembling and disassembling filaments (Mitchison and Cramer, 1996). The cellular cytoskeleton is composed of an actin cytoskeleton, microtubules and intermediate filaments. In all animal cells the actin cytoskeleton also participates in maintaining the cell's shape (Lodish et al., 2003). Actin forms bundles and network-like structures that are connected by actin binding proteins. The latter are further anchored onto membranes, or membrane proteins, or to different cytoskeletal elements (Winder and Ayscough, 2005). During morphological changes the structure of the actin cytoskeleton is reorganized (Lodish et al., 2003). There are several important examples of processes that involve the dynamic actin cytoskelton. These examples include: morphogenetic movements, endocytosis, T-cell dependent immune responses, the development and remodeling of the nervous system, in addition to wound healing (Matus, 2000, Engqvist-Goldstein and Drubin, 2003, Huang and Burkhardt, 2007).

2.1 FORMS OF ACTIN

Actin is one of the most abundant cellular and most conserved proteins in eukaryotes. There are also actin-like proteins in prokaryotes, but they seem to have slightly different functions to those of actin in eukaryotes (Shih and Rothfield, 2006 and Michie and Löwe, 2006).

Actin exists in cells both as a monomeric, globular shaped form (G-actin, Figure 1A) with a molecular mass of ~42 kDa and as filamentous forms (F-actin, Figure 1B). Actin binds ADP or ATP in addition to divalent cations of magnesium (Mg^{2+}) or calcium (Ca^{2+}) (Strzelecka-Golaszewska, 2001). Several three dimensional crystal structures of actin have been solved for example in complex with DNAseI (Kabsch et al., 1990), with different gelsolin segments (McLaughlin et al., 1993, Kazmirski et al., 2002, Choe et al., 2002, Burtnick et al., 2004), and with profilin (Schutt et al., 1993). Structures of uncomplexed actin in ADP and ATP bound forms have also been determined (Otterbein et al., 2001, Graceffa and Dominguez, 2003). To date atomic resolution structures of actin filament are not available, but the Holmes model (Holmes et al., 1990) of the actin filament (Figure 1B) introducing the double-stranded helical structure is still in good agreement with the current knowledge of actin (Aguda et al., 2005).

The nomenclature of the filament ends is based on the arrow-head-like appearance of the filaments, which originates from the myosin subfragment S1 decoration along the filament (Schröder et al., 1993). One end is referred to as the pointed end and the other as the barbed

end. An important feature of actin is that ADP-G-actin and ATP-G-actin have different affinities for the two filament ends. ATP-G-actin has the highest affinity for the barbed end of the filament, and under conditions found in living cells. Thus the filament elongation occurs most often at the barbed end. In a newly formed filament, the actin bound ATP undergoes a rapid hydrolysis of ~2 s (Blanchoin and Pollard, 2002) to ADP-Pi followed by the release of the inorganic phosphate (Pi), which is slow ~6 min (Carlier and Pantaloni, 1986, Melki et al., 1996, Blanchoin and Pollard, 1999). At the pointed end of the filament, the ADP-G-actin dissociation dominates over the association processes. This generates a situation where actin filaments have equal rates for the barbed end polymerization and the pointed end depolymerization, known as an actin treadmilling. Thus the length of the filament remains unchanged, though the filament continuously shifts in the direction in which the barbed end is pointed. Favorable electrostatic environments facilitate the head-to-tail polymerization of actin, whereas the physiological ionic concentration promotes the filament formation. The characteristic biochemical properties of both filament ends are based on their chemical and functional directional orientations (Pollard et al., 2000, Pollard and Borisy, 2003, Moseley and Goode, 2006a).

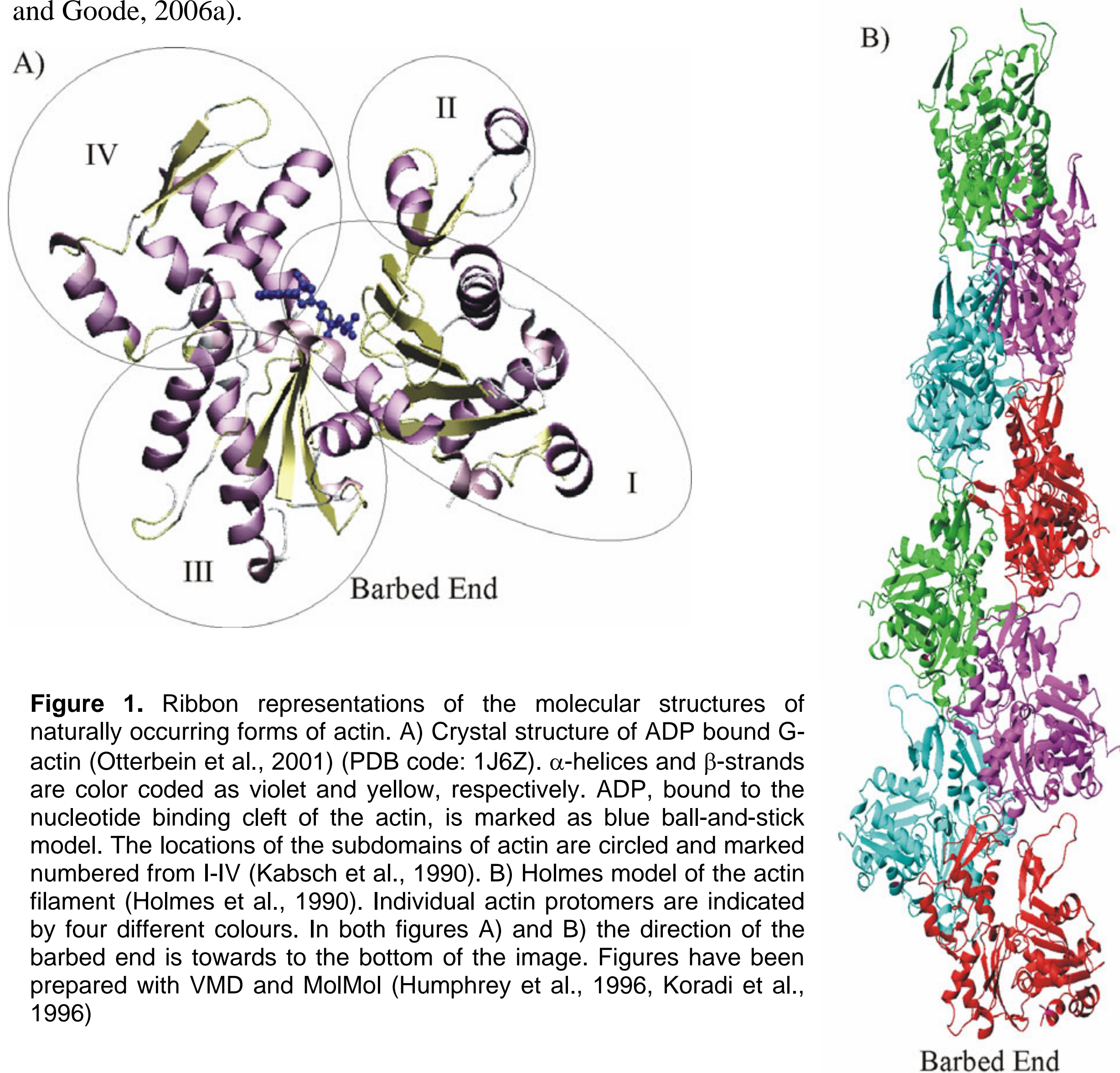

Figure 1. Ribbon representations of the molecular structures of naturally occurring forms of actin. A) Crystal structure of ADP bound G-actin (Otterbein et al., 2001) (PDB code: 1J6Z). α-helices and β-strands are color coded as violet and yellow, respectively. ADP, bound to the nucleotide binding cleft of the actin, is marked as blue ball-and-stick model. The locations of the subdomains of actin are circled and marked numbered from I-IV (Kabsch et al., 1990). B) Holmes model of the actin filament (Holmes et al., 1990). Individual actin protomers are indicated by four different colours. In both figures A) and B) the direction of the barbed end is towards to the bottom of the image. Figures have been prepared with VMD and MolMol (Humphrey et al., 1996, Koradi et al., 1996)

In muscle cells, actin is mainly found in relatively stable filamentous forms, whereas in non-muscle cells significant amounts exist in the monomeric form (Pollard et al., 2000). The monomeric actin pool acts as a power source for accelerated dynamics of the cell, thus the rate of the treadmilling is dependent on the actin monomer concentration. The term, critical concentration of actin, is used to define the actin monomer concentration, at which the polymerization and depolymerization rates are equal. Depending on the cell type, the net turnover rate *in vivo* can be 200 times faster than that *in vitro* (Pollard et al., 2000). Accelerated actin dynamics are regulated by more than 60 protein families (Pollard et al., 2000).

2.2 MECHANISMS OF ACTIN REGULATION

Several proteins that regulate actin dynamics have been characterized. Some of them indirectly affect the actin dynamics. One example of this is the activation of binding proteins. However, many of the regulatory proteins interact directly with either the G-actin or with the F-actin. The molecular weights of the actin binding proteins range widely, and their three dimensional structures vary significantly. The following sections discuss the findings of some of the many recent studies on the main regulation mechanisms of actin including: nucleation, monomer sequestering, filament elongation, filament barbed end capping and filament unraveling (Pollard et al., 2000, dos Remedios et al., 2003, Pollard and Borisy, 2003, Staiger and Blanchoin, 2006).

2.2.1 NUCLEATION

The initiation of a completely new filament is the most time-consuming process, since the formation of actin dimers is very unfavorable. This is due to the fact that the dissociation process overrides the association process (Sept and McCammon, 2001) and that the actin monomers preferentially join onto the existing filaments instead of onto other monomers. According to current knowledge, three types of proteins promote nucleation in cells: The actin related protein (Arp) 2/3 complex (by forming branched filaments), formins (by forming linear filaments) and the Spire-family proteins (by forming completely new filaments).

The Arp2/3 complex is activated by the nucleation-promoting factor proteins (NPF). All NPFs have the characteristic Arp2/3 binding region including connector (C) and acidic (A) regions, but the structural organization and protein with which they interact are diverse. This diversity is an indication of various upstream signaling pathways and the different functions of several NPFs (Welch and Mullins, 2002). The Arp2/3 complex is composed of seven subunits, two of which, Arp2 and Arp3 resemble the G-actin’s structure. Activated Arp2/3 complex is generally supposed to overcome the kinetic barrier for actin-dimer formation, by mimicking the dimer by itself and thus makes the actin-actin dimer formation unnecessary (Robinson et al., 2001). Arp2/3 remains embedded at the pointed end of the branched filament. The existing (mother) filament thus becomes branched by the Arp2/3 complex and

the resulting new filament changes the direction of the elongation by 70° compared to the original direction of the mother filament, which is still oriented towards the cell protrusion (Higgs and Pollard, 2001, Pollard, 2007).

The formins are multi-domain proteins that contain a forming homology 2 (FH2) domain that bind as doughnut-shaped dimers to the barbed end of the filament. Moreover, FH2 competes with the barbed end capping proteins. Consequently, when formin binding prevails over that of the barbed end capping proteins the elongation rate increases. Formin homology 1 (FH1) domain recruits the profilin-actin monomer complex at the barded end, which accelerates filament elongation (Faix and Grosse, 2006, Kovar, 2006, Pollard, 2007, Goode and Eck, 2007).

The metazoan Spire-family proteins are also composed of several domains. The central region of the spires comprises four G-actin binding Wiskott-Aldrich syndrome protein homology domain 2 (WH2) domains. The linker regions between the third and fourth Wiskott-Aldrich syndrome protein homology domain 2 domains promote actin assembly formation by stabilizing the actin monomer-monomer complex. The actin monomers bound to the first and second Wiskott-Aldrich syndrome protein homology domain 2 domains are brought into contact with the initial dimer. In contrast to both the Arp2/3 complex and formins that stabilize the lateral contact of the actin dimer, spire proteins stabilize longitudinal contacts (Baum and Kunda, 2005, Kerkhoff, 2006).

2.2.2 MONOMER SEQUESTERING/FILAMENT ELONGATION

Actin monomer sequestering proteins, profilins and thymosin β_4, maintain the G-actin pool in living cells and thus enable actin filament growth. Profilins are well conserved, low molecular weight (~15 kDa), single domain ubiquitous proteins that exhibit high structural homology to different species and isoforms (Nodelman et al., 1999). They form 1:1 complexes with the G-actin and catalyze the actin bound nucleotide exchange from ADP to ATP. The filament elongation at the barbed end is stimulated by profilins, and the elongation rate of profilin bound ATP-G-actin equals that of free ATP-G-actin, an association that is diffusion limited. The main role of profilin is to facilitate polymerization, although the depolymerization effect (Bubb et al., 2003) and the copolymerization with the F-actins have been reported as well (Witke, 2004, Yarmola and Bubb, 2006). Thymosin β_4 is the most abundant actin sequestering protein. Unlike profilin, thymosin β_4, is purely an actin sequestering protein that prevents the G-actin association to both the barbed and pointed ends of the filament. Thymosin β_4 is a small unstructured protein, with a molecular weight of only ~5 kDa. It also exists as an actin binding module, namely the Wiskott-Aldrich syndrome protein homology domain 2 (WH2 domain), in large proteins. NMR data has revealed that thymosin β_4 achieves its three dimensional structure upon binding to the G-actin (Domanski et al., 2004). The ADF/cofilins and the twinfilins have been shown to sequester the G-actin as well. However,

in contrast to profilin and thymosin β_4, they have a binding preference for the ADP-G-actin over that of ATP-G-actin (Ojala et al. 2002).

2.2.3 FILAMENT BARBED END CAPPING

The newly developed filaments are rapidly capped by capping proteins, which can exist at high cellular concentrations (~1.0 μM) depending on the source (Pollard et al., 2000) and have a high affinity (K_d ~0.1 nM) for the filament barbed end (Schafer, 1996). When capped, the filament end becomes unresponsive to growth and unraveling. The capping protein and the Ca^{2+} dependent gelsolin protein families are examples of actin filament barbed end capping proteins.

Heterodimeric capping proteins are composed of α- and β-subunits, with molecular weights of 32-36 kDa and 28-32 kDa, respectively (Wear and Cooper, 2004). Skeletal muscle homology for non-muscle capping protein is CapZ (Kilimann and Isenberg, 1982, Castella et al., 1986), and the protein found in *Dictyostelium* is named as Cap32/34 (Schleicher et al., 1984). Significant sequence homologies have not been found between proteins belonging to different families. Regardless of the lack of sequence similarity between the α- and β-subunits, the three dimensional crystal structure of the skeletal CapZ revealed that both subunits take on very similar structures (Yamashita et al., 2003), which assume a mushroom-like shape. The same crystal structure study revealed that the C-terminal regions of the subunits were flexible and critical for the filament binding. Yamashita et al. (2003) suggested their two-to-two binding mechanisms on the actin filament acted as tentacles. That is each domain of CapZ interacts with one actin at the filament barbed end within the same filament, while the flexible C-termini of α- and β-subunits work as tentacles strengthening the interaction by bending towards the filament. The tentacle model was supported by further investigations, in which mutational studies on chicken capping protein were conducted (Wear et al., 2003). Furthermore, a study on *Saccharomyces cerevisiae* (Kim et al., 2004) found that the flexible C-terminal tails of both subunits are important, but the α-subunit is the critical component during the filament binding phase. Cryo-electron microscopy studies provided a low-resolution structure for the actin filament-capping protein (Narita et al., 2006), and clarified the proposed tentacle model of binding. Initially the α-tentacle binds by establishing an electrostatic interaction with both actin protomers at the barbed end. Subsequently, the β-tentacle binds to the hydrophobic cleft of the lower actin protomer.

The members of gelsolin protein family comprise either three or six structurally homologous domains (G1-G6) with molecular weights of 12-15 kDa. Gelsolins with six repeats are separated at their N-terminal (G1-G3) and their C-terminal (G4-G6) halves by a linker comprising ~50 residues. Domains within the halves are connected by linkers of varying lengths (McGough et al., 2003). In addition to their capping activity, gelsolins promote the Ca^{2+} dependent G-actin sequestering, nucleating and filament severing activities

(Kwiatkowski, 1999 and Silacci et al., 2004). The G1 domain has been shown to bind the G-actin in a Ca^{2+} independent manner. Consequently, the corresponding G-actin binding domain at the C-terminal half namely G4, is actually activated by Ca^{2+}. The G2 contains a filament binding site and the construct comprising G1-G3 is able to sever (McGough et al., 2003) and G1-G2 gelsolin fragment is capable of capping the actin filaments (Way et al., 1992). The C-terminal half of the gelsolin is assumed to function as a sensor for cellular Ca^{2+} concentration. The C-terminal helix of G6, the latch region, in particular is critical for Ca^{2+} regulation. The 'helix latch hypothesis' obtained from studies of the crystal structure of full length gelsolin, has been studied by mutational experiments as well (Lin et al., 2000, Lueck et al., 2000, Burtnick et al., 2004). According to the 'helix latch hypothesis', the actin filament binding site at the G2 domain in the absence of Ca^{2+} is blocked by the C-terminal tail of gelsolin. Upon activation with Ca^{2+} the latch region is moved and thereby re-orientation of the domains is induced. Individual domains have been shown to bind to Ca^{2+} after unlatching (Choe et al., 2002, Burtnick et al., 2004, Kazmirski et al., 2002). The mutations (in the G2-domain) Asp187Asn or Asp187Tyr disrupt the Ca^{2+} binding site, which results in the cleavage of gelsolin by furin. This, in turn, leads to the accumulation of amyloid fibrils and is manifested as a hereditary disease known as familial amyloidosis of the Finnish type (FAF) (Kazmirski et al., 2002). The gelsolin also acts as a substrate for the apoptopic enzyme caspase-3, which separates gelsolin's N- and C-halves from each other. The over-expression of the Ca^{2+} independent N-half in cells leads to apoptosis (Kothakota et al., 1997).

2.2.4 FILAMENT UNRAVELING

One of the most essential events in the dynamic regulation of actin, *in vivo*, is filament unraveling. Filaments do not continuously grow. Instead their building blocks, the actin monomers, are recycled by depolymerization at the pointed ends of the filaments or by the severing of filaments into shorter segments. The actin depolymerizing factor (ADF/cofilins) are the main regulators of these processes (Lappalainen et al., 1998).

3. ACTIN DEPOLYMERIZING FACTOR HOMOLOGY DOMAINS

Actin depolymerizing factor homology (ADF-H) domain is an actin binding module that characterizes the ADF-H family of proteins. The protein family can be divided in four sub-families based on both their function and domain architecture (Lappalainen et al., 1998). Some of the members bind solely to the monomeric form of actin, others to the filamentous form and yet others to both forms of actin. Members of two sub-families have only a single ADF-H domain, whereas in others there are either two ADF-H domains or one domain along with other functional modules (Lappalainen et al., 1998). The founding sub-family consists of ADF/cofilins, from which a huge amount of information has already been obtained, including cell biological and biochemical data in addition to structural information. The other sub-

families are: the actin binding protein 1 (Abp1)/drebrin, coactosin-like protein (CLP)/coactosin and twinfilin sub-families (Lappalainen et al., 1998, II).

3.1 BIOCHEMICAL PROPERTIES OF ADF-H DOMAINS

3.1.1 ACTIN DEPOLYMERIZING FACTOR/COFILINS

ADF/cofilins were first isolated and characterized from chicken brain in 1980 (Bamburg et al., 1980). Due to historical reasons, the members of ADF/cofilins have many different names, depending on the source from which the respective protein was isolated. The history of ADF/cofilins and their rather confusing nomenclature has since been clarified by Bamburg (Bamburg, 1999).

In yeasts only a single isoform of ADF/cofilins exists, which is essential for cell survival (Moon et al., 1993). On the other hand, vertebrates have two or three different characterized isoforms of ADF: cofilin-1 (absent in birds), cofilin-2 and ADF. Cofilin-2 is found in muscle cells whereas cofilin-1 and ADF are expressed in non-muscle cells. ADF is expressed in neuronal and epithelial cells and cofilin-1 in most embryonic and adult mouse cells (Vartiainen et al., 2002). The function of the mammalian ADF and cofilin-1 (non-muscle ADF/cofilins) is pH dependent. At normal physiological pH (7.35), cofilin-1 and ADF both disassemble filaments. At higher pH, ADF is significantly more active in disassembling filaments than cofilin-1. At lower pH (pH ~6.5) the differences between the two are small (Vartiainen et al., 2002, Yeoh et al., 2002, Chen et al., 2004). The muscle isoform, cofilin-2, has 5-10 times higher affinity for binding to the ATP-G-actin than the non-muscle isoforms. Moreover, the difference between binding affinities for the ADP-G-actin and the ATP-G-actin is smaller for the non-muscle ADF/cofilins (Vartiainen et al., 2002). In spite of the biochemical differences amongst ADF/cofilins, the mammalian non-muscle ADF and cofilin-1 have been shown to have overlapping roles in cell motility and cytokinesis (Hotulainen et al., 2005).

ADF/cofilins bind both to the G-actin and the F-actin. They have a higher affinity for the ADP-actins over the ATP-actin, with K_d's 0.1-0.4 μM and 6.2-8 μM at low ionic concentration, respectively (Maciver and Weeds, 1994, Carlier et al., 1997, Blanchoin and Pollard, 1998, Yeoh et al., 2002). Once bound to the ADP-G-actin, ADF/cofilins inhibit the actin bound nucleotide exchange from ADP to ATP (Carlier et al., 1997, Blanchoin and Pollard, 1999). ADF/cofilins accelerate the actin treadmilling by severing the ADP-actin filaments and simultaneously increase the number of filament ends. In addition, they increase the recycling of actin monomers by releasing them through depolymerization of the filament pointed end, while concomitantly increasing the G-actin pool of the cell by forming a 1:1 complex with the G-actin (Carlier et al., 1997, Lappalainen and Drubin, 1997a). However, ADF/cofilins do not perform direct recycling of the G-actin from the depolymerized pointed

end to the barbed end as might be expected, because the addition of ADP-G-actins to actin filament's barbed ends is not favored. Synergy between ADF/cofilins and profilins has been shown to accelerate the actin treadmilling (Didry et al., 1998, Blanchoin and Pollard, 1998).

It has been suggested that the hydrolysis of the F-actin bound ATP to ADP works as a timer for the filament unraveling. This is because the disassembly of ADP-F-actin is more efficient than that of the ATP-F-actin (Pollard et al., 2000). ADF/cofilins have also been assumed to enhance the release of inorganic phosphate from the intermediate state of the hydrolysis product, ADP·Pi (Blanchoin and Pollard, 1999). Muhlrad et al., observed antagonistic effects between Pi and ADF/cofilins on F-actin binding. At physiological Pi concentrations, the rate, but not the extent, of yeast cofilin binding to the F-actin is decreased. On the other hand, the extent of binding of Pi to the F-actin-cofilin complex was reduced (Muhlrad et al., 2006). Different theories have been proposed that explain the underlying mechanism for the F-actin and ADF/cofilin interaction that leads to filament severing and depolymerization. The ADF/cofilins bind actin filaments in a cooperative manner (McGough et al., 1997). Cooperative interaction has been shown to vary between different ADF/cofilins and actin isoforms where the flexibility of the actin enhances severing (Blanchoin and Pollard, 1999, De La Cruz, 2005). It was suggested that ADF/cofilins formed cross-bridges and disrupted the interaction between the longitudinal actin protomers (McGough et al., 1997, Galkin et al., 2001, Galkin et al., 2003, Kudryashov et al., 2006). According to the original theory, ADF/cofilins bind to the side of the F-actin and thereon weaken the contacts between adjacent actin monomers by twisting the filament by 5° (McGough et al., 1997). Another theory suggests that ADF/cofilins are capable of selecting the most favorable conformation during the actin filament fluctuations rather than inducing conformational changes (Galkin et al., 2001). It was assumed that the actin filament was severed at the point where ADF/cofilin binds (McGough et al., 1997, Maciver, 1998, Galkin et al., 2001, Galkin et al., 2003). According to differential scanning calorimetric studies, cofilin stabilizes the G-actin, in addition to the F-actin at saturating concentration, whereas at a sub-saturating concentration the stability of the F-actin is decreased (Dedova et al., 2004, Bobkov et al., 2006). These authors concluded that the filaments with the bound cofilin are sequestered whereas the undecorated part is disassembled (Dedova et al., 2004, Bobkov et al., 2006). Furthermore, Bobkov et al. suggested that the filament is destabilized by the allosteric long-range effect of ADF/cofilin (Bobkov et al., 2006).

ADF/cofilins are typically down-regulated by the phosphorylation of the first serine of the N-terminus. This is usually the third residue in the primary structure's sequence. Thus Ser-3 phosphorylation suppresses the actin binding. ADF/cofilins are phosphorylated in reactions mediated by the LIM and TESK kinases (Bamburg, 1999, Ono, 2003). The phosphorylated cofilin has not been found in yeast (Lappalainen et al., 1997b). The three dimensional structure of Ser-3 phoshorylated ADF remains unchanged (Blanchoin et al., 2000). Moreover, it seems that the phosphate group forms a steric hindrance by preventing the hydrogen bond formation between ADF's N-terminus and the actin, rather than by causing conformational

changes to ADF (Wriggers et al., 1998, Blanchoin et al., 2000). Phosphorylation is reversed by protein phosphatases 1 (PP1), protein phosphatases 2A (PP2A) and specific slingshot serine phosphatases (Ambach et al., 2000, Niva et al., 2002). It has also been shown that the cell membrane associated phoshoinositol $PI(4,5)P_2$ molecules interact with ADF/cofilin while impairing the actin binding (Ono, 2003).

Actin-interacting protein 1 (Aip1) collaborates with ADF/cofilin by enhancing the actin filament disassembling activity. The 64-66 kDa Aip1 also co-sediments with actin filaments in addition to capping the barbed end of filament that have been severed by ADF/cofilins and thereby prevents re-annealing of the disassembled filaments (Ono, 2003).

3.1.2 ACTIN BINDING PROTEIN 1/DREBRINS

Abp1p was first identified in yeast (Drubin et al., 1988) and drebrins from chicken embryo (Shirao and Obata, 1985). Mammalian drebrins are multi-domain proteins with a molecular weight of 115 or 125 kDa, depending on the splicing variant. One isoform is found in the brain, mainly in the denritic spines, whereas the other isoform is non-neuronal. Drebrin participates in the regulation of the cell shape via the rearrangement of cytoskeletal actin filaments and it is also important in neuronal development (Majoul et al., 2007).

Abp1 proteins are composed of several functional domains. They play an important role in endocytosis and activate the Arp2/3 complex. The yeast Abp1p (MW ~65.5 kDa) contains an acidic domain in its C-terminus (Goode et al., 2001). Since mammalian Abp1 proteins lack the acidic domain, they have been considered as a functional link between the actin cytoskeleton and the endocytic machinery (Qualmann et al., 2000). In contrast to the G-actin binding Wiscott-Aldrich syndrome protein/Neural Wiscott-Aldrich syndrome protein (WASp/N-WASP); another Arp2/3 activating protein family, the yeast Abp1p activates Arp2/3 and binds to the F-actin. It has been suggested that Abp1s direct the Arp2 and Arp3 domains to the correct orientation, thus enabling them to perform nucleation and also strengthens the association of the Arp2/3 to the actin filament. This mechanism corresponds with that used by cortactins (Olazabal and Machesky, 2001). Abp1/drebrins have not been shown to bind to the G-actin, nor have they been shown to cap, sever, or depolymerize the F-actin (Ishikawa et al., 1994).

3.1.3 TWINFILINS

Twinfilins were first characterized from pudding yeast during a genome database search of cofilin-like proteins (Goode et al., 1998). Later they were characterized in the mouse and drosophila (Vartiainen et al., 2000, Wahlström et al., 2001). All known twinfilins bind to actin monomers, by forming a 1:1 complex. Yeast twinfilin localizes onto the cortical actin patches, but not onto the cytoplasmic actin cables. The cellular localization and tissue distribution

properties of twinfilins have been reviewed in more detail by Palmgren et al. (Palmgren et al., 2002).

Mouse twinfilin-1 is the isoform expressed during the embryonic stage of development. In adult mice twinfilin-1 is expressed in most tissues and most strongly in the liver and kidney. However, it is not expressed in the skeletal muscles. In contrast twinfilin-2 is expressed in heart and at lower levels in skeletal muscles (Vartiainen et al., 2003).

Similar to that of ADF/cofilin, twinfilins have a binding preference for the ADP-G-actin over that for the ATP-G-actin. At physiological ionic concentrations and at pH 7.5 affinities for the ADP-G-actin and the ATP-G-actin are 0.05 μM and 0.47 μM, respectively (Ojala et al., 2002). The isolated C-terminal domain of twinfilin (TwfC) binds to the G-actin with similar affinity compared to the full-length twinfilin. In comparison, the N-terminal domain of twinfilin (TwfN) exhibits only one-tenth the affinity of the C-terminus. The actin binding activity of the twinfilin is inhibited by phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) (Palmgren et al., 2001). However, down-regulation by phosphorylation has not been reported.

The Cap1/2p gene encodes the heterodimeric barbed end capping protein in yeast. Deletion of this gene from the yeast genome disrupts twinfilin's normal localization onto the cortical actin patches (Palmgren et al., 2001). This localization is also unsuccessful with the mutated twinfilin, in which the actin binding site had been disrupted by the mutation of the critical residues for actin monomer binding. Twinfilin's cap1/2p binding site was found to be localized at the C-terminal tail of twinfilin in mutation studies in yeast and mouse protein isoforms (Falck et al., 2004). The affinity of cap1/2p to the actin filament barbed end remains unchanged in the presence of inactivated twinfilin (Falck et al., 2004). A three dimensional model has been built for the twinfilin-capping protein complex by using data from small angle X-ray scattering studies (SAXS) (Falck et al., 2004).

3.1.4 COACTOSIN-LIKE PROTEIN/COACTOSINS

Coactosin was first isolated from the actin-myosin complex from *Dictyostelium discoideum* in 1993. It is a 17 kDa protein, which interacts with the F-actin *in vitro* without affecting actin polymerization (de Hostos et al., 1993). Coactosin binds actin filaments weakly at the physiological ionic concentration (de Hostos et al., 1993). Coactosin represses the capping activities of the barbed end capping proteins, S1 fragment of severin, which is homologous to mammalian gelsolin and cap32/34, expressed in *Dictyostelium discoideum* (Röhrig et al., 1995). Initially the DNA sequence for homologous human coactosin-like-protein (CLP) was reported as a sequence flanking a deletion on chromosome 17, which was characteristic of Smith-Magenis syndrome (Chen et al., 1997), and also as a 5-Lipoxygenase (5-LO) binding protein with the yeast two-hybrid screening (Provost et al., 1999).

Coactosin-like protein mRNA is expressed strongly in placenta, kidney, lungs, spleen and peripheral leucocytes, and weakly in brain, liver, pancreas, heart, lymph node and bone marrow. However, mRNA expression was not found in the skeletal muscle or in the thymus (Provost et al., 2001b).

CLP/coactosins interact with the F-actin but not with the G-actin (Provost et al., 2001b). A co-sedimentation study showed one CLP molecule binds to two actin subunits. Despite this, cross-links were observed for only one actin monomer with a zero-length chemical cross-linker (Provost et al., 2001b). Mouse and human CLPs have very similar primary structures, with only 5 differing amino acids, and they also exhibit similar biochemical properties (Doucet et al., 2002). F-actin binding is independent of Ca^{2+} and pH but it is decreased with the increasing K^+ concentration (Provost et al., 2001b, Doucet et al., 2002).

The enzyme 5-LO (MW ~75 kDa) is primarily expressed in various leukocytes, which can migrate to sites of inflammation. 5-LO catalyzes leukotriene biosynthesis using arachidonic acid as a starting material. Leukotrienes are mediators in some inflammatory conditions such as: asthma, atherosclerosis vascular diseases and also cancers. In the cell, 5-LOs are activated by Ca^{2+}, phosphatidylcholine, some glycerides and CLP through the C2-like domain of 5-LO (Rådmark et al., 2007). Direct interaction between CLP and 5-LO *in vitro* independent of Ca^{2+} occurs at the molar ratio of 1:1. The interaction has also been observed in mammalian cells (Provost et al., 2001a). Amino acid residues of human CLP critical for 5-LO activation have been localized at the C-terminus of the last α-helix (Provost et al., 2001a). F-actin binding and 5-LO binding properties of CLP can be uncoupled as shown in a study on mutated proteins (Rakonjac et al., 2006). CLP has not only been shown to interact with 5-LO, but also to enhance 5-LO enzymatic activity (Rakonjac et al., 2006). In resting cells 5-LO and CLP can be found in the cytosolic compartment and after stimulation by ionophores both proteins are present in the nuclear fraction (Rakonjac et al., 2006).

3.2 STRUCTURES OF ADF-H DOMAINS

3.2.1 ARCHITECTURAL ORGANIZATION

Structural architecture of the ADF-H domain sub-families is represented in Figure 2. ADF-H domains can exist 1) as single domain molecules, as in ADF/cofilins and CLP/coactosins, 2) as modules of larger multi-domain proteins, where other domains have distinct interacting partners such as Abp1/drebrins and 3) as two-domain proteins where two ADF-H domains are separated by a linker, such as twinfilins (Lappalainen et al., 1998).

Although ADF/cofilins and CLP/coactosins are both small single ADF-H domain proteins with a similar molecular weight range of 15-21 kDa, their biochemical properties are different from each other (see sections 3.1.1. and 3.1.4)

Twinfilins, with a molecular weight of ~40 kDa, are composed of tandem repeats of ADF-H domains, connected by a short linker region and followed by a short extended C-terminal tail region. TwfC binds to the ADP-G-actin with an affinity ten-fold that of TwfN, which is comparable to the affinity of full-length twinfilin (Ojala et al., 2002). A linker region has not been shown to be involved in binding, but the tail region increases the binding affinity.

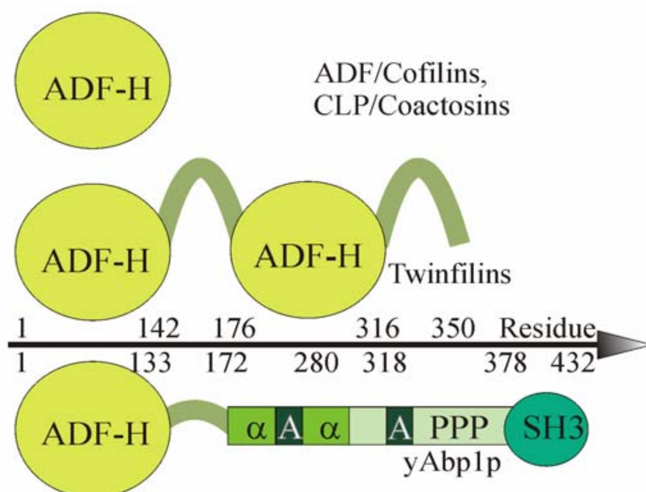

Figure 2. Architectural organization of each category of ADF-H domains. Abbreviations used in the figure: actin depolymerizing factor homology (ADF-H), helical (α), proline-rich (PPP), acidic motif (A), Src homology domain (SH3), Domain boundaries/structures of yeast Abp1p are according to Quintero-Monzon, et al. (Quintero-Monzon et al., 2005).

Abp1 and drebrins are large homologous proteins with a high sequence conservation carried in their N-terminal halves (Kessels et al., 2000). Abp1/drebrins contain one N-terminal ADF-H domain that is responsible for the F-actin binding. However, another shorter and charged helical region has also been observed to participate in the F-actin interaction (Goode et al., 2001). In mouse Abp1 and rat embryonic DrebrinE; the ADF-H domain, the charged helical region and the linker between them are required for the correct localization in cells (Xu et al., 2006). In addition Abp1 has a C-terminal SH3 domain, which is absent in drebrins (Kessels et al., 2000). Binding properties of the isolated ADF-H domains have not been published.

3.2.2 THREE DIMENSIONAL STRUCTURES OF ADF-H DOMAINS

Isolated ADF-H domains are composed of ~150 amino acids. They form themselves into compact three dimensional structures with five central β-strands surrounded by four α-helices. Until today, 12 structures of ADF-H domains have been published and deposited in the PDB. The first published structure of an ADF-H domain was porcine destrin, also called ADF1 by some authors (Hawkins et al., 1993, Hatanaka et al., 1996) in 1996. After that the structures of *Saccaromyces cerevisiea* cofilin (Fedorov et al., 1997), *Acantamoeba* actophorin (Leonard et al., 1997) were published. Later *Arabidopsis thaliana* ADF-1 (Bowman et al., 2000), *Homo sapiens* cofilin-1 (Pope et al., 2004), *Gallus gallus* cofilin-2, the muscle isoform (Gorbatyuk et al., 2006) and *Schizosaccharomyses pombe* cofilin-1 were published

(Andrianantoandro and Pollard, 2006). The structure of the isolated ADF-H domain obtained from *Saccharomyces cerevisiae* Abp1p has also been determined, which is so far the only known structure belonging to Abp1/drebrins (Quintero-Monzon et al., 2005). The structure of the isolated N-terminal domain of *Mus musculus* twinfilin-1 has been elucidated (Paavilainen et al., 2002).

All known ADF-Hs have a very similar fold overall in that they have a five-strand mixed β-sheet surrounded by four α-helices. Root mean square deviation (RMSD) of the CA-atoms between the different structures of the members range from ~1 Å to ~3.5 Å. Considering the low sequence identities of 15-25%, RMSD values are low. Regardless of the high structural similarities, the different ADF-H domains are able to distinguish between the different forms of actin. In addition, many of the ADF-H domains have other binding partners. The three dimensional structure of TwfN differs from other ADF-H domains mainly at its β-extension region, which is bent towards the C-terminus of the α3-helix. This configuration causes a steric hindrance, which prevents the F-actin from binding (Paavilainen et al., 2002).

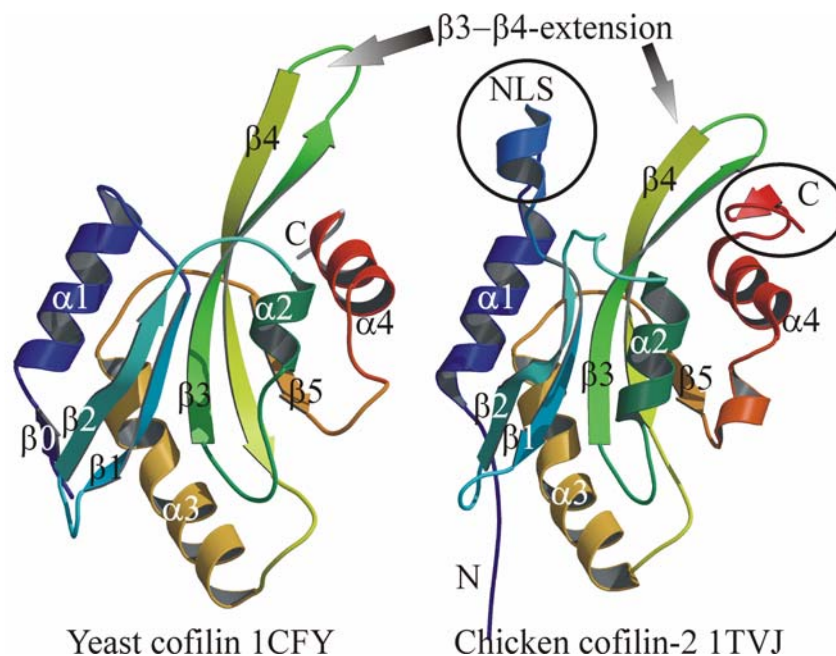

Figure 3. Ribbon representations of the 3D structures of non-vertebrate (yeast) and vertebrate cofilins (Fedorov et al., 1997, Gorbatyuk et al., 2006). Secondary structures are color-coded according to structural alignments between the two proteins. NLS and the C-terminus are circled, which are the vertebrate specific regions. β4-β5-extension regions, in which length and orientation are varied in different ADF-H domains, are marked with grey arrows. Structures were prepared by Molscript and Raster3D (Kraulis, 1991, Merritt and Murphy, 1994)

The amino acid sequences of the vertebrate ADF/cofilins are longer (~166 residues) than the non-vertebrate proteins (~142 residues). There are two vertebrate specific inserts: an additional α-helix between the α1-helix and the β2-strand, and an extended C-terminus with two additional β-strands (Figure 3). The first insertion has been characterized as a nuclear localization signal (NLS), which is critical for the nuclear translocation of cofilin under heat-shock (Iida et al., 1992). ADF/cofilins are associated with both nuclear and cellular actin

(Nishida et al., 1987). Actin does not have NLS and it has been shown that ADF/cofilin is required for the nuclear localization of actin (Pendleton et al., 2003). The exact biological role for the nuclear localization of the ADF/cofilins and actin is unclear. NLS can be found in the structures of destrin, human cofilin-1 and chicken cofilin-2 (Hatanaka et al., 1996, Pope et al., 2004, Gorbatyuk et al., 2006). However, NLS is absent in currently known non-vertebrate and plant ADF/cofilins. Structural data reveal that the yeast Abp1p and even the vertebrate TwfN lack the NLS sequence. Based on the sequence alignment studies, the vertebrate Abp1 also lacks NLS, though no three dimensional structure is available. It seems that the vertebrate ADF/cofilins form one class of proteins and the second class consists of non-vertebrate ADF/cofilins together with twinfilins and Abp1/drebrins from all organisms.

3.2.3 BINDING INTERFACES

Currently there are no experimentally determined high resolution structures of the ADF-H-actin complexes available. All the proposed models for the ADF/cofilins-F-actin complex have been derived from cryo-electron microscopic data (McGough et al., 1997, Galkin et al., 2001, Galkin et al., 2003). In addition, a computational simulations model has been built using the gelsolin-G-actin structure as a template to depict the yeast cofilin-G-actin complex (Wriggers et al., 1998). This model is based on the assumption that cofilins and gelsolins share the same binding sites on the G-actin.

The actin interaction sites on ADF/cofilins are the G/F-site and the F-site (Ono, 2003). The G/F site participates in interactions with both the G- and F-actin whereas the F-site only interacts with the F-actin. The binding interface is formed between the G/F-site and the cleft between subdomains 1 and 3 of the G-actin and also interfaces with the ‘upper’ actin of the filament (Galkin et al., 2001, Ono, 2001). The F-site of the ADF/cofilins interacts with the subdomains 1 and 2 of the ‘lower’ actin of the filament (Galkin et al., 2001, Ono, 2001). These sites have also been identified in mutagenesis studies (Lappalainen et al., 1997b, Moriyama and Yahara, 2002), synchrotron foot printing studies (Guan et al., 2002) and NMR chemical shift perturbation studies (Pope et al., 2004) (Figure 4). However, in a recent footprinting study it was proposed that cofilin interacted with subdomains 1 and 2 of the G-actin (Kamal et al., 2007).

Putative G/F- and F-sites of other ADF-H domains have also been studied. TwfN may have a larger interaction interface with actin, since the corresponding residues at the F-site in the region of the β-extension of yeast cofilin have been shown (Figure 4) to be involved in G-actin binding (Paavilainen et al., 2002). The orientation of the β-extension is responsible for the larger TwfN-G-actin interface (see Figure 4). A Lys75Ala mutation at the region of putative F-site of CLP impairs the affinity for F-actin binding, though the binding affinity for 5-LO remains unchanged (Provost et al., 2001b). In contrast, a mutation of the conserved amino acid, Lys131 to Ala, diminished the interaction for binding with 5-LO, and it was the F-actin binding that was preserved on this occasion (Provost et al., 2001a). The important F-

actin binding regions of yeast Abp1p correspond to the F- and G/F-sites of yeast cofilin, but the G/F-site is smaller. Interestingly, a mutation in the C-terminal helix (Lys134Ala) did not inhibit the F-actin binding, but instead interfered with the activation of the Arp2/3 complex (Quintero-Monzon et al., 2005).

Jiang et al. (1997) have shown the importance of the conserved tyrosine residues in maize ADF3 (ZmADF) studies. They analyzed all available ADF/cofilin sequences and observed that there were only nine amino acids, which were fully conserved among ADF/cofilins. By mutating conserved tyrosine residues Tyr67 and Tyr70 or Tyr103 (corresponding to Tyr64, Tyr67 and Tyr101 in the sequence of yeast cofilin), these authors found that the Tyr67/70Phe mutant had lost the ability to bind to the F-actin, though its G-actin binding remained unchanged (Figure 4). The interactions with the G-actin and F-actin were strongly reduced in the mutant Tyr103Phe. Structural alignment data show that these two tyrosines are conserved in all ADF-H domains that are able to bind to the monomeric form of actin.

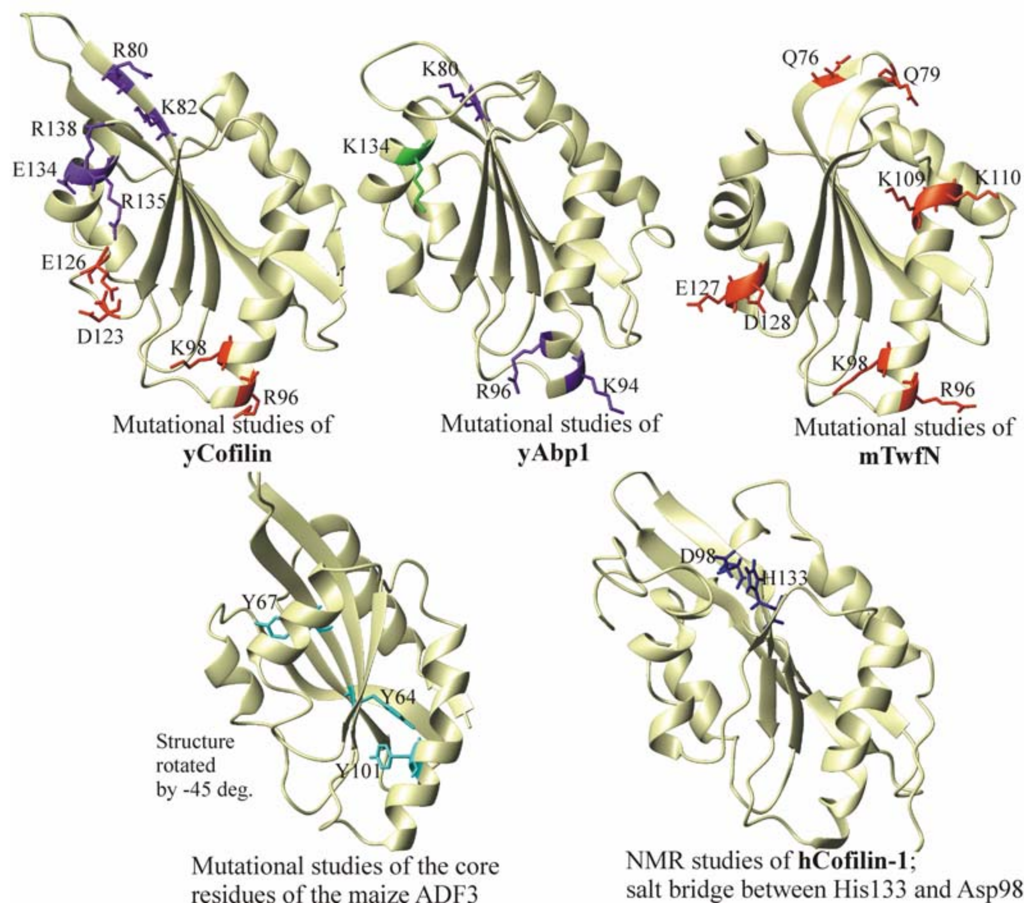

Figure 4. Effective mutation amongst different ADF-H domains. Upper row: Residues important for F-actin binding and G-actin binding are shown in violet and orange, respectively (Lappalainen et al., 1997b, Quintero-Monzon et al., 2005, Paavilainen et al., 2002). The residue involved in the Arp2/3 activation is colored green. Lower row: Conserved tyrosine residues found at the core of the ADF/cofilins and reported to change actin binding properties of the Maize ADF3 are colored with cyan (Jiang et al., 1997). Mutated, critical residues are drawn to the corresponding structure of the yCofilin. Salt bridge of hCofilin-1 between the residues His133 and Asp98 (blue), which have been proposed to determine the pH sensitivity of the ADF/cofilins. (Pope et al., 2004). Figures made by using MolMol (Koradi et al., 1996)

In the structure of human cofilin-1 and chicken cofilin-2, the vertebrate specific extended C-terminus is tightly packed (see Figure 3) against the β-extension region (Pope et al., 2004, Gorbatyuk et al., 2006). It has been suggested that the C-termini of ADF-H domains play some role in the choice between the F-actin severing and depolymerization by ADF/cofilins (Moriyama and Yahara, 1999, Ono, 2001). Mutational studies on different isoforms of *C. elegans*, UNC-60A and UNC-60B, have demonstrated that single point mutations on the C-terminus impair the affinity to the F-actin due to a surprising increase in the disassembling activity (Ono et al., 2001). The C-termini of other ADF-Hs, such as Abp1p, CLP and twinfilin, are thought to interact with other binding partners (Provost et al., 2001b, Falck et al., 2004, Quintero-Monzon et al., 2005). In general, it seems that proteins with a combination of 'low enough' F-actin binding and 'high enough' G-actin binding affinities are capable of performing the F-actin depolymerization.

A study using chemical shift perturbations (CSP) obtained by NMR spectroscopy showed that the salt bridge between residues His133 and Asp98 of human cofilin (Figure 4) is responsible for the pH sensitivity (Pope et al., 2004). It was suggested by the authors of this study that the structure of human cofilin-1 is more strongly stabilized by several non-covalent bonds than is the case in human ADF1. Consequently, the breakage of the salt bridge has a stronger effect on the human ADF1, which manifests as pH sensitivity (Pope et al., 2004) (Figure 4).

4. AIMS OF THE STUDY

The first objective of the study was to determine the structure of the F-actin binding protein CLP. The structure of CLP was studied in order to understand their low binding affinities in comparison to those of other ADF-H domains. As the sequence identity of mouse CLP with that of other ADF-H domains is only ~15 %, it was uncertain whether CLP/coactosins actually belong to the family of ADF-H domains. In general the structural, biochemical and cell biological information obtained from CLP/coactosins, were limited. Moreover, the biological role of CLP/coactosins was unclear (I and II).

The second aim of the study was to reveal the structural basis of twinfilin's two-domain architecture and resolve the three dimensional structure of the C-terminal domain. The name 'twinfilin' refers to twins, and indeed, it was anticipated that the TwfC adopts a three dimensional structure identical to that of the previously determined TwfN. The two-domain architecture of twinfilins appeared to have no identifiable function, even as an evolutionary mistake (III and IV).

The third aim, was to study if laccases, the cross-linking enzymes, can use proteins as substrates in the absence of any auxiliary substances and if the structural environment of the protein substrate is significant in terms of laccases function and activities. The ultimate goal was to determine, which amino acids participate in a plausible cross-linking reaction and whether they are located in structured or unstructured parts of the protein. Previous studies, laccases had shown that laccases cross-link with small substrates, and only cross-link with some proteins in the presence of small molecular weight mediators. The three dimensional structure of CLP contains both rigid and flexible regions with putatively reactive aromatic amino acids for laccase cross-linking (V).

The overall goal of all studies in this thesis was to acquire new structural information in order to understand and explain how the functional differences can be linked to structural differences within the family of ADF-H domains.

5. MATERIALS AND METHODS

Table I. Protein expression and purification for NMR experiments and mass spectrometric analysis.

Protein construct	Vector	Labels	Purification	Additional methods for mass spectrometric analysis
Mouse CLP (1-142)	pRat5	N^{15} N^{15}, C^{13}	See I	Gel filtration was performed in order to separate dimers from monomeric CLP and from higher oligomers (See V)
Mouse truncated CLP (1-131)	pRat5	-	As the wild type (1-142)	
Mouse TwfC (176-316)	pRat4	N^{15} N^{15}, C^{13}	See III	

Table II. NMR spectra used for structure determination

Spectrum	Mouse CLP (1-142)	Mouse twinfilin (176-316)
Assignments		
2D ^{15}N-HSQC	x	x
2D ^{13}C-HSQC	x	x
2D ^{13}C-(CT)-HSQC	x	x
3D HNCA	x	x
3D HN(CO)CA	x	x
3D HNCACB	x	x
3D CBCA(CO)NH	x	x
3D HNCO	x	x
3D HN(CA)CO	x	x
3D HC(C)H-COSY	x	x
3D H(C)CH-TOCSY	x	x
3D (H)C(CO)NH	x	x
3D H(C)(CO)NH	x	x
2D(HB)CB(CGCDCE)HE		x
2D (HB)CB(CGCD)HD		x
3D DE-MQ-(H)CC$_m$H$_m$-TOCSY	x	x
Distance restraints		
3D NOESY-^{15}N-HSQC	x	x
3D NOESY-^{13}C-HSQC	x	x
Residual Dipolar Couplings		
2D ^{15}N-HSQC TROSY	x	
3D HNCO(α/β-C´C$^{\alpha}$-J)	x	

Spectra were processed using VNMR 6.1C (Varian Inc., Palo Alto, CA) and analyzed using Sparky (Goddard and Kneller, 2002) (I and III). Methods used for structure determination, were based on the nuclear Overhauser effect (NOE) data (TwfC) and NOE data together with

residual dipolar coupling (RDC) data (CLP), are described in IV and II, respectively. All the figures have been prepared using MolMol (Koradi et al., 1996), VMD (Humphrey et al., 1996), Molscript (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

We chose mouse CLP to laccase cross-linking studies, because during the structure determination, mouse CLP was found to be structurally very stable, easy to handle, produce and purify in large quantities (V).

6. RESULTS

6.1 STRUCTURAL STUDIES OF MOUSE COACTOSIN-LIKE PROTEIN (I and II)

The three dimensional structure of the mouse CLP was found to be constructed of five internal β-strands, four of which (β1-β4) are anti-parallel and one, β5, which runs parallel to β4 (Figure 1 in II). The β-strands are surrounded by four α-helices. Regardless of the low sequence homology (~15 %) with other ADF-H domains, the overall fold is typical for ADF-H domains. This finding confirmed the mouse CLP as the fourth member of ADF-H family (II). The closest structural similarities are between the yeast cofilin (1CFY) and chicken cofilin-2 (1TVJ) (Fedorov et al., 1997, Gorbatyuk, et al., 2006), with RMSD values of 2.2 Å and 2.3 Å, respectively.

Structural comparisons with other ADF-Hs reveal that the mouse CLP tends to have a different packing mechanism to other ADF-H domains. We found that two conserved amino acids amongst the ADF-H domains, Tyr68 and Tyr101, were respectively replaced by phenylalanine and threonine. In addition, the ensemble of structures and the T_2 relaxation data revealed that the β-extension region composed of β3- and β4-strands is highly flexible and the C-terminal part of mouse CLP is completely unstructured (Figure 5).

6.2 STRUCTURAL STUDIES OF MOUSE C-TERMINAL DOMAIN OF TWINFILIN (III and IV)

At the beginning of the structure determination process, a problem concerning domain boundaries was encountered. The structural C-terminal ADF-H domain is located in the middle of the protein. Therefore two different constructs of TwfC, used previously in biochemical studies, were studied with two-dimensional ^{15}N, ^{1}H correlation spectra. Construct I) comprised residues 169-322 and construct II) residues 169-350. Spectra from both constructs were unsatisfactory and an aggregation problem was encountered during the measurements. However, the assignment of the main chain signals was successful. Based on the backbone assignment, and also the backbone dynamics as probed by longitudinal and spin relaxation rates of ^{15}N, it was possible to identify those amino acids that were outside the

domain boundaries. The newly identified construct (residues 176-316) was prepared according to the NMR data and used for further structural studies.

Before this structural study, twinfilins were treated as actin monomer binding proteins and structurally identical to the N-terminal domain of twinfilin. The TwfC possesses a typical ADF-H domain fold (Figure 1a in IV). The RMSD value between the ensemble of NMR structures and ^{15}N T_2 relaxation time measurements indicates that only the first five residues are flexible. In contrast to CLP, the β-extension is relatively rigid. Surprisingly, the β-extension regions in TwfN and TwfC adopt completely different orientations from each other (Figure 1b. in IV). Hence a steric hindrance caused by the β-extension of TwfN explaining the absence of F-actin binding properties, is no longer valid for TwfC. Furthermore, the three dimensional structure of TwfC resembles the structure of cofilin.

Based on these structural discoveries, the functional properties of TwfC were further studied. Interestingly, TwfC is also functionally similar to the yeast cofilin. In addition to its ability to bind to the ADP-G-actin (Ojala et al., 2002), TwfC co-sediments with the F-actin in a cofilin-like manner (IV).

6.3 CROSS-LINKING STUDIES OF LACCASE USING COACTOSIN-LIKE PROTEIN AS A MODEL COMPOUND

The study presented in V revealed that laccase has the ability to oxidize particular proteins without auxiliary substrates. The native, full-length CLP was polymerized effectively during the laccase treatment by forming dimers, trimers and tetramers. However, when the truncated form of CLP (lacking the flexible tail containing one tyrosine residue) underwent the same treatment with laccase no signs of the oxidation reaction were observed. These results and the fact that tyrosine was the most effective of the single amino acid substrates for laccase, suggest that the ability of laccase to cross-link with proteins is dependent on the flexible structural environment around reactive tyrosine residues.

Monomers, dimers and trimers were separated by gel filtration from the laccase treated CLP solution for the further mass-spectral analysis of the dimers.

7. DISCUSSION

Calculated structures are models, which offer an excellent opportunity to hypothesize the structural basis for the function of a particular protein. ADF-H domains are interesting from this point of view, because there are several structures available whose binding properties are substantially modified by only very small structural deviations. At first sight, ADF-H domains seem structurally equal with a very similar overall fold, i.e. a five-strand mixed β-sheet

surrounded by four α-helices. Some particular sites in the three dimensional structures that might explain distinctive behavior between different ADF-H domains are highlighted in the following text.

Orientation and dynamics of the β-extension

In the structures of the ADF-H domains the extension formed by β3- and β4-strands adopts different orientations and the length of the extension varies as well. This region is critical for binding to actin for all of the ADF-H family members as shown by mutagenesis studies (Lappalainen et al., 1997b, Provost et al., 2001b, Quintero-Monzon et al., 2005, Dai et al., 2006, IV). It can be supposed that the various β-extensions have different dynamic properties amongst the ADF-H domains.

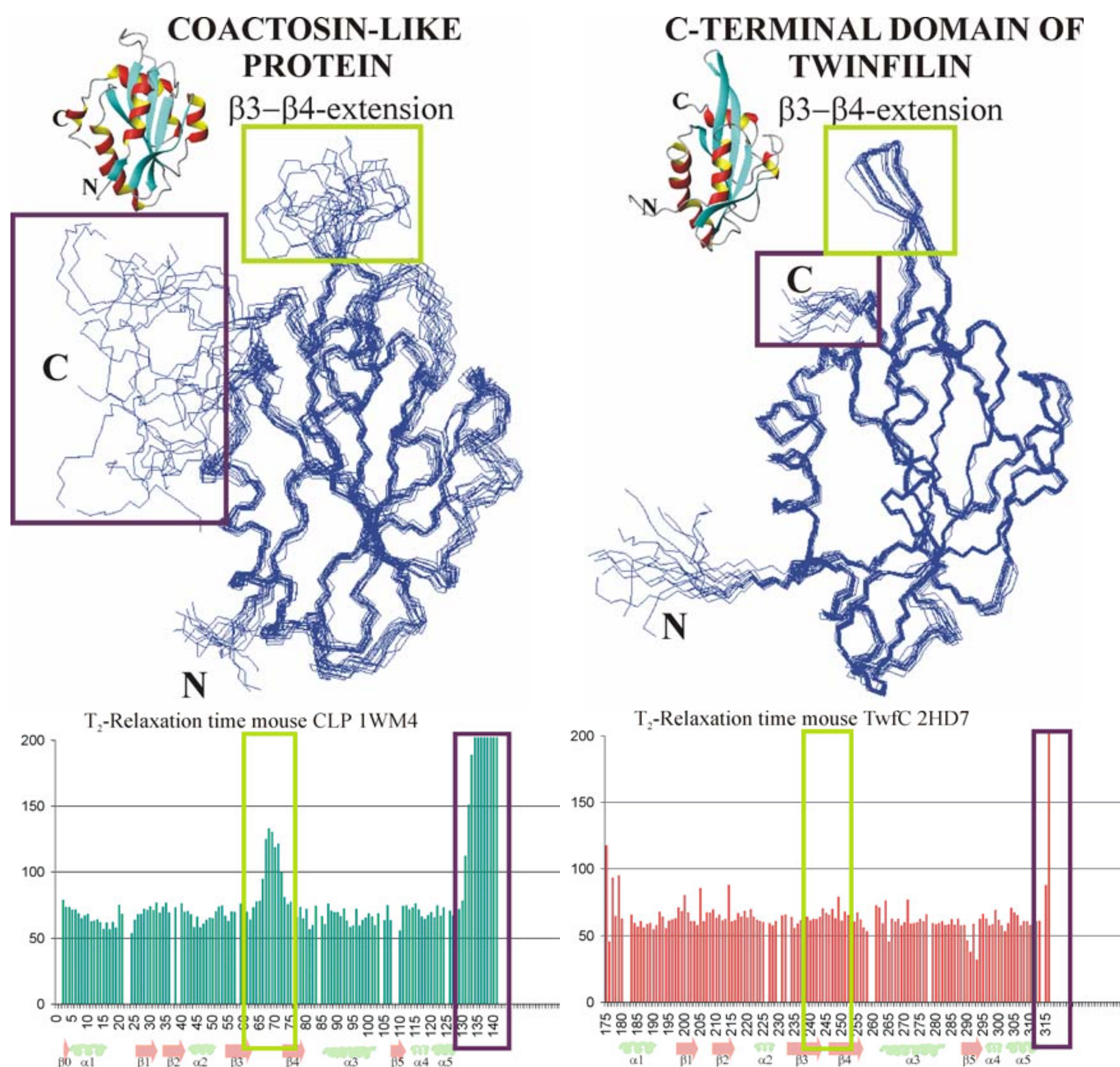

Figure 5. An example of how flexibility of the protein can be represented by RMSD of the ensemble of NMR structures, using TwfC with a stable β-extension and CLP with a mobile β-extension. Supportive T_2 relaxation distribution is also presented. Main chain representation of the lowest energy structures for mouse CLP and for TwfC are superimposed on one another. Flexibility at the F-actin binding interface of coactosin can explain the weak interaction with the F-actin. Figures of the molecules were prepared with MolMol (Koradi et al., 1996).

Analysis of three available NMR structure ensembles of CLPs indicates that RMSD values are highest for the C-terminal tails and for the β-extension sites, which indicates high mobility in those particular regions. The RMSD values of β-extensions in the structures of vertebrate cofilins (1Q8G, 1TVJ) and TwfC (2HD7) are comparable to other internal loop regions in these proteins, which indicate more restricted internal mobility in their respective β-extensions. It is noteworthy that molecular dynamics can be efficiently probed by measuring longitudinal and transverse relaxation rates in addition to ^{15}N heteronuclear NOEs. Experimentally measured spin relaxation time (T_2) data of human cofilin-1 (Pope et al., 2004), human CLP (Dai et al., 2006), TwfC (our unpublished data, Figure 5) and mouse CLP (II) (Figure 5) support the RMSD analysis and suggests that the loosely defined β-extension is a result of elevated local dynamics rather than a lack of distance restraints (NOEs). These data show that the flexibility is only slightly increased in human cofilin-1 and TwfC, whereas it is substantially increased in CLP. Hence the flexibility at the F-actin binding interface of CLP might explain the weak interaction.

```
HELIX STRAND                               β0      α1          αNLS NLS  β1           β2
pud. yeast     Cof 1CFY ...MSRSGVAVA.DESLTAFNDLKLGKKYK..........FILFGLNDAKTEIVVK..ETST...   47
fis. yeast   Cof-1 2I2Q ...MSFSGVKVS.PECLEAFQELKLGKSLR..........YVVFKMNDTKTEIVVE..KKST...   47
Amoeba Actophorin 1AHQ ......SGIAVS.DDCVQKFNELKLGHQHR..........YVTFKMNASNTEVVVE..HVGGP..   44
A. thaliana ADF-1 1F7S MANAAASQMAVH.DDCKLRFLELKAKRTHR..........FIVYKIEEKQKQVVVE..KVGQP..   50
Human        Cof-1 1Q8G ....MASGVAVS.DGVIKVFNDMKVRKSSTPEEVKKRKKAVLFCLSEDKKNIILEEGKEILVGD   60
Chicken      Cof-2 1TVJ ....MASGVTVN.DEVIKVFNDMKVRKSSTPEEIKKRKKAVLFCLSDDKKQIIVEEAKQILVGD   60
Pig(Human)Destrin 1AK6 ....MASGVQVA.DEVCRIFYDMKVRKCSTPEEIKKRKKAVIFCLSADKKCIIVEEGKEILVGD   61
Pud. Yeast    Abp1 1HQZ .MALEPIDYTTHSREIDAEYLKIVRGSDPTT..........WLIISPNAKK.EYEPE..STGS...   51
Mouse          CLP 1WM4 ....MAT..KIDKEACRAAYNLVRDDGSAVI..........WVTFRYD..GATIVPG..DQGA...   44
Human          CLP 1WNJ ....MAT..KIDKEACRAAYNLVRDDGSAVI..........WVTFKYD..GSTIVPG..EQGA...   44
Mouse         TwfN 1M4J ..MSHQTGIQAS.EDVKEIFARAR.NGKYR..........LLKISIE..NEQLVVG..SCSP...   45
Mouse         TwfC 2HD7 ....QGVAFPIS.RDAFQALEKLSKKQLN..........YVQLEIDIKNETIILA..NTEN...   45

                                 α2                β3  β-Extension  β4              α3...
pud. yeast     Cof 1COF .....DPSYDA.FLEKLPENDCLYAIYDFEYEINGNEGKRSKIVFFTWSPD.TAPVRSKMVYA  102
fis. yeast   Cof-1 2I2Q .....DKDFDT.FLGDLPEKDCRYAIYDFEFNLG..EGVRNKIIFISWSPD.VAPIKSKMVYS  100
Amoeba Actophorin 1AHQ .....NATYED.FKSQLPERDCRYAIFDYEFQVD..GGQRNKITFILWAPD.SAPIKSKMMYT   98
A. thaliana ADF-1 1F7S .....IQTYEE.FAACLPADECRYAIYDFDFVTA.ENCQKSKIFFIAWCPD.IAKVRSKMIYA  104
Human        Cof-1 1Q8G VGQTVDDPYAT.FVKMLPDKDCRYALYDATYETK..ESKKEDLVFIFWAPE.SAPLKSKMIYA  118
Chicken      Cof-2 1TVJ IGDTVEDPYTA.FVKLLPLNDCRYALYDATYETK..ESKKEDLVFIFWAPE.SAPLKSKMIYA  118
Pig(Human)Destrin 1AK6 VGVTITDPFKH.FVGMLPEKDCRYALYDASFETK..ESRKEELMFFLWAPE.LAPLKSKMIYA  118
Pud. Yeast    Abp1 1HQZ .......SFHD.FLQLFDETKVQYGLARVSPPG....SDVEKIIIIGWCPD.SAPLKTRASFA  100
Mouse          CLP 1WM4 .......DYQH.FIQQCTDDVRLFAFVRFTTGD..AMSKRSKFALITWIGE.DVSGLQRAKTG   95
Human          CLP 1WNJ .......EYQH.FIQQCTDDVRLFAFVRFTTGD..AMSKRSKFALITWIGE.NVSGLQRAKTG   95
Mouse        N-twf 1M4J PSDSWEQDYDSFVLPLLEDKQPCYVLFRLDS....QNAQGYEWIFIAWSPD.HSHVRQKMLYA  102
Mouse        C-twf 2HD7 ......TELRD.LPKRIPKDSARYHFFLYKHSHE..GDYLESVVFIYSMPGYTCSIRERMLYS   98

                                                           Flexible
                       ...α3          β5         α4        β6   β7
pud. yeast     Cof 1COF SSKDALR.RALN.....GVSTDVQGTDFSEV.SYDSVLERVSRGAGSH                 143
fis. yeast   Cof-1 2I2Q SSKDTLR.RAFT.....GIGTDIQATDFSEV.AYETVLEKVTRK                     137
Amoeba Actophorin 1AHQ STKDSIK.KKLV.....GIQVEVQATDAAEI.SEDAVSERAKKDVK                   137
A. thaliana ADF-1 1F7S SSKDRFK.RELD.....GIQVELQATDPTEM.DLDVFRSRAN                       139
Human        Cof-1 1Q8G SSKDAIK.KKLT.....GIKHELQANCYEEVKDRCTLAEKLGGSAVISLEGKPL          166
Chicken      Cof-2 1TVJ SSKDAIK.KKFT.....GIKHEWQVNGLDDIKDRSTLGEKLGGNVVVSLEGKPL          166
Pig(Human)Destrin 1AK6 SSKDAIK.KKFQ.....GIKHECQANGPEDL.NRACIAEKLGGSLIVAFEGCPV          165
Pud. Yeast    Abp1 1HQZ ANFAAVANNLFK.....GYHVQVTARDEDDLDENELLMKISNAAGA                  141
Mouse          CLP 1WM4 TDKTLVK.EVVQ.....NFAKEFVISDRKELEEDFIRSELKKAGGANYDAQSE          142
Human          CLP 1WNJ TDKTLVK.EVVQ.....NFAKEFVISDRKELEEDFIKSELKKAGGANYDAQTE          145
Mouse        N-twf 1M4J ATRATLK.KEFGGG...HIKDEVFGTVKEDVS.LHGYKKYLLSQ                    142
Mouse        C-twf 2HD7 SCKSPLL.EIVERQLQMDVIRKIEIDNGDEL.TADFLYDEVHPKQ                  141
```

Figure 6. Structure based alignment of different ADF-H domains. α-helices and β-sheets are marked green and violet, respectively. Two highly conserved tyrosine residues amongst ADF/cofilins and twinfilins are framed violet and an NLS site is framed green. Structure based alignments have been performed with the program Dali (Holm and Sander, 1993)

Side chains at the α3-helix

The α3-helix is relatively conserved between the different known ADF-Hs. The structural sequence alignment analysis showed the most significant deviations in the primary sequence were found for the CLPs and Abp1p (Figure 6). These deviations might partially explain the low G-actin binding affinity of CLP and Abp1.

Stabilization of the long α3-helix

The importance of the conserved tyrosine residues (Tyr64, Tyr67 and Tyr101) for actin binding has been reported (Jiang et al., 1997). These authors predicted that the hydroxyl group of Tyr64 on the β3-strand establishes a hydrogen bond with the main chain carbonyl of Tyr101 on the α3-helix, thus stabilizing the correct orientation of the long α3-helix (Jiang et al., 1997). Structural alignment shows all the ADF-H domains that are able to bind to a monomeric form of actin, have tyrosines at these positions. In CLPs, Tyr64 is replaced by phenylalanine and Tyr101 is replaced by a structurally very different amino acid threonine. Similarly, Tyr101 of the Abp1p was replaced by phenylalanine. Therefore, it might be assumed that the replacement of Tyr101 affects the G-actin binding whereas the replacement of Tyr64 weakens the CLP's F-actin binding ability.

C-terminal flexible tail

Many ADF-H domains have a C-terminal flexible tail of varying length. The tail remained invisible in crystal structures, whereas in solution the RMSD of an ensemble of structures is large in this region. The longest tail was found in twinfilin (~35 residues) and it has been shown to interact with the barbed end capping protein. The tail region of CLP is ~10 residues long and highly disordered. The length of the tail region in non-vertebrate ADF/cofilins is limited to a few amino acids, whereas in vertebrate isoforms the longer tail adopts a β-hairpin-like structure (see Figure 3).

The orientation and nature of the C-terminal α4-helix

Differences have been found in the orientation and amino acid composition of the C-terminal α4-helix. It has been suggested that α4-helices of the ADF/cofilins determine the mode of disassembling activities (severing vs. depolymerization). Members of the ADF-H domains can interact through the α4-helix with other binding partners than actin. The amino acid composition in the α-helix of yeast cofilin has been shown to play a critical role in the actin filament binding (Lappalainen et al., 1997b). In contrast, the Lys134Ala mutation in this region of yeast Apb1p causes an impaired Arp2/3 activation (Quintero-Monzon et al., 2005). It has also been shown that the CLP mutant Lys131Ala is effective for the binding to 5-LO (Provost et al., 2001a). It can be assumed that the last α4-helix is a key element in the selection of binding partners in different ADF-H domains (Figure 7).

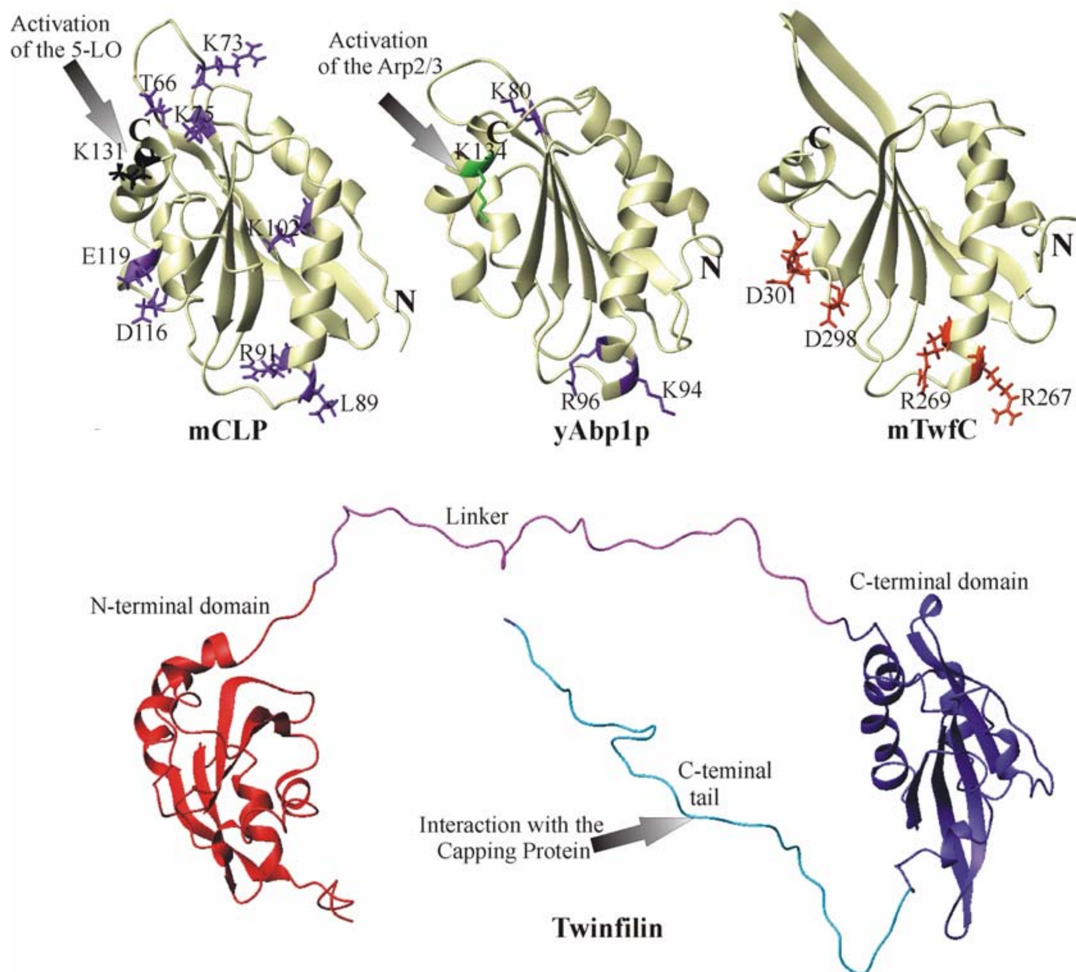

Figure 7. Upper row: Results from the mutational studies of the CLP (Dai et al., 2006, Provost et al., 2001a and 2001b), Abp1p (Quintero-Monzon et al., 2005) and TwfC (IV). Residues important for the F-actin binding and the G-actin binding are indicated, by violet and orange, respectively. CLP mutation in the α4-helix that reduces the 5-LO activation, and the Abp1p mutation that diminishes the Arp2/3 activation are colour-coded black and green, respectively. Lower row: Ribbon representation of the full-length twinfilin. Individually determined structures of the TwfN (Paavilainen et al., 2002) and TwfC (IV) are connected with a linker (35 amino acids) and followed by a long tail region that has been shown to interact with the heterodimeric capping protein (Falck et al., 2004). Linker and tail regions are drawn on the same scale as the structural domains. C-terminals, α-helices and tails that have been shown to activate or interact with binding partners other than actin, are indicated by arrows. Figures were prepared with MolMol (Koradi et al., 1996).

Twinfilin

For a long time twinfilin was regarded as a G-actin binding protein (Goode et al., 1998, Ojala et al., 2002, Paavilainen et al., 2002, Vartiainen et al., 2004). Very recently, mammalian twinfilins were found to cap the actin filament barbed ends (Helfer et al., 2006, IV). The two-domain structural organization is essential for the capping activity because neither domain by itself is capable of capping actin filaments. It is even possible to construct an engineered capping protein from two cofilin-2 molecules that are connected by the twinfilin's linker (IV). Engineered tandem cofilin-2 has a more efficient capping protein activity in comparison to the native twinfilin. This is consistent with a stronger binding affinity of the native cofilin-2 for

the F-actin compared to that of TwfC. The order of the N- and C-terminal domains can also be swapped without affecting the capping activity (IV).

The two domains of twinfilin are connected by the relatively long linker region (35 residues). Structurally and functionally related proteins for twinfilins are gelsolins, which are also composed of more than one domain. The smallest possible gelsolin fragment capable of capping the actin filament has been shown to be composed of G1 and G2 domains (Way et al., 1992). It can be assumed that at least a two-domain architecture is required for capping activity. Such an assumption would explain twinfilin's two-domain architecture.

Sequence identities of TwfN and TwfC to other ADF-H domains are ~25 % and 15 %, respectively. After structure determination of TwfC, it is evident that the two domains of twinfilins are not actually twins, or at the very least they are not identical twins. However, as domains can be supposed to be descendants of a common ancestor of cofilin, they are preferably siblings. The name sibfilin can describe better the nature and relationship of these domains.

8. CONCLUSION

The two newly determined three dimensional structures of mouse CLP and the C-terminal domain of mouse twinfilin have been presented in this thesis. Both proteins belong to distinct sub-families of the ADF-H domain family and their exact biological role is unclear. The structure of CLP revealed that CLP/coactosins are the fourth member of ADF-H domain family (II). A detailed analysis of the new structure and comparison with other ADF-H domains reveal several structural distinctions. These are mainly localized in the β3-β4–extension region and the C-terminal parts of CLP. Clear differences were detected at the hydrophobic core region, which have previously been shown to stabilize and create a correct orientation for the α3-helix. The correct orientation of the α3-helix is critical for actin binding. The structural findings led us to make a hypothesis of the CLP's lower binding affinity for the F-actin.

The three dimensional structure of TwfC we elucidated disagreed with our initial assumption of structural similarity between TwfC and TwfN. Instead it revealed a surprisingly similar three dimensional structure to that of the yeast cofilin. This rather unexpected discovery inspired the further functional characterization of TwfC. Indeed, TwfC possess similar (slightly weaker) depolymerization and severing activities compared to cofilin. Finally, the full-length twinfilin was found to cap the barbed end of the actin filament. Neither domain caps the F-actin by itself. Therefore, the two domain architecture is essential for achieving the capping activity. Moreover, the order of twinfilin domains can be swapped without losing its capping properties (IV). The two domain architecture of twinfilin is explicable in terms of its capping function.

At the present time, no high-resolution structure of ADF-H domain in complex with actin has been resolved. Consequently, the mechanism of interaction between the ADF-H domain and actin is unknown as yet. In the future, the high-resolution NMR structure of ADF-H domain-actin complex will be a challenging task due to a fundamental problem in obtaining isotopically labeled actin. However, as shown in this thesis, NMR can be efficiently used for elucidating construct design of the protein sample. A well-behaving construct can not only overcome problems encountered in the structure determination by NMR, but it can also serve as an invaluable module for crystallization of more complex structures beyond the current NMR size-limit.

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