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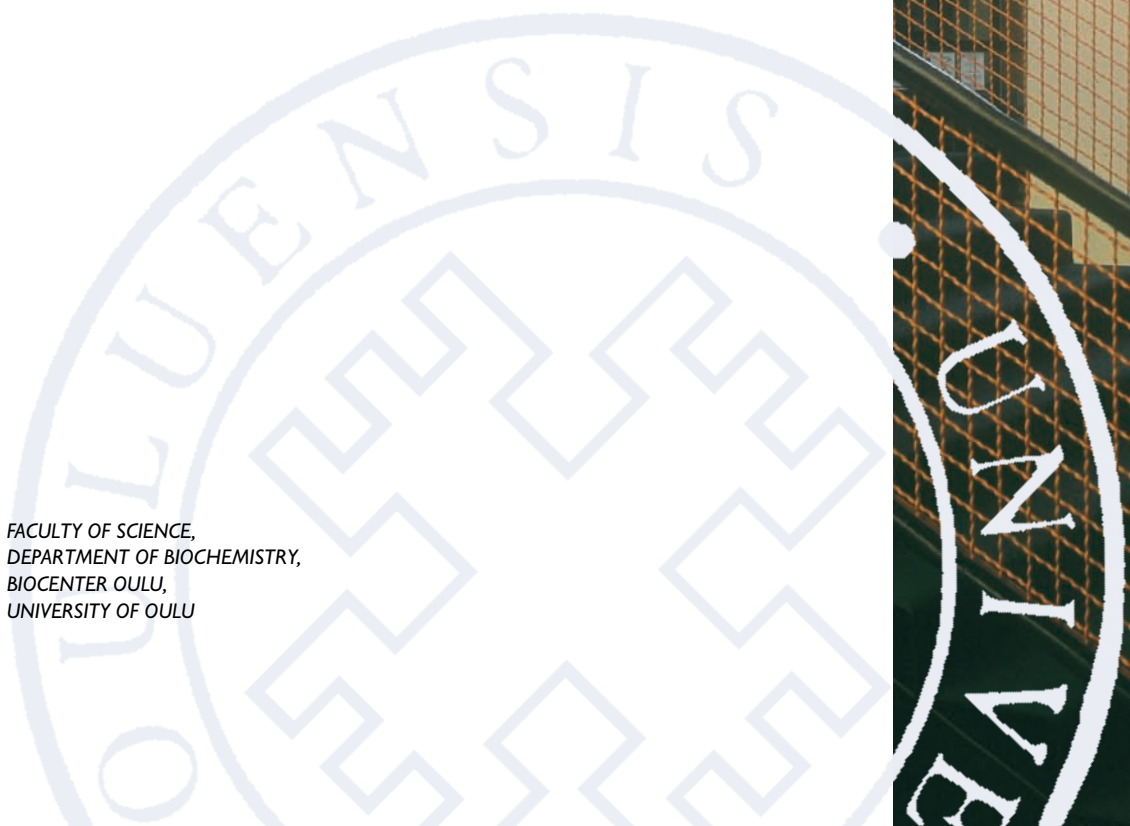
Regina Pudas

STRUCTURAL AND
INTERACTION STUDIES ON
THE CARBOXY-TERMINUS
OF FILAMIN, AN ACTIN-
BINDING PROTEIN

FACULTY OF SCIENCE,
DEPARTMENT OF BIOCHEMISTRY,
BIOCENTER OULU,
UNIVERSITY OF OULU

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REGINA PUDAS

**STRUCTURAL AND INTERACTION
STUDIES ON THE CARBOXY-
TERMINUS OF FILAMIN, AN ACTIN-
BINDING PROTEIN**

Academic dissertation to be presented, with the assent of the Faculty of Science of the University of Oulu, for public defence in Auditorium TA105, Linnanmaa, on December 5th, 2006, at 10 a.m.

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Supervised by
Professor Jari Yläne

Reviewed by
Docent Pekka Lappalainen
Professor Juha Rouvinen

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Faculty of Science, Department of Biochemistry, University of Oulu, P.O.Box 3000, FI-90014
University of Oulu, Finland, Biocenter Oulu, University of Oulu, P.O. Box 5000, FI-90014
University of Oulu, Finland
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Abstract

Filamins are large dimeric proteins that cross-link actin into three-dimensional bundles or orthogonal networks. In addition to an actin-binding domain, each filamin monomer contains 24 immunoglobulin-like domains separated by flexible regions between domains 15–16 and 23–24. Dimerisation of filamin occurs through the Ig-like domain 24. Filamins bind to a variety of molecules. They provide a link between the plasma membrane and the cytoskeleton through interactions with transmembrane receptors, and at the same time, serve as a platform for signalling molecules. Filamins are involved in several human diseases affecting the central nervous system, vascular system and muscle. In this study the structure of the the carboxy-terminus of filamin was resolved and details of filamins interaction with a platelet surface protein important in haemostasis were analysed.

An x-ray structure of the Ig-like domain 24 of human filamin C was solved at the resolution of 1.43 Å. The asymmetric unit of the crystal contains one monomer; a crystallographic dimer is formed by 2-fold axis symmetry. Point mutation studies confirmed that the dimer seen in the crystal is also present in solution. The structure showed that the dimerisation mode of human filamin is completely different from that in the *Dictyostelium discoideum* amoeba filamin analogue. Human filamin dimerises through β -strands C and D, and the *Dictyostelium* protein through β -strands B and G located on the opposite edge of the β -sandwich. Based on the sequence homology between vertebrate filamins it was proposed that the interface seen in human filamin is common for all vertebrate filamins.

The structure of human filamin C Ig-like domains 23–24 was solved by combining the techniques of x-ray crystallography and small angle x-ray scattering (SAXS). This structure provides further insight into the organization of the domains in the carboxy-terminal part of filamin molecule.

One of the first structural examples of the interaction of filamin with a ligand was provided by this study. The x-ray structure of filamin A domain 17 in complex with the alpha subunit of the GPIb-V-IX receptor was solved at a resolution of 2.3 Å. The interaction between filamin and the GPIb α -V-IX receptor is important for maintaining the integrity and shape of blood platelets, as well as for regulating the receptor adhesive function. This study also revealed that the Ig-like domain 17 represents a major binding site of filamin to GPIb α . The K_d of the interaction, determined by calorimetric studies, was 11 μ M. The specificity of the filamin A 17 - GPIb α interaction is mainly determined by hydrophobic contacts.

Keywords: actin-binding protein, cytoskeleton, filamin, immunoglobulin

To Roza and Askhat

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Oulu, October 2006

Regina Pudas

List of original articles

This thesis is based on the original articles, which are referred to in the text by their Roman numerals:

- I Pudas R, Kiema T-R, Butler PJG, Stewart M & Yläne J (2005) Structural basis for vertebrate filamin dimerization. *Structure* 13(1): 111-119.
- II Nakamura F*, Pudas R*, Heikkinen O, Permi P, Kilpeläinen I, Munday AD, Hartwig JH, Stossel TP & Yläne J (2006) The structure of the GPIb-filamin A complex. *Blood* 107(5): 1925-1932.
- III Sjekloća L, Pudas R, Sjöblom B, Konarev P, Carugo O, Rybin V, Kiema T-R, Svergun D, Yläne J & Djinović-Carugo K (2006) Crystal structure of human filamin C domain 23 and small angle scattering model for filamin C 23-24 dimer. Manuscript.

*These authors contributed equally to this work.

Abbreviations

aa	Amino acid
ABD	Actin binding domain
ABP	Actin binding protein
CH	Calponin homology
GAP	GTPase activating protein
GP	Glycoprotein
GEF	Guanine nucleotide exchange factor
GST	Glutathione S-transferase
GTPases	Guanosine triphosphatases
GPCR	G protein coupled receptors
H2	Hinge 2
Ig-like	Immunoglobulin-like
IPTG	Isopropyl-1-thio- β -D-galactopyranoside
ITC	Isothermal titration calorimetry
LGMD	Limb girdle muscular dystrophy
N-WASP	Neuronally enriched Wiskott-Aldrich syndrome protein
PBS	Phosphate-buffered saline
PNH	Periventricular nodular heterotopia
SAD	Single-wavelength anomalous dispersion
Tev	Tobacco etch virus
vWF	von Willebrand factor

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1 Introduction

Filamins were discovered as a family of non-muscle actin-binding proteins. They are elongated cytoplasmic homodimeric proteins that cross-link filamentous actin into three-dimensional structures (rev. in van der Flier & Sonnenberg 2001, Stossel *et al.* 2001, Feng & Walsh 2004, Popowicz *et al.* 2006). Monomer chains of filamin comprise an actin-binding domain (ABD) and 24 immunoglobulin (Ig)-like domains.

Besides actin cross-linking, filamin functions involve anchoring of transmembrane proteins and membrane stabilisation, as well as providing a scaffold for various signalling molecules. Recently it became clear that filamin also has functions related to protein trafficking that affect the selective insertion of membrane receptors, transporters, ion channels, enzymes and cell adhesion molecules into distinct plasma membrane domains. Moreover, the important role of filamin has been demonstrated in several human diseases affecting the brain, bone and cardiovascular system, as well as muscle fibres (rev. in Krakow *et al.* 2004, Robertson *et al.* 2005).

Filamin functions through binding to a vast variety of molecules, and for a significant part of the binding partners, the interaction site resides in C-terminal Ig-like domains 16-24 of filamin. Domain 24 of filamin is necessary and sufficient for dimerisation and serves as a platform for a number of molecules participating in the regulation of filamin function. The accumulation of ligands on the Ig-like domain 24 allows them to interact with each other in close proximity thus facilitating cross-talk between different signaling pathways (Stossel *et al.* 2001). Furthermore, the mutation in the FLNC gene, which causes a human myofibrillar myopathy, was mapped to domain 24 (Vorgerd *et al.* 2005).

In the present study the structural details of the carboxy-terminus of filamin were unveiled. The x-ray structure of the human filamin C dimerisation domain was solved, which revealed a different dimerisation mechanism from that in *Dictyostelium discoideum* amoeba filamin, also called gelation factor protein (McCoy *et al.* 1999, Popowicz *et al.* 2004). Based on the significant similarities in protein sequences it was proposed that the dimerisation mode found in human filamin is common for all vertebrate filamins. We were able to generate a low resolution model for the tandem filamin domains 23-24 by small angle x-ray scattering (SAXS).

The interaction of filamin A with the alpha subunit of the GPIb-V-IX receptor complex is essential for platelet adhesion to the von Willebrand Factor, has a significant

influence on platelet integrity and shape under high shear, and is important for normal signal transduction reactions involved in platelet activation (Williamson *et al.* 2002). Given the physiological importance of this interaction, it was characterised in more detail in the present study. The GPIb α interaction site was mapped to the Ig-like domain 17 of human filamin A, and an x-ray structure of the GPIb α -filamin A 17 complex was solved. The amino acid residues responsible for this interaction were identified on filamin and the general model for the filamin A – GPIb-V-IX complex interaction was proposed. The structure presents an example of filamin-ligand binding and may be used as a general model for such interactions.

Overall, the present study provides a deeper insight into filamin structure and presents mechanistic details of filamin interactions.

2 Review of the literature

2.1 Actin cross-linking proteins

The actin cytoskeleton of eukaryotic cells plays a central role in many cell functions such as the maintenance of asymmetric (polarised) cell shape, cell division, adhesion, contraction, motility, signal transduction, protein sorting and phagocytosis. To perform these functions, the organization of the actin cytoskeleton must be tightly regulated, both in terms of space and time. The complex and dynamic properties of the actin cytoskeleton are regulated at multiple levels by a variety of proteins that control actin polymerisation, sever actin filaments, cross-link actin filaments into networks or bundles, or act as molecular motors (Pollard & Cooper 1986, Carlier & Pantaloni 1997, Ayscough 1998, Dos Remedios *et al.* 2003).

Proteins that cross-link F-actin are important for maintenance of the viscoelastic properties of the cytoplasm and the integrity of the plasma membrane associated cytoskeleton, as well as the generation of cell locomotion in conjunction with motor proteins and proteins modulating actin assembly and disassembly (Rivero *et al.* 1996, McGough 1998). The assembly of actin bundles or networks is dictated by structural differences between the actin crosslinking proteins. Small crosslinking proteins, in which the two actin binding sites are in close proximity, tend to pack actin filaments tightly into bundles, whereas large flexible proteins in which the two binding sites are distant, direct the formation of loose assemblies (Matsudaira 1991, Otto 1994, Puius *et al.* 1998).

Generally, the cross-linking of actin requires at least two actin-binding sites, one per each filament. The largest family of F-actin-crosslinking proteins is characterised by a conserved 250 aa actin binding domain (ABD), that consists of two tandem calponin-homology (CH) domains (de Arruda *et al.* 1990, Stradal *et al.* 1998). CH domains are so named because the muscle regulatory protein calponin has been adopted as the family prototype (Castresana & Saraste 1995). However, calponin itself does not bind actin through its CH-region (Gimona & Mital 1998, Leinweber *et al.* 1999).

A classification of actin binding and cross-linking proteins is based on domain composition (Stradal *et al.* 1998, Gimona *et al.* 2002, Korenbaum & Rivero 2002). Proteins involved in F-actin cross-linking generally fall into three subclasses (Puius *et al.*

1998): the first subclass has the simplest organisation, with two tandem ABDs on the same polypeptide chain (fimbrin, plastin); the second subclass (α -actinin, spectrin, dystrophin, plakin families, utrophin) form non-covalent dimers via a dimerisation interface composed of variable numbers of a three helix coiled-coil domain, called a spectrin repeat (Djinovic-Carugo *et al.* 2002); the third subclass, which includes filamins, is characterised by a dimerisation interface composed of an antiparallel seven-stranded β -barrel adopting an Ig-like fold.

2.2 Overall structure of filamins

Filamins are a family of large, high molecular weight, homodimeric actin-crosslinking proteins, which organize actin filaments into parallel arrays or three-dimensional webs, depending on the relative actin/filamin ratio and the source of purified filamin, and link them to cellular membranes (Niederman *et al.* 1983, Weihing 1985, Hou *et al.* 1990). Filamins anchor a variety of transmembrane proteins to the actin cytoskeleton and provide a scaffold for many cytoplasmic signalling molecules (reviewed in van der Flier & Sonnenberg 2001, Stossel *et al.* 2001).

Filamin was first identified in chicken gizzard and rabbit peripheral blood as a protein capable of inducing actin polymerization, (Wang *et al.* 1975, Hartwig & Stossel 1975). The term 'filamin' refers to the ability of this protein to form filamentous arrays in cultured cells along stress fibres *in situ* (Wang *et al.* 1975). Similar proteins were then discovered in macrophage cells (Hartwig & Stossel 1981), thyroid gland (Roustan *et al.* 1982), lower eukaryotic organisms like *Dictyostelium discoideum* amoeba (Condeelis *et al.* 1982) and around skeletal muscle Z lines (Gomer & Lazarides 1983). No filamin-like proteins were identified either in yeast or plants. Studies on avian filamins isolated at different stages of myogenesis (Gomer & Lazarides 1983) suggest the possibility of switching filamin isoforms during myogenesis, however, similar studies on mammalian filamins (van der Ven *et al.* 2000) imply that only the muscle filamin isoform is expressed during the myofibril assembly from its earliest stage.

Filamin appears in electron micrographs as an extended Y-shaped flexible homodimer, with subunits connected to each other at one end (Tyler *et al.* 1980, Hartwig & Stossel 1981) with a molecular weight of 280 kDa per subunit and approximate dimensions of 3-5 nm wide and 162 \pm 16 nm long (Hartwig & Stossel 1981, Matsudaira 1991) (Fig. 1).

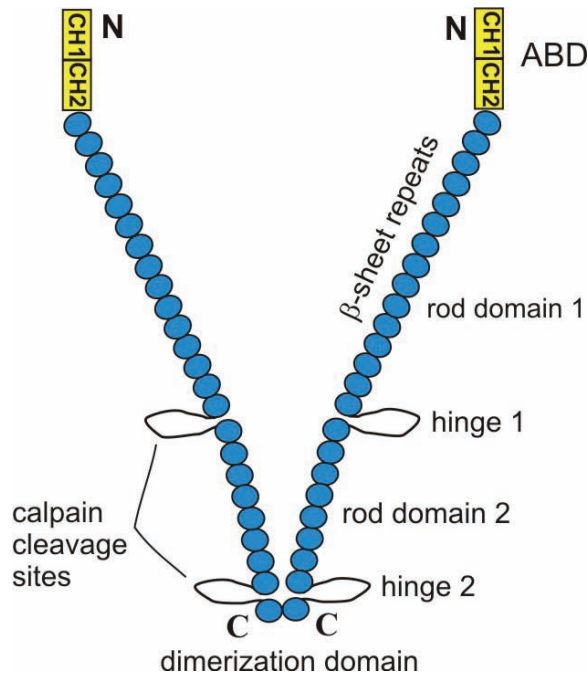


Fig. 1. Overall structure of filamins.

2.3 Detailed structure of filamin

2.3.1 Actin-binding domain of filamin

The N-terminus of each filamin monomer is composed of two CH domains that make up the 274 amino acid ABD. Each CH domain is composed of six helical segments and the relative orientation of the two CH domains to each other is dictated by a hydrophobic core composed of residues contributed by the amino and carboxyl termini and the helix connecting the two CH domains. The actin-binding site is formed by the conserved hydrophobic region corresponding to the last 15 aa residues in the C-terminus of the CH1 domain. The CH2 domain enhances its binding affinity, but it alone does not bind F-actin (Bañuelos *et al.* 1998).

2.3.2 Structure of filamin repeats

The filamin rod region consists of 24 Ig-like domains of 96 aa each, also called filamin repeats, which are interrupted by one or two flexible non-modular ‘hinge’ regions (Fig. 1). Except for those hinge regions, the linkers between filamin repeats are short and rich in proline. All vertebrate filamins have a 38-aa long stretch between repeats 23 and 24, designated ‘hinge 2’. Hinge 1 between repeats 15 and 16 is lacking in some filamin splice variants. The hinge regions are particularly sensitive to proteolysis by calpain, a calcium-dependent protease (Davies *et al.* 1978, Weihing 1988).

Each Ig-like repeat has dimensions of $17\text{\AA} \times 22\text{\AA} \times 48\text{\AA}$ (Fucini *et al.* 1997) and is a β -sandwich, which belongs to the E-set superfamily of Ig-like folds (Murzin *et al.* 1995). Such an arrangement implies two β -sheets of four and three β -strands (sheet I: D-E-B-A; sheet II: G-F-C). However, the 6th dimerisation domain of the *Dictyostelium discoideum* filamin has a different fold, with strand A missing and an additional carboxy-terminal strand H present (McCoy *et al.* 1999, Popowicz *et al.* 2004).

2.3.3 Dimerisation of filamin

Dimerisation is crucial for the actin cross-linking function of filamins and occurs through the most C-terminal domain (Davies *et al.* 1980, Hock *et al.* 1990). It has been shown that the Ig-like domain 24 alone is sufficient for filamin dimerisation (Himmel *et al.* 2003). Heterodimerisation has been shown to occur *in vivo* between isoforms A and B (Sheen *et al.* 2002), although experiments *in vitro* failed to confirm it (Himmel *et al.* 2003). However, filamin B and C could form heterodimers *in vitro* (Himmel *et al.* 2003).

When the current work was started only the structure of the gelation factor protein – filamin homologue from the *Dictyostelium discoideum* amoeba was known (McCoy *et al.* 1999, Popowicz *et al.* 2004).

The *Dictyostelium* gelation factor structure comprising repeats 4-6 shows two tail-to-tail dimerising chains that overlap only at repeat 6 in an antiparallel way (Fucini *et al.* 1999). However, the parallel arrangement of the filamin chains rod regions implied by electron micrographs of human filamin A (Hartwig & Stossel 1981) suggests the existence of an alternative dimerisation mechanism. One model for molecular organisation of filamin based on a bivalent leaf spring has been proposed by Gorlin *et al.* in 1990 (Gorlin *et al.* 1990).

2.4 Filamin protein family

The human filamin family comprises three members: filamin A, filamin B and filamin C, encoded by genes FLNA, FLNB and FLNC, respectively. FLNA is located on chromosome Xq28 (Gorlin *et al.* 1990); FLNB on chromosome 3p14.3 (Takafuta *et al.* 1998, Xu *et al.* 1998); and FLNC on chromosome 7q32-q35 (Maestrini *et al.* 1993).

The three filamin isoforms show 70-80% identity over their entire sequence with the exception of two hinge regions, which show greater divergence. Filamin C contains a unique 81 aa insertion within repeat 20 that shows no sequence similarity to any other repeats in this family (Xie *et al.* 1998). The exon-intron structure of all three human filamin gene paralogues is highly conserved, but the gene organization does not correlate with the domain structure of the respective proteins (Chakarova *et al.* 2000).

Splice variants of all three human filamin isoforms add more complexity to the filamin protein family. Variants lacking hinge 1 (Δ H1) of filamins B and C (Xu *et al.* 1998, Xie *et al.* 1998), the 8 aa region in repeat 15 of filamin A (Maestrini *et al.* 1993, Patrosso *et al.* 1994), the 41-aa region between repeats 19 and 20 of both filamin A ($A_{\text{var-1}}$) and B ($B_{\text{var-1}}$) and the four carboxy-terminal domains of the cardiac-specific filamin B ($B_{\text{var-2}}$ and $B_{\text{var-3}}$) (van der Flier *et al.* 2002) have been identified.

In addition to humans, other vertebrate species have three filamin genes sequenced thus far that contain 24 Ig-like domains (Ensembl Database (Birney *et al.* 2004)). Invertebrates, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have only two filamin genes, one of which shows an apparent similarity to vertebrate filamins, and the other is completely different from any of them. For example, *Drosophila* filamin-240 (*cher*) results from the *cheerio* locus and contains 20 repeats, of which repeats 6-9, corresponding to human filamin, are deleted. Its truncated version known as filamin-90 results from alternative splicing and contains only 9 C-terminal repeats (Li *et al.* 1999, Sokol & Cooley 1999, Guo *et al.* 2000). The similarity of *cher* to vertebrate filamins is obvious (Fig. 6B, I). However, the *Drosophila* filamin *jbug* is less similar to vertebrate filamins and it contains three calponin-homology domains at the N-terminus. *C.elegans* filamin (encoded in chromosome IV, wormbase gene ID Y66H1B.2) is similar to vertebrate filamin, but other filamin-like proteins in *C.elegans* (encoded in the X chromosome, wormbase gene ID C23F12.1) resemble the *jbug* of *Drosophila*. Slime mould *Dictyostelium discoideum* filamin homologue gelation factor (ABP-120) (Noegel *et al.* 1989) contains 6 repeats and *Entamoeba histolytica* EhABP-120 contains only 4 repeats (Vargas *et al.* 1996).

All three filamin isoforms are widely expressed in most human tissues; however some clear differences in expression patterns between isoforms are present. Filamin A is expressed more in, for example, heart and lung, blood vessels and haematopoietic cells, and filamin B in kidney and pancreas (Takafuta *et al.* 1998, Sheen *et al.* 2002). Only isoforms A and B are present in the central nervous system (Sheen *et al.* 2002). Splice variants of filamin B show an expression pattern different from that of wild type. Thus, while wild type is predominantly expressed in prostate, uterus, lung, liver, thyroid, stomach, lymph node, small intestine and spleen, its Δ H1 variant is mostly present in Daudi cells and spinal cord. Placenta, bone marrow, brain, umbilical vein endothelial cells, retina and skeletal muscle contain both forms (Xu *et al.* 1998). $B_{\text{var-1}}$ is detectable in heart, lung and skeletal muscle (van der Flier *et al.* 2002). Moreover, filamin B is expressed in human growth plate chondrocytes and in the developing vertebral bodies in the mouse (Krakow *et al.* 2004). The expression of filamin C has been originally shown to be restricted to skeletal and cardiac muscle (Maestrini *et al.* 1993), but a later study revealed the presence of the wild type as well as the Δ H1 variant in many other tissues not analysed previously, like stomach, uterus, prostate, retina, spinal cord and bone marrow (Xie *et al.* 1998). However, filamin C is the only one isoform expressed in

mature myotubes and in adult muscle fibres. In mouse primary skeletal muscle cells, filamin C gradually replaces isoform A during the earliest stages of skeletal muscle differentiation (Chiang *et al.* 2000).

In mammalian striated muscle, filamin C is enriched in Z-disks, particularly at the edges, and, in addition, it is present in intercalated disks of cardiac muscle and in myotendinous junctions of skeletal muscle (van der Ven *et al.* 2000). Only 3 % of muscle filamin is associated with plasma membrane (Thompson *et al.* 2000). In chicken smooth muscle filamin has been found in dense plaques and dense bodies (Tachikawa *et al.* 1997).

It has to be mentioned though, that in early studies no distinction was made between filamin isoforms (Wang *et al.* 1975, Hartwig & Stossel 1975, Stossel & Hartwig 1975). What is known to be filamin isoforms today was previously referred to as ‘filamin’ or ‘actin-binding protein’ when found in different tissues (Wallach *et al.* 1978, Bechtel 1979, Koteliansky *et al.* 1981). The difference between the filamin isoforms localization was not clear. Filamins were referred to as a family of proteins for the first time 10 years after their discovery (Weihsing 1985, Pollard & Cooper 1986).

In cultured cells filamin localizes to cortical actin cytoskeleton on the cell periphery and along densely bundled stress fibres (Langanger *et al.* 1984), but it is also found in focal adhesions (Zamir 2001) and in membrane ruffles of migrating cells (Vadlamudi *et al.* 2002). In dividing cells, filamin is concentrated in the cleavage furrow, where it remains associated at the mid-body region until the completion of cell division (Nunnally *et al.* 1980).

Although filamin resides and executes most of its functions in the cytoplasm, recent data shows evidence of its involvement in nuclear-related functions (Ozanne *et al.* 2000, Loy *et al.* 2003).

2.5 Role of filamin and its interaction partners in cell functioning

Filamin functions in general can be sorted into three groups: (1) organising the actin cytoskeleton; (2) providing a link between extracellular matrix, plasma membrane and actin cytoskeleton through interaction with a number of transmembrane receptors; (3) serving as a platform for a variety of signalling molecules and thus playing an important role in signal transduction between the cell membrane and cell interior (Fig. 2). The interaction partners of filamins are listed in Table I, and these three functions are discussed below.

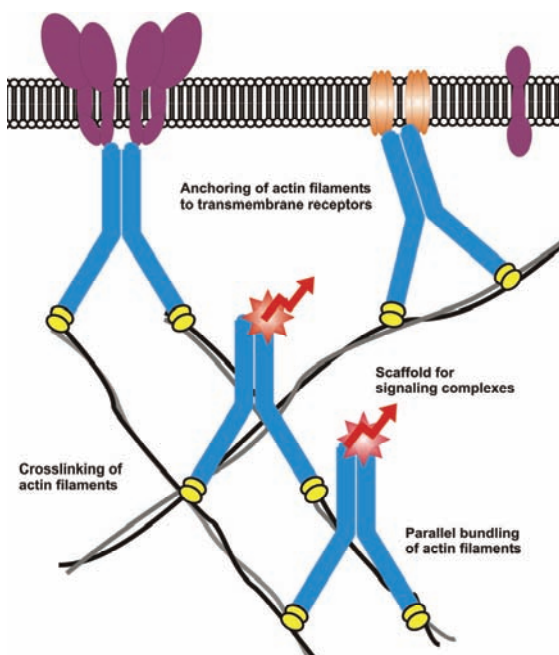


Fig. 2. Filamin functions.

2.5.1 Interaction with actin and actin organisation

Filamins are known to induce gelation of actin (Brotschi *et al.* 1978, Bennett *et al.* 1984). Gelation is due to the cross-linking of actin filaments into orthogonal networks or bundles. Filamin binds to F-actin *in vitro* with a K_d value of about 0.5-2 μM (Hartwig & Stossel 1981). Actin-filamin networks formed *in vitro* break irreversibly when subjected to high shear stress, resembling the breakage of covalently cross-linked actin networks (Brotschi *et al.* 1978, Janmey *et al.* 1990). However, the mechanism of how filamin promotes actin filament branching is not understood completely (Stossel *et al.* 2001). It is clear, that filamin must dimerise and have intact N-terminal ABD domains.

Interestingly, increasing the filamin-actin molar ratio leads to the formation of tighter networks (Niederman *et al.* 1983). When this ratio is high (1:10-50), parallel bundles of actin filaments form (Hartwig & Stossel 1981), otherwise the stoichiometry of 1:150-740 produces high-angle branching of filamentous actin (Hartwig *et al.* 1980, Hartwig & Shevlin 1986). This result suggests that the intracellular ratio of filamin to actin could influence the organisation of actin inside the cell. However, the organisation of actin into networks or bundles is dependent also on the structure of the filamin variants. Variants, lacking the hinge 1 region, which thus have restricted flexibility, might form tight actin fibres instead of three-dimensional networks (Gorlin *et al.* 1990, Xu *et al.* 1998).

Furthermore, the viscoelastic properties of filamin-actin networks are dependent on the presence of the hinge region 1. *In vitro* experiments with the recombinant filamin lacking this hinge region demonstrated a linear stiffening and a consequent breakage of the cross-linked actin structures at much lower stresses than in the presence of a hinge 1 in filamin (Gardel *et al.* 2006)

In addition, filamin from rabbit macrophages, amphibian oocytes and human uterus reveals greater actin gelation potency than filamin from chicken gizzard (Gorlin *et al.* 1990).

Filamin possesses a dual mechanical function in controlling the architecture of F-actin networks (Tseng *et al.* 2004). At the edge of the cell, filamin in low concentrations links F-actin into orthogonal networks forming X, Y and T junctions (Flanagan *et al.* 2001), thus providing conditions for fast remodelling of the actin cytoskeleton, as well as certain stiffness which is needed to resist pushing forces produced by polymerising actin against the cell membrane (Pollard & Borisy 2003). In contrast, in higher concentrations, filamin partly provides stretchability to F-actin contractile bundles located in the ventral side of adherent cells.

2.5.2 Linking the plasma membrane and cytoskeleton: interaction with transmembrane proteins

An actin network stabilises the cell periphery and the attached plasma membrane. Filamin connects actin filaments to transmembrane receptors, thus providing membrane surface stabilisation. The membrane association of filamin is achieved by direct binding of the C-terminal part of filamin to transmembrane proteins like the glycoprotein Ib-V-IX – von Willebrand factor receptor complex (Meyer *et al.* 1997), β -integrins (Calderwood *et al.* 2000), tissue factor, the protease receptor initiating the coagulation cascade (Ott *et al.* 1998), ion channels like Kv4.2 (Petrecca *et al.* 2000) and Kir2.1 (Sampson *et al.* 2003), as well as caveolin 1, a cholesterol-binding integral membrane protein (Stahlhut & van Deurs 2000). Filamin also selectively and directly interacts with G protein coupled receptors (GPCR), such as dopamine D2 (Li *et al.* 2000) and D3 receptors (Lin *et al.* 2001), the calcitonin receptor (Seck *et al.* 2003), Ca^{2+} -sensing receptor (Awata *et al.* 2001), glutamate receptor (Enz 2002), or opioid receptor (Onoprishvili *et al.* 2003); and with tyrosine-kinase associated receptors, like Fc γ RI (Ohta *et al.* 1991) (Table I). The association of integral membrane receptors with the cytoskeleton through filamin is thought to be of key importance in regulating adhesion and spreading, as well as transmembrane signalling.

Table 1. Interaction partners of human filamins

Protein	Filamin	Repeat	Reference
Cell adhesion and signalling receptors			
GPIIb α of GPIIb-V-IX (vWF receptor) complex	FLN A, B	17-19	(Fox 1985b), (Cranmer <i>et al.</i> 1999)
Integrins * β 1A, β 1D, β 2, β 3, β 7	FLN A, B, B _{var-1} , *C	20-24 *20-21	(Sharma <i>et al.</i> 1995), (Loo <i>et al.</i> 1998), (Calderwood <i>et al.</i> 2000) (van der Flier <i>et al.</i> 2002) *(Gontier <i>et al.</i> 2005)
Gamma- and delta-sarcoglycans	FLNC	23-24 (incl.H2)	(Thompson <i>et al.</i> 2000), (Wheeler & McNally 2003)
CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1	FLNA	23-24 (incl.H2)	(Klaile <i>et al.</i> 2005)
GPCR			
Dopamine D2, 3 receptor	FLNA	16-19	(Li <i>et al.</i> 2000), (Lin <i>et al.</i> 2001)
Metabotropic glutamate receptor type 7 (mGluR7)	FLNA	21-22	(Enz 2002), (Enz & Croci 2003)
Acetylcholine receptor (AChR)	FLNA	n/d	(Bloch <i>et al.</i> 1989), (Shadiack & Nitkin 1991)
μ -opioid receptor	FLNA	H2-24	(Onopriashvili <i>et al.</i> 2003)
Calcium-sensing receptor (CaR)	FLNA	14-16 (incl. H1)	(Awata <i>et al.</i> 2001), (Hjälml <i>et al.</i> 2001), (Rey <i>et al.</i> 2005)
G-protein coupled calcitonin receptor (CTR)	FLNA	20-21	(Seck <i>et al.</i> 2003)
Thyroid-stimulating hormone receptor (TSH-R)	FLNB	n/d	(Leedman <i>et al.</i> 1993)
Ion channels			
Potassium channel Kv4.2	FLNA	Between 20-24	(Petrecca <i>et al.</i> 2000)
Potassium channel Kir2.1	FLNA	23-24 (incl.H2)	(Sampson <i>et al.</i> 2003)
Pacemaker channel HCN1	FLNA	23-24 (incl.H2)	(Gravante <i>et al.</i> 2004)
Receptor tyrosine kinases			
Insulin receptor	FLNA	22-24	(He <i>et al.</i> 2003)
Tyrosine-kinase associated receptors			
Fc γ RI	FLNA	n/d	(Ohta <i>et al.</i> 1991)
Other transmembrane receptors			
Tissue factor	FLNA	24	(Ott <i>et al.</i> 1998)
Toll-receptor	FLNA	C-term (incl.24)	(Edwards <i>et al.</i> 1997)
(D)-Presenilin 1, 2	FLNA, B FLNA (dm)	22-24	(Zhang <i>et al.</i> 1998), (Guo <i>et al.</i> 2000)
Furin receptor	FLNA	13-14	(Liu <i>et al.</i> 1997)
Cholesterol-binding integral membrane protein Caveolin 1	FLNA, B, C	22-24 (incl.H2)	(Stahlhut & van Deurs 2000)
Prostate specific membrane antigen (PSMA)	FLNA	23-24	(Anilkumar <i>et al.</i> 2003)

Protein	Filamin	Repeat	Reference
Type II transmembrane serine protease epithin (MT-SP1, matriptase)	FLNA, B*	14-24, *16-24	(Kim <i>et al.</i> 2005)
Small GTPases and related proteins			
RalA, RhoA, Rac1, Cdc42	FLNA	24 (+part of H2)	(Ohta <i>et al.</i> 1999)
Trio, Rho guanine nucleotide-exchange factor (GEF)	FLNA	23-24	(Bellanger <i>et al.</i> 2000)
Ras-GAP (GTPase activating protein)	FLNC	15-17	(Lypowy <i>et al.</i> 2005)
FilGAP, Rac GTPase activating protein	FLNA	21-24	(Ohta <i>et al.</i> 2006)
Kinases and their activators			
ROCK/Rho kinase	FLNA	24	(Ueda <i>et al.</i> 2003)
p21-activated kinase (Pak1)	FLNA	23	(Vadlamudi <i>et al.</i> 2002)
Protein kinase C α (PKC α)	FLNA	1-4 and H2-24	(Tiggess <i>et al.</i> 2003)
Protein kinase B α (PKB α)	FLNC	20 (insertion)	(Murray <i>et al.</i> 2004)
SEK1 (MKK-4, JNKK)	FLNA	21-23	(Marti <i>et al.</i> 1997)
Phosphatases			
SHIP-2	FLNA, B, C	22-24	(Dyson <i>et al.</i> 2001) (Dyson <i>et al.</i> 2003)
Nuclear transcription factors			
Androgen receptor	FLNA, C	16-19	(Ozanne <i>et al.</i> 2000), (Loy <i>et al.</i> 2003)
FOXC1	FLNA	4-6 and 17-part of 20	(Berry <i>et al.</i> 2005)
PEBP/2CBF	FLNA	hinge 2-24	(Yoshida <i>et al.</i> 2005)
Adaptor proteins			
FAP52 (PACSIN 2 or syndapin II)	FLNA	14-16 (incl.H1)	(Nikki <i>et al.</i> 2002)
FATZ-1 (myozenin-1, calsarcin 2)	FLNA, B, B _{var-1} , *C	19-24, *20-24	*(Faulkner <i>et al.</i> 2000), (Gontier <i>et al.</i> 2005)
Calsarcin 3	FLNC	20-24	(Frey & Olson 2002)
Myotilin	FLNC	19-21	(van der Ven <i>et al.</i> 2000) (Hauser <i>et al.</i> 2000)
FILIP (Filamin A interacting protein)	FLNA	15-18 (incl.H1)	(Nagano <i>et al.</i> 2002)
Migfilin	FLNA, C	21	(Tu <i>et al.</i> 2003) (Wu 2005)
FLBP-1 (Filamin binding LIM protein)	FLNB	10-14	(Takafuta <i>et al.</i> 2003)
Pdlim2, PDZ/LIM-domain containing protein (Mystique)	FLNA	n/d	(Torrado <i>et al.</i> 2004)
Adapter protein Tc-mip (truncated c-maf inducing protein)	FLNA	16-17	(Grimbert <i>et al.</i> 2004)
Xin, F-actin, Mena and VASP-binding protein	FLNC	20 (insertion)	(van der Ven <i>et al.</i> 2006)
Smad	FLNA	20-24	(Sasaki <i>et al.</i> 2001)

Protein	Filamin	Repeat	Reference
Other intracellular proteins			
TRAF2, tumour necrosis factor (TNF) receptor-associated factor-2	FLNA	15-19	(Leonardi <i>et al.</i> 2000)
LL5 β , phosphatidylinositol (3,4,5)-triphosphate sensor	FLNC	n/d	(Paranavitane <i>et al.</i> 2003), (Kishi <i>et al.</i> 2005)
Tumour suppressor BRCA2	FLNA	21-24	(Yuan and Shen 2001)
cv heat shock protein (cvHSP)	FLNA	23-24	(Krief <i>et al.</i> 1999)
HBV core protein (viral)	FLNB	23-24 (incl.H2)	(Huang <i>et al.</i> 2000)
Granzyme B	FLNA	20-24	(Browne <i>et al.</i> 2000)
KY-protein	FLNC	20-22	(Beatham <i>et al.</i> 2004)
Nephrocystin	FLNA, B	15-16 (incl.H1)	(Donaldson <i>et al.</i> 2002)

2.5.2.1 Integrins

Integrin adhesion receptors provide a linkage between the extracellular matrix and cytoskeleton and transmit signals across the plasma membrane in both directions. This connection is important for cell adhesion and cell shape regulation during cell migration and matrix remodelling.

Integrins are type I transmembrane proteins expressed on the cell surface as distinct non-covalently associated heterodimers of α and β subunits, each of which contains a large extracellular ligand-binding domain, a single transmembrane domain and a short cytoplasmic domain. There are several α and β subunits, the combination of which defines the binding specificity of integrin to extracellular molecules (rev. in Hynes 1987, Hynes 1992, Calderwood *et al.* 2000).

β -integrin cytoplasmic tails are necessary and sufficient to link integrins to the actin cytoskeleton (Burrige & Chrzanowska-Wodnicka 1996). Filamin binding to integrin requires all but the last three carboxy-terminal β -subunit residues, and the integrin binding site on filamin has been mapped to the C-terminal 477 residues (Loo *et al.* 1998), which includes the dimerisation site. However, recent results have mapped that major binding site of the integrin $\beta 7$ cytoplasmic subunit on the Ig-like domain 21 of filamin, although domain 19 also shows some binding affinity (Kiema *et al.* 2006). In the same study it was shown that filamin A competes with talin, another large actin-binding protein, for binding to β -integrin tails and that this competition can impact the talin-dependent integrin activation process, the mechanism of which was previously poorly understood.

The increased binding of filamin to β -integrin cytoplasmic tails has been shown to reduce the ability of the cells to become polarized and to initiate membrane protrusions, two of the earliest steps leading to cell migration, but had little effect on focal adhesion formation and fibronectin matrix assembly (Calderwood *et al.* 2001). This is consistent with previous studies that overexpression of filamin decreases locomotion (Cunningham *et al.* 1992). Therefore, the integrin-filamin association appears to play an important role in the regulation of cell motility.

In cells under high shear stress, filamin A is recruited to β 1-integrin-containing focal adhesions, which results in cell stiffening and increased resistance of the cell to physical strain. This observation elucidates a role of filamin in signal transduction through interaction with integrin (Glogauer *et al.* 1998).

2.5.2.2 Glycoprotein Ib alpha

The interaction between filamins A and B with the glycoprotein (GP) Iba subunit of the von Willebrand factor (vWF) receptor complex GPIb-V-IX is one of the best characterised.

GPIb-V-IX is a major transmembrane receptor complex present on the platelet surface at about 25 000 copies per platelet (Berndt *et al.* 1985). It consists of four distinct subunits, of which GPIb α (135 kD), and GPIb β (25 kD) are associated by a disulphide bond and non-covalently linked to GPIX (22 kD) and GPV (82 kD), with the stoichiometry of 2:2:2:1, respectively (Du *et al.* 1987, Fox *et al.* 1988, Modderman *et al.* 1992). Mutations in the GPIb-V-IX complex cause bleeding disorders: platelet-type von Willebrand disease and Bernard-Soulier syndrome (Miller & Castella 1982, Lopez *et al.* 1998).

GPIb α is the largest and functionally most significant subunit of the complex, since its interaction with vWF is critically important for platelet adhesion to the blood vessel wall at sites of injury and facilitates the ability of thrombin at low concentrations to activate platelets. The cytoplasmic domain of GPIb α consists of 96 aa, extending from residues 515 to 610. The interaction of GPIb α with vWF triggers another important hemostasis function – shear-induced platelet aggregation (Kroll *et al.* 1996).

How the binding of vWF to the GPIb-V-IX complex induces intracellular changes, is not known. The cytoplasmic domain of GPIba associates tightly with filamin, thus providing a link between the platelet membrane and the underlying actin cytoskeleton (Fox 1985b), an association that is important for maintaining the cytoskeletal architecture of resting platelets.

The binding of GPIb α to filamin has been demonstrated by immunoblot assays (Okita *et al.* 1985, Fox 1985a) and confirmed by yeast two-hybrid screens and pull-down assays (Meyer *et al.* 1997, Takafuta *et al.* 1998, Xu *et al.* 1998). *In vitro* binding studies have identified repeats 17-19 of filamins A and B as the minimal binding domain for the hydrophobic aa sequence Thr⁵³⁶-Phe⁵⁶⁸ of GPIb α (Andrews & Fox 1992). Deletion mutants of GPIb α lacking aa stretches 551-570 (Englund *et al.* 2001), 557-568 and 569-579 (Williamson *et al.* 2002) and 560-570 (Feng *et al.* 2005) completely abolished its interaction with filamin A in CHO cells. It appears that the stretch of amino acids between Trp⁵⁷⁰ and Ser⁵⁹⁰ is essential for maintaining the cytoskeletal association in the intact cell by interacting with submembranous filamin (Cunningham *et al.* 1996). Furthermore, Trp⁵⁷⁰ and Phe⁵⁶⁸ have been identified as critical residues for maintaining GPIb-V-IX receptor anchorage to the membrane skeleton and in maintaining cell adhesion to a vWF matrix under high shear stress (Cranmer *et al.* 2005).

The GPIb α -filamin interaction has a significant influence on platelet integrity and shape. Moreover it is important for regulation of the GPIb-V-IX receptor adhesive

function (Mistry *et al.* 2000, Englund *et al.* 2001, Williamson *et al.* 2002). These studies emphasise the need to examine more precisely the interaction between the GPIIb α cytoplasmic domain and filamin.

2.5.3 Scaffold for signalling molecules

Cell signalling takes place in different compartments, which dynamically interact with each other. It is, however, not clear how signalling molecules are targeted to different cellular compartments (Meder & Simons 2005). The submembranous F-actin cytoskeleton is connected to the plasma membrane by proteins interlinking actin filaments and integrated membrane proteins. F-actin recruits signalling molecules into the submembranous area, where signalling complexes build up and function. Filamin serves as a scaffold for a variety of intracellular signalling molecules and is involved in the regulation of their signalling pathways. These include several small G proteins (RhoA, Rac1, Cdc42); and their effectors like mitogen-activating protein kinase kinase-4, MKK4 or SEK1, a kinase that activates stress activated protein kinase (SAPK) to phosphorylate c-Jun (Marti *et al.* 1997) in programmed cell death; and TRAF2, the association with which involves filamin in the tumour necrosis factor receptor and Toll receptor signalling pathways and implies a role of filamin in inflammatory signal transduction (Leonardi *et al.* 2000) Filamin is linked to a number of signalling processes including those regulated by the kinases of the *Src* family, p56(lck) and PKC α (Goldmann 2002, Tigges *et al.* 2003) (see Table 1 for more interaction partners).

Cdc42, Rac1 and RhoA, members of the Rho family (Ras-superfamily) of small GTPases, have received a lot of attention for their distinct effects on the actin cytoskeleton, which in fibroblasts lead to the formation of filopodia, lamellipodia and membrane ruffles, and stress fibres, respectively. Filamin is indirectly involved into regulation of actin dynamics through its interaction with p21-activated kinase (Pak21) (Vadlamudi 2002) – the downstream effector of Rac1 and Cdc42. This interaction results in the formation of ruffles, lamellipodia and filopodia (Sells *et al.* 1997) and regulates cell motility (Sells *et al.* 1999).

RhoA, Rac1, Cdc42 and RalA have been shown to bind filamin, however, only the binding of RalA is GTP-dependent (Ohta *et al.* 1999), indicating that filamin has a specific effector function for RalA. Moreover, filamin is necessary for RalA-activated filopodia formation. Considering the facts that RalA activation increases the amount of cytoskeletal filamin, and that the filamin-RalA binding site corresponds to filamin dimerisation repeat 24, a possible mechanism of filopodia formation could be proposed, which involves the concentration of filamin, triggered by RalA activation, at the sites of actin cross-linking, to where actin fragments are recruited by Cdc42 and its effector N-WASP (Miki *et al.* 1998, Ohta *et al.* 1999). Yet, this might be just one mechanism controlling cell shape and mechanics.

The Rho GEF Trio, shown in *Drosophila* and *C.elegans* to be involved in axon guidance and cell migration in the nervous system (Liebl *et al.* 2000, Steven *et al.* 1998), binds to filamin and this association is necessary for Trio-mediated membrane ruffle formation.

It has been proposed that Rho GTPases bind to filamin and wait for activators like Trio to turn them on and for other effectors to turn them off (Bellanger *et al.* 2000). However, recent data revealed a mechanism of negative regulation of cell movement, involving competition of the CEACAM1 protein with RalA for binding of the 24th repeat of filamin and thus inhibiting RalA mediated signalling (Klaile *et al.* 2005).

A mechanism for the regulation of cell polarity was recently proposed with filamin playing a central role through its interaction with a new GAP for Rac GTPase named FilGAP (Ohta *et al.* 2006). According to Ohta and colleagues, FilGAP complements Trio to control the activity of filamin-associated Rac to affect cell polarization. The interaction of filamin with FilGAP results in suppressed lamellae formation at the leading edge in response to activated Rho kinase (ROCK).

Consequently, filamin provides a scaffold for a variety of signalling proteins, being involved in the integration of the external stimuli, thus becoming a key component in regulation of actin cytoskeleton dynamics.

2.6 Role of filamins in cell migration

Cell migration is a multistep process that requires the integration and coordination of complex biochemical and mechano-chemical signals. A key event in the response to those signals is actin polymerization in the direction of migration to provide the protrusion force for the extension of the leading edge as lamellipodia or filopodia. The ability to organize F-actin into arrays and networks brings filamin forward as an essential component for stabilization of the cell surface, alteration of cell shape, organisation of protrusive activity and efficient movement *in vivo* (Flanagan *et al.* 2001).

Loss of filamin A expression in cells results in altered morphology and impaired migration. Thus, the cultured melanoma cell line M2, which is deficient in human filamin A, exhibits extensive, continuous blebbing of the plasma membrane, resulting from global cytoskeletal disorganisation. Although blebs on a spreading cell edge are a feature of a certain cell state, such blebbing is markedly prolonged in cells lacking expression of filamin (Cunningham 1995). Mutant cells are unable to support the formation of lamellae and lack translocational motility. However, the cell restores its appearance and ability to migrate after stable transfection with filamin mRNA (Cunningham *et al.* 1992).

Furthermore, filamin A deficient cells do not accumulate actin in the sites of force application, which suggests the role of filamin in mechanoprotection (Glogauer *et al.* 1998).

Dictyostelium amoeba cell lines depleted of filamin, show defects in pseudopod formation which results in impaired motility, chemotaxis and phagocytosis (Cox *et al.* 1992, Cox *et al.* 1996, Ponte *et al.* 2000). These cells show also increased sensitivity to osmotic shock (Rivero *et al.* 1996). *Dictyostelium* slugs with the gene encoding filamin disrupted show impaired photo- and thermosensory responses (Fisher *et al.* 1997). These altered properties could reflect a reduced strength of the cortical cytoskeleton.

Several proteins are known to regulate cell migration by interacting with filamins. Filamin links the peripheral actin cytoskeleton to the plasma membrane and is targeted by a variety of different signalling molecules, like FILIP (Nagano *et al.* 2002, Nagano *et al.*

2004), ribosomal S6 kinase (Woo *et al.* 2004), protein kinase C α (Tigges *et al.* 2003), SEACAM1 (Klaile *et al.* 2005) and small GTPases from the Ras family (Ridley 2001), that implicate filamin in cell shape remodelling, adhesion and motility.

2.7 Role of filamins in regulation of tissue and organ development

With the well established role of filamin in the maintenance of cell shape and motility as well as in the integration of cell signals, it would be reasonable to speculate that molecular pathology of the filamin gene would result in alteration of its functions leading to the abnormalities in cell appearance and locomotion (Cunningham *et al.* 1992); and, moreover, contribute to defects and malformations during embryonic, foetal and postnatal development (Robertson 2005).

The first human phenotype associated with mutations in the FLNA gene was a periventricular nodular heterotopia (PNH) (Eksioglu *et al.* 1996, Fox *et al.* 1998), a disorder where a subset of neurons fail to migrate from the subventricular zone to the neocortex during foetal development (Barkovich & Kjos 1992). PNH is an X-linked dominant inherited disorder and characterised by seizures and cardiovascular malformation in heterozygous females and prenatal death in hemizygous males (Eksioglu *et al.* 1996, Poussaint *et al.* 2000). However, later results revealed a few cases of males with the PNH (Sheen *et al.* 2001, Gerard-Blanluet *et al.* 2006, Masruha *et al.* 2006). PNH is caused by FLNA gene mutations and results from different genetic mechanisms like missense mutations, protein truncation and splicing mutations, which cause phenotypes of different severity (Guerrini *et al.* 2004) (Table 2). However, it should be pointed out that the major phenotypic appearances of PNH are not associated with FLNA gene mutations, suggesting that key roles belongs to other genes (rev. in Parrini *et al.* 2006).

The mechanisms by which filamin A affects neuronal migration are unclear. Certain proteins, for example FILIP, might have a role in regulating the level of filamin A in neurons of the subventricular zone (Nagano *et al.* 2002). However a recent study (Lo Turco & Bai 2006) identified a critical point of neuronal migration control as a newly recognized morphological stage of neuronal migration – the multipolar stage, which is regulated in part by filamin A. Disruption of this stage by mutations in the proteins involved cause distinct neuronal migration disorders.

Such features of PNH as cardiovascular anomalies indicate a critical role for filamin A beyond the central nervous system, in hemostasis and vascular remodelling (Fox *et al.* 1998, Kakita *et al.* 2002). For example, in response to extracellular ligands filamin A is recruited to tissue factor (Ott *et al.* 1998), the protease receptor which has multiple roles in vascular development, angiogenesis and tumour cell metastasis (Bugge *et al.* 1996) and this interaction is associated with the reorganisation of actin filaments.

Mutations in the FLNA gene were identified, affecting craniofacial structures, skeleton, brain, viscera and urogenital tract in X-linked human disorders belonging to the otopalatodigital syndrome (OPD) spectrum (including OPD1 and 2, frontometaphyseal dysplasia (FMD) and Melnick-Needles syndrome (MNS)) (Robertson *et al.* 2003, Hidalgo-Bravo *et al.* 2005, Stefanova *et al.* 2005) (Table 2). Unlike mutations causing PNH, OPD mutations are localized to certain sites (Table 2). These mutations implicate

filamin A in signalling pathways that mediate organogenesis in multiple tissues during embryonic development and do not overlap with PNH-spectrum disorders.

Filamins A and B seem to have partially overlapping and partially specific functions in the central nervous system (Sheen *et al.* 2002). Both proteins are implicated in neuronal migration during cortical development, but filamin B possibly has a greater role during neuronal proliferation, while filamin A is involved in multiple stages of development. The possibility of heterodimer formation between filamin isoforms A and B in the periventricular zone suggests that in a subset of neurons filamin B may compensate for the lack of filamin A (Sheen *et al.* 2002).

Mutations in the *FLNB* gene result in human skeletal disorders: spondylarthritis (SCT), Larsen syndrome and perinatal lethal atelosteogenesis (AO) I and III (Krakow *et al.* 2004, Farrington-Rock *et al.* 2006, Bicknell *et al.* 2006) (Table 2). These data indicate a role of filamin in vertebral segmentation, joint formation and endochondral ossification. Like mutations in *FLNA*, mutations in *FLNB* produce a diverse range of phenotypes, depending on the nature and location of the mutation. Although the role of filamin A in skeletogenesis has been demonstrated (Robertson *et al.* 2003), mutations in the genes encoding isoforms A and B do not have the same biological consequences, and their expression pattern in cartilage is distinct (Krakow *et al.* 2004).

The limb-girdle muscular dystrophies (LGMD) comprise a heterogeneous group of hereditary diseases characterised by muscle weakness with histologic signs of necrosis and regeneration in muscle (Bushby 1999). It was hypothesized that filamin C might be involved in LGMD disease through its interactions with γ - and δ -sarcoglycans, members of dystrophin-glycoprotein complex (Thompson *et al.* 2000). But, although the LGMD1 locus was mapped to chromosome 7q (Speer *et al.* 1999, Gamez *et al.* 2001), and, subsequently, to the 7q32-32.2 region, where the *FLNC* gene would be an obvious candidate for the disease (Palenzuela *et al.* 2003), defects in *FLNC* or its protein product were not detected by Palenzuela and colleagues. However, a truncation mutation in the *FLNC* gene was found to cause a myofibrillar myopathy (MFM) (Vorgerd *et al.* 2005), a human neuromuscular disease resulting in weakening of limb muscles (Engel 1999). This mutation results in a truncated Ig-like domain 24 in filamin C, preventing the protein from dimerising properly, thus disturbing the fine balance between filamin C and its interacting partners on both the Z-disk and cell membrane and causing filamin aggregation in myofibrils (Vorgerd *et al.* 2005).

Table 2. Human diseases caused by mutations in *FLN* genes.

Disease	Filamin isoform/ protein domain	Protein consequence from DNA mutation	Reference
PNH	A/IG1, 15 22, 24; A/CH2, IG4, 11,12, 13, 21; A/IG11, 15, 23, 24; A/IG22, 24	16 mutations, including splice site mutations; truncations; deletions (24 – fsX2617); insertions (24 fsC X 2600)	(Parrini <i>et al.</i> 2006)
PNH	A/IG23	Deletion L2439-G2445	(Zenker <i>et al.</i> 2004)
PNH	A/IG7, 21	2 truncations	(Sheen <i>et al.</i> 2005); (Sheen <i>et al.</i> 2001)
PNH	A/CH1, IG2, IG4, IG24 (P2641L)	7 substitutions	(Guerrini <i>et al.</i> 2004); (Gomez-Garre <i>et al.</i> 2006); (Masruha <i>et al.</i> 2006); (Sheen <i>et al.</i> 2001); (Hehr <i>et al.</i> 2006); (Gerard-Blanluet <i>et al.</i> 2006)
OPD (variable phenotype)	A/IG14	R1635-V1637del	(Stefanova <i>et al.</i> 2005)
OPD1	A/CH2	3 substitutions	(Robertson <i>et al.</i> 2003); (Hidalgo-Bravo <i>et al.</i> 2005)
OPD2	A/CH2; A/IG14	8 substitutions; Deletion G1614-Y1616	(Robertson <i>et al.</i> 2003); (Robertson <i>et al.</i> 2003)
FMD	A/IG14	I1620del	(Robertson <i>et al.</i> 2003)
FMD	A/IG 14, 15, 23	4 substitutions	(Robertson <i>et al.</i> 2003); (Zenker <i>et al.</i> 2004); (Zenker <i>et al.</i> 2006)
MNS	A/IG10	3 substitutions	(Kristiansen <i>et al.</i> 2002); (Robertson <i>et al.</i> 2003)
SCT	B/IG5, 6, 14, 22 B/IG20	4 truncations S2137 frame shift	(Krakow <i>et al.</i> 2004)
Larsen Syndrome	B/IG14; B/CH2, IG14, 15	N1571del; Multiple substitutions	(Krakow <i>et al.</i> 2004); (Zhang <i>et al.</i> 2006); (Bicknell <i>et al.</i> 2006)
AOI	B/CH2, B/IG14, 15	Multiple substitutions, deletions	(Krakow <i>et al.</i> 2004); (Farrington-Rock <i>et al.</i> 2006)
AOIII	B/CH2, IG6, B/IG14, 15	Multiple substitutions	(Krakow <i>et al.</i> 2004); (Farrington-Rock <i>et al.</i> 2006)
MFM	C/IG24	Truncation W2710X	(Vorgerd <i>et al.</i> 2005)

*Abbreviations: OPD – otopalatodigital syndrome; FMD – frontometaphyseal dysplasia; MNS – Melnick-Needles syndrome; SCT – spondylocarpotarsal syndrome; AOI and III – atelosteogenesis I and III; MFM – myofibrillar myopathy.

2.8 Regulation of filamin function

Filamin functions are regulated on many levels by proteolysis (Davies *et al.* 1978, Truglia & Stracher 1981), phosphorylation by serine/threonine kinases (Ohta & Hartwig 1996), binding of phospholipids (Goldmann *et al.* 1999) and Ca^{2+} binding (Rosenberg *et al.* 1981).

It has been shown *in vitro* that filamin binding to phosphatidylinositolphosphate phospholipids (PIPs) results in inhibition of F-actin gelation and the loss of the actin-binding activity by filamin (Furuhashi *et al.* 1992).

A dynamic phosphorylation/dephosphorylation process regulates the interaction of filamin with other cytoskeletal elements. Filamin is translocated from the membrane to the cytosol in response to the bradykinin receptor – an endothelium dependent vasodilator, involved in the mechanism of pain. The process of filamin translocation is dependent upon phosphorylation of the C-terminus of filamin by the Ca^{2+} /calmodulin-dependent (CaM) kinase II and inhibited by the cAMP-dependent protein kinase pathway (Wang *et al.* 1996). The phosphorylation of filamin by CaM kinase II also (CaM) decreases its actin binding (Ohta & Hartwig 1995). *In vivo* filamin phosphorylation by p56lck tyrosine-kinase, the lymphocyte-specific member of the *Src*-family, regulates its actin cross-linking function, as well as binding to surface receptors and lipid membranes (rev. in Goldmann 2001). The phosphorylation of filamin by serine-threonine protein kinase A leads to an increase in its resistance to proteolytic cleavage by calpain (Zhang *et al.* 1988, Chen & Stracher 1989); and phosphorylation by protein kinase C, regulated by local Ca^{2+} concentrations, influences the interaction of filamin with integrins, thus regulating the association of the cortical actin with the plasma membrane and affecting cellular adhesion and migration (Tigges *et al.* 2003). Pak1 and Ribosomal S6 kinase phosphorylate filamin in processes which involve membrane ruffle formation and migration of human melanoma cells, respectively (Vadlamudi *et al.* 2002, Woo *et al.* 2004). Dephosphorylation of filamin by treatment with *E.coli* alkaline phosphatase in platelets results in the loss of its ability to crosslink F-actin (Zhuang *et al.* 1984). Dephosphorylation by calcineurin, a calcium/calmodulin-dependent threonine/serine phosphatase, protects platelet filamin from calpain degradation (Garcia *et al.* 2006).

The unique example of negative regulation of filamin is presented by FILIP, which inhibits filamin function by suppressing cell motility and the formation of lamellipodia and induces degradation of filamin in a calcium-dependent manner, contributing to the malformation pathology in PNH (Nagano *et al.* 2002).

In vivo, assembly and disassembly of the actin cytoskeleton in platelets is controlled by the intracellular concentration of free calcium. Ca^{2+} is thus involved in cell shape change and pseudopod formation during platelet activation (Rosenberg *et al.* 1981). Recently, the first mechanism for the regulation of filamin interaction with F-actin has been proposed, providing an explanation for why a large part of cellular filamin stays free of F-actin *in vivo* (Nakamura *et al.* 2005). It involves direct interaction between filamin and Ca^{2+} -bound calmodulin, which dissociates F-actin from filamin and inhibits its ability to crosslink actin filaments.

3 Aims of the present study

The carboxy-terminus of filamin is required for dimerisation and provides a binding site for a great variety of molecules of different functions.

The importance of the interaction between the GPIb-V-IX receptor and filamin is well documented. GPIb α , through binding to filamin A, anchors the GPIb-IX-V complex to the cytoskeleton and is important for the receptor adhesive function.

The aims of the present study were:

1. To determine the structure of the carboxy-terminal dimerisation domain of filamin (I, III)
2. To map the binding site between GPIb α and filamin and to determine the structure of this complex (II)

4 Materials and methods

The materials and methods have been described in more detail in the original articles referred to by their Roman numerals (I-III).

4.1 cDNA cloning (I-III)

All oligonucleotides were purchased from Sigma Genosys.

The fragments encoding Ig-like domains 24 and 23-24 (including H2) of human filamin C (residues 2633-2725 and 2503-2725, respectively, Swiss-Prot accession number Q14315) have been amplified from a human skeletal muscle cDNA library (Matchmaker, Clontech, Invitrogen, Palo Alto, CA). The oligonucleotide pairs for amplification were for filamin C 24: ATATAT**GAAGACACCATG** GGA TCA GAT GCC AGC AAG GTG GTG ACT CGG GGC CCT GGG CTG (5'-primer) and ATATATAT**GCGGCCGC** TTA TCA AGG GAC CTT GAC TTT GAA GGG GCT TC (3'-primer); for filamin C 23-24: ATATAT**GAAGACACCATG** GGG GAC CCA GGC TTG GTG TC (5'-primer), 3'-primer is the same as for filamin C 24 (*BbsI*, *NcoI* and *NotI* restriction sites are shown in bold). PCR products were subcloned into *NcoI-NotI* sites of a modified pETM24d plasmid (Novagen) containing an N-terminal His6-tag followed by the cleavage sequence for the tobacco etch virus protease (Tev).

The fragment encoding the Ig-like domain 17 of human filamin A (residues 1863-1955) was subcloned into plasmid pGEX4T-3 (Amersham Biosciences) containing cleavage sites for thrombin and Tev. The oligonucleotide pair for filamin A 17 was: ATATAT**GAAGACACCATG** GTC AAC TGT GGC CAT GTC ACT GCC TAT GGG CCT GGC CTC AC (Primer 5'); ATATATAT**GCGGCCGC** TTA GTC GTC ACC TGT GAC CCG AGC AGT GAA GGG GCT G (primer 3') (*BbsI*, *NcoI* and *NotI* restriction sites are shown in bold).

The fragments encoding Ig-like domains 19 and 17-19 of human filamin A (residues 2045-2140 and 1863-2140, respectively) and subcloned into the pGEX4T-1 plasmid (Amersham Biosciences) containing thrombin and Tev cleavage sites were provided by Dr. F. Nakamura (Harvard Medical School, Boston, MA).

All inserts were verified by DNA sequencing.

4.2 Site-directed mutagenesis (I)

Two mutant constructs of filamin C domain 24 were produced to identify the aa residues critical for filamin dimerisation. In one of them Met2669 was changed to Asp (M2669D), and in the second one, a stretch of 8 residues (aa 2666-2673, NMMMVG^{VH}) was replaced with 8 residues from human filamin A domain 20 (QDMTAQVT) (Mut20). Mutations were performed with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA), in a modified pET24d plasmid (Novagen).

Full length human filamin A constructs, containing the same mutations were produced to check the effect of the dimerisation on cell migration. One of them contained the point mutation of Leu2590 to Asp, and in the second one a stretch of 8 residues (aa 2587-2594, NMLLVG^{VH}) was replaced with the 8 residues from human filamin A domain 20 described above. Mutations were performed with a QuikChange II XL Site Directed mutagenesis kit (Stratagene), in plasmid pREP4 (Invitrogen).

4.3 Recombinant proteins expression and purification (I-III)

In order to produce recombinant proteins, the constructed expression vectors were transformed to *E. coli* BL21(DE3) and BL21 cells, for His6- (filamin C 24, 23-24) and GST-tagged (filamin A 17) proteins respectively. In all experiments Luria Bertani plates and broth were used (Sambrook & Russel 2001).

For His6-tagged proteins, protein expression was induced for 3 h at 37 °C in the presence of 0.4 mM IPTG. Cells were collected by centrifugation, washed with Lysis buffer (10 mM imidazole, 50 mM Na Phosphate, 0.3 M NaCl, pH 8.0) and stored frozen at -70 °C until used. For the initial steps of purification, a French press and ultracentrifugation at 150000 g was used. Then the supernatant fractions were applied to a Ni²⁺-NTA agarose matrix and the protein was allowed to bind for 3-4 h. The matrix with protein bound was extensively washed with Lysis and Wash (20 mM imidazole, 50 mM Na Phosphate, 0.5 M NaCl, pH 8.0) buffers until the A₂₈₀ was <0.1. Proteins were eluted with Elution buffer (250 mM imidazole, 50 mM Na Phosphate, 0.3 M NaCl, pH 8.0), which was changed to 20 mM TrisCl, pH 8.0 by applying the flow-through to PD10 columns (Amersham Biosciences). The protein was cleaved with Tev protease at 30 °C overnight. Further purification was achieved by cation exchange chromatography using a Fractogel EMD SO⁻³ matrix (Merck). After this step, the purity of filamin C 24 was sufficient for crystallisation experiments, but for further purification of filamin C 23-24 a size exclusion chromatography was performed on Superdex 75 16/60. The final concentrations achieved were: filamin C 24 – 40-42 mg/ml (20 mM TrisCl, pH 8.0); filamin C 23-24 – 43 mg/ml.

GST-tagged Filamin A 17 purification was carried out initially as above, except using a Glutathione-Sepharose matrix (Amersham Biosciences). The binding time was 2-2.5 h. The wash buffer was phosphate-buffered saline (PBS) (pH 7.4) and the elution buffer was 15 mM reduced glutathione, 50 mM TrisCl, pH 8.0. The buffer was changed to 20 mM TrisCl (pH 8.0) with PD10 columns (Amersham Biosciences), and the protein cleaved with Tev protease at 30 °C overnight. Size exclusion chromatography on a Superdex 75

26/60 column (Amersham Bioscience) completed the purification. A concentration of 26.5 mg/ml was achieved (20 mM TrisCl, 50 mM NaCl, 1 mM DTT, pH 8.0).

Double labelled $^{13}\text{C}/^{15}\text{N}$ filamin A 17 was produced for the NMR experiment by growing *E.coli* BL21 cells that harbour a pGEX4T-3 expression vector (Amersham Biosciences) with the FLNA17 gene in 1.0 l of M9 minimal medium containing $^{15}\text{NH}_4\text{Cl}$ and ^{13}C D-glucose as sole sources of nitrogen and carbon. Expression and purification were the same as described above, and the concentration of the final sample was 19 mg/ml in 50 mM Na Phosphate, pH 7.5, 10 mM DTT.

4.4 Production of selenomethionine-labelled recombinant filamin C24 (I)

To generate filamin C domain 24 labelled with selenomethionine (SeMet), the corresponding expression vector was transformed to a methionine auxotrophic *E.coli* B834(DE3) (Leahy *et al.* 1992, Wood 1966) strain. The bacteria were first grown for 6 h at 37 °C in Luria Bertani broth supplemented with 100 µg/ml kanamycin. The cells were collected by centrifugation, washed with 20 ml of L-Met minimal medium and resuspended in 20 ml L-Met minimal medium [L-Met MM: 48 mM $\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$; 22 mM KH_2PO_4 ; 9 mM NaCl; 0.4 % (w/v) glucose; 2 mM MgSO_4 ; 0.1 mM CaCl_2 ; 8 mM $(\text{NH}_4)_2\text{SO}_4$; 1 x MEM vitamin solution (Gibco BRL), 0.1 g/l D-biotin, 0.5 mM L-Met], containing 100 µg/ml kanamycin and cultured overnight (16-17 h) at 37 °C. This overnight culture was centrifuged and the cells were washed with 20 ml of SeMet minimal medium containing 0.5 mM SeMet (Calbiochem) then resuspended in 5 ml of the same medium, which was used as inoculum for 1.0 l of SeMet MM in presence of 100 µg/ml kanamycin. Growth was performed for 7 h at 37 °C until the A_{600} reached 0.9, and expression was initiated with 1.0 mM IPTG for 20 h at 20 °C. The bacterial cells were harvested, washed with cold Lysis buffer and stored at -70 °C until used. Protein was purified as native.

4.5 Protein analysis (I-III)

The monodispersity of the purified protein samples (filamin C 24 wild type and mutant constructs, filamin C 23-24) was analysed by dynamic light scattering (DLS). DLS experiments were done with the DynaPro Instrument equipped with Dynamics 5.0 software (Protein Solutions). The concentration of samples was in the range of 1.8 mg/ml (for filamin C 23-24) and 3 mg/ml (for filamin C 24 wt and mutants) and the experiments were performed at 15 °C and 4 °C in 20 mM Tris-HCl (pH 8.0).

The secondary structure of purified protein samples (filamin C 24 wt and mutants, filamin A 17) was analysed by circular dichroism (CD) spectroscopy. Experiments were performed in the far UV region (190-250 nm) at 20 °C with a Jasco J-715 spectropolarimeter. Protein concentrations were in the range of 0.2-0.3 mg/ml measured in 5 mM Na Phosphate (pH 7.4).

In order to estimate a monomer-dimer ratio for filamin C 24 a sedimentation equilibrium analytical ultracentrifugation was performed. An Optima XLA (Beckman Coulter) instrument with an An-60Ti rotor was used and the experiment was carried out at +4 °C and a scanning wavelength of 229 nm. The protein sample was diluted in 140 mM NaCl, 10 mM Na Phosphate (pH 7.4). More detailed information is presented in (I).

Analytical gel-filtration was performed for purified protein samples at RT on a Superdex 75 10/30 column (filamin C 24 wt and mutants, filamin C 23-24) (Amersham Biosciences). Gel-filtration standards from BioRad Laboratories were used.

Mass spectrometric analyses were carried out using a MALDI-TOF mass spectrometer (Voyager DE-STR, Applied Biosystems) to identify the degree of Se-Met labelling in filamin C 24, the level of isotope incorporation in ¹³C/¹⁵N filamin A 17, as well as to confirm the presence of both protein and ligand in the complex crystals of Filamin A 17-GPIb α 556-577.

Proteins were separated on an SDS-polyacrylamide slab gel according to their molecular weight (Laemmli 1970) using Coomassie Blue staining and low molecular weight standards (Amersham Biosciences) for calibration.

The concentrations of the purified protein samples were estimated by measuring the UV-absorbance at 280 nm together with the theoretical extinction coefficient for 280 nm, calculated from the protein sequence by the ProtParam computation tool (Gasteiger *et al.* 2005) from the ExPasy server (www.expasy.org).

4.6 Isothermal titration calorimetry (II)

A thermodynamic analysis of the interaction between filamin A domain 17 and GPIb α was carried out by isothermal titration calorimetry (ITC) using a VP-ITC device (MicroCal, Northampton, MA) at 25 °C. Samples for calorimetry experiments were exhaustively dialysed against 100 mM Na Phosphate (pH 7.4). Prior to the titration experiments, the samples were degassed for 8 min under vacuum (ThermoVac, MicroCal). The GPIb α peptide (1.2 mM) in a 250 μ l rotating syringe was titrated into a sample cell containing filamin A17 (80 μ M) by using one injection of 2 μ l followed by 18 injections of 7 μ l. Injections lasted 14 s with 240 s intervals in between. The heat generated by peptide dilution was determined in separate experiments by injecting a 1.2 mM GPIb α solution into a 100 mM Na Phosphate (pH 7.4) buffer-filled sample chamber. All data were corrected for the heat of peptide dilution. Data were fitted by using χ^2 (chi-sqr) minimisation on a model assuming a single set of sites to calculate the binding affinity K_a . Data acquisition and analysis were performed by using Origin version 5.0 (MicroCal) software.

4.7 Crystallisation (I, II)

Initial crystallisation screening was performed using the sparse matrix screening method described in (Jancarik & Kim 1991) at 20 °C by hanging-drop and sitting-drop vapour diffusion methods. The drop contained 1.0 μ l of protein solution and 1.0 μ l of mother

liquor. The crystallisation conditions were optimised by altering protein and precipitant concentration as well as by microseeding (for review, see Bergfors 2003).

Crystallisation of filamin C 24 was achieved using a hanging drop vapour diffusion method in which the recombinant protein was mixed with the reservoir solution (v/v 1:1) containing 1.6 M Na citrate, 0.1 M HEPES (pH 7.5). The crystals that appeared overnight were tetragonal pyramids up to 0.8 mm long. For the SeMet-labelled protein, there was a tendency for better quality crystals to form in lower Na citrate concentrations (1.4-1.5 M) and well-diffracting crystals were obtained only after setting drops with freshly purified and concentrated protein. The crystals were smaller, appearing as tetragonal pyramids along with rosettes.

Co-crystallization experiments of filamin A 17 with GPIIb α 556-577 (peptide was provided by Dr. F. Nakamura) were performed mixing approximately equimolar amounts of protein and peptide (1.6-1.7 mM), and small brush-shaped crystals were obtained in 1.75 M ammonium phosphate (pH 8.2). Since the quality of those crystals was not appropriate for the diffraction experiments and the optimisation screens did not give any reasonable results, the microseeding method was applied. A brush-shaped crystal was crushed with a micropipette tip and transferred into a stock mother liquor solution (10 μ l). A series of dilutions were made (1:10, 1:100, 1:200, 1:500, 1:1000) and hanging drops were set from this dilution series with lower precipitant concentrations (1.25 M ammonium phosphate, pH 8.2). Large, rod-shaped crystals were obtained in a few days.

4.8 X-ray diffraction data collection and processing (I, II)

Diffraction data from native and SeMet filamin C 24 crystals were collected from flash-frozen crystals at 100 K using 10 % glycerol as a cryoprotectant. The data set from the native protein was collected from a single crystal using the oscillation method with 1° rotations per frame at a wavelength of 0.8020 at the beamline X13 at the EMBL outstation, DESY, Hamburg, Germany. For SeMet crystals a multiwavelength anomalous dispersion (MAD) experiment was performed on the beamline BW7A (EMBL, DESY). The detector used was a 165 mm MAR CCD (MarResearch).

Diffraction data from filamin A 17 – GPIIb α 556-577 crystals were collected from flash-frozen crystals at 100 K using 20 % glycerol as a cryoprotectant. The dataset was collected from a single crystal at a wavelength of 0.9795 Å at the beamline ID23-1, ESRF, Grenoble, France. A diffraction pattern could be obtained from the crystal only after the annealing procedure (removing the crystal from the nitrogen stream, soaking in the mother liquor and flash-freezing again). The detector used was a 225 mm MarMosaic (Mar-USA, *Inc.*).

In all cases the data was processed and scaled using the XDS program package (Kabsch 1993).

4.9 Structural determination, model building, refinement and validation (I, II)

The primary phases for SeMet labelled filamin C domain 24 (PDB id 1V05) were determined by the single-wavelength anomalous diffraction (SAD) method using data measured at the peak wavelength of Se x-ray absorption. The initial positions of the Se atoms were located with the CNS 1.0 program package (Brunger *et al.* 1998), which was also used for density modification. The electron density map produced was used for automatic model building by means of Arp/Warp 5.0 (Perrakis *et al.* 1999). The 86 residues model obtained was used for solving a native dataset. Residues Ser2718, Pro2719 and the C-terminal Pro2725 have been built manually by means of program *O* (Jones *et al.* 1991). During crystallographic refinement with the maximum likelihood target ten percent of the reflections were reserved for calculating the free R factor. Double atomic conformations were found for residues Cys2679 and Asn2689. The three N-terminal residues seen in the density map belong to the expression vector (Gly-Met-Ala).

The structure of the filamin A 17 – GPIb α 556-577 complex (PDB id 2BP3) was solved by the molecular replacement method (Rossman & Blow 1962) with the program Phaser (Storoni 2004) using as a search model a structure of filamin C 24. For creating the search model, aa sequences of filamin A 17 and filamin C 24 were aligned in ClustalX1.8 (Thompson *et al.* 1994), and according to that alignment all residues that were not identical or glycines were mutated to alanines in program *O*. Phaser (Storoni *et al.* 2004) was used to find a molecular replacement solution. A rigid body refinement using Refmac5.2 was performed after each run of Phaser. Arp/Warp 6.1.1 was able to build an almost complete asymmetric unit: partial chains A and B of filamin A 17 and one peptide chain. The rest of the model building and adjustments to electron density were performed manually in *O*.

Structural refinement for both structures was done with Refmac5 (version 5.2 for 2BP3) (Murshudov *et al.* 1997). Water molecules were partly added automatically using the solvent-building mode in Arp/Warp and partly added manually. Double conformations (for 1V05) were refined and recorded using SHELXPRO (Sheldrick & Gould 1995).

Structures were validated with programs WHATIF (Vriend 1990) and Procheck (Laskowski *et al.* 1993), and the locations and types of structural motifs were determined by DSSP (Kakita *et al.* 2002). Monomer interaction (for 1V05) and protein – ligand interaction (for 2BP3) were analysed using the server <http://www.biochem.ucl.ac.uk/bsm/PP/server> (Jones & Thornton 1996) and in case of 2BP3 also using LIGPLOT (v. 4.4.2) (Wallace *et al.* 1995).

4.10 Structural comparisons

To identify highly conserved residues in the dimerisation domain for vertebrate filamins, an aa sequence alignment for all sequences available was performed with ClustalX (V.1.8 and 1.83) (Thompson *et al.* 1994) with the weight matrix Gonnet PAM250.

The structure of filamin C 24 was compared by superimposition studies with known structures. For the comparisons the following PDB entries were used: gelation factor rod domain 4 (20 NMR structures, PDB id 1KSR) (Fucini *et al.* 1997) and gelation factor rod domains 5 and 6 (PDB id 1QFH) (McCoy *et al.* 1999). Superimpositions were performed in *O* with the *lsq* option, and only C α atoms were used. During superimposition the loop regions were excluded. Molecules were compared by specifying each β -strand and by calculating a root-mean-square (rms) deviation between 69 atoms.

Structures of filamin C 24 and filamin A 17 were then compared by superimposition with the known structure of the gelation factor protein and filamin. For comparisons the structures mentioned above were used, as well as structures of the gelation factor rod domains 4,5 and 6 (PDB id 1WLH) (Popowicz *et al.* 2004), filamin A domain 21 (PDB id 2BRQ) (Kiema *et al.* 2006) and filamin C domains, 14, 16, 17 and 22 (20 NMR structures, PDB ids 2D7M, 2D7N, 2D7O and 2D7P, respectively) (Tomizawa *et al.* 2006). Superimpositions were performed in Pymol (v.0.99) (DeLano 2002) with the *align* command and only C α atoms were used.

All images presenting structural information were generated by Pymol (DeLano 2002) and Grasp (Nicholls *et al.* 1991). The surface potential was calculated by Grasp.

4.11 Small angle x-ray scattering (III)

The small angle x-ray scattering (SAXS) experiment was performed at the X33 SAXS beamline of the EMBL on the storage ring DORIS III (DESY, Hamburg, Germany). The details of the experiment are given in (III).

4.12 Other methods

NMR spectra were measured from the ¹⁵N- and ¹⁵N/¹³C-labelled filamin A domain 17 protein with the Varian Unity INOVA spectrometers at 500 MHz and 800 MHz (Varian, Palo Alto, CA). Experiments were performed by Outi Heikkinen (Laboratory of Organic Chemistry, Department of Chemistry, and Institute of Biotechnology, University of Helsinki).

In vitro and *in vivo* filamin A 17 – GPIb α peptide binding, reconstitution and competition assays, as well as electron microscopy were performed by Dr. Fumihiko Nakamura (Hematology Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA) and described in (II).

5 Results

5.1 Studies on the filamin C dimerisation domain 24

5.1.1 Crystal structure of filamin C 24 (I)

The structure of Ig-like dimerisation domain 24 of filamin C was solved by the single-wavelength anomalous diffraction (SAD) method with SeMet labelled crystals. The space group was defined as $P6_122$. For the final model a restrained isotropic refinement was performed to a resolution of 1.43 Å, yielding an R_f of 18.6 % and R_{free} of 20.5 %.

The stereochemistry of the structure is good, 93.6 % of the residues are in the most favoured regions in the Ramachandran plot and 6.4 % in additionally allowed regions (I, Table I). In the crystal form the asymmetric unit contains one monomer, and a crystallographic dimer related by a 2-fold axis is formed (I, Fig. 3). Each monomer has an expected Ig-like β -sandwich fold with β -strands A,B, D and E forming a four-stranded antiparallel β -sheet and strands C, F and G – a three-stranded sheet (I, Fig. 2A).

5.1.2 Structural comparisons for filamin C 24 (I)

When the present study was initiated only three structures of filamin type Ig-like domains (Structural Classification of Proteins, (Murzin *et al.* 1995) were available (Fucini *et al.* 1997, McCoy *et al.* 1999). All three were from *Dictyostelium discoideum* filamin, also called ABP-120 or gelation factor.

The results of structural comparisons between all those structures are presented in the original article I. Strand-by-strand superimposition in *O* and calculation of rms deviation between 69 atoms of the corresponding β -strands revealed a spectacular difference between the dimerisation domains of filamin C and gelation factor protein (rms deviation equals 1.6 Å, 2.2 Å and 8.3 Å between filamin C 24 and domains 4, 5 and 6 of gelation factor) (I).

In recent years several x-ray and NMR solution structures of separate repeats of filamin were reported. The results of a structural comparison between filamin C 24 and all structures currently available are presented on table 3.

Table 3. Structural comparisons between filamin C 24 and other filamin and gelation factor protein structures available. Superimpositions were performed in Pymol by command align with no strand-by-strand alignment. Only C α atoms were used.

Structure 1 PDB id	Structure 2 PDB id	# of residues	rmsd c- α
Filamin C 24 1VO5	Gelation factor 4 1KSR	84	2.432
- " -	Gelation factor 5 1QFH	76	1.041
- " -	Gelation factor 6 1QFH	59	3.034
- " -	Gelation factor 4 1WLH	79	1.196
- " -	Gelation factor 5 1WLH	76	1.123
- " -	Gelation factor 6 1WLH	60	3.082
- " -	Filamin C 14 2D7M	77	1.501
- " -	Filamin C 16 2D7N	30	0.850
- " -	Filamin C 17 2D7O	79	1.271
- " -	Filamin C 22 2D7P	80	2.235
- " -	Filamin A17 2BP3	79	1.023
- " -	Filamin A 17 2AAV	85	1.504
- " -	Filamin A 19 unpublished	81	0.803
- " -	Filamin A 21 2BRQ	78	0.775
- " -	Filamin C 23 2NQC	73	0.803

The results of structural comparisons show that the biggest difference in rms deviation occurs between filamin C domain 24 and gelation factor domain 6*. This difference emerges from the variations in topology (Fig. 3; I, Fig. 2A and D) which implies that *Dictyostelium* domain 6 does not have strands A and A', but has an additional strand H, absent from human filamin C 24.

5.1.3 Dimerisation mode of human filamin C (I)

The most striking difference found was in the way the *Dictyostelium* and human filamin dimerise. In *Dictyostelium* a dimerisation interface is formed mainly by β -strands B and G, whereas in filamin the C 24 structure it involves strands C and D on the opposite edge of the β -sandwich (I, Fig. 2A and D).

The filamin C 24 dimerisation interface occupies 19 % (1109 Å²) of the accessible surface area of each monomer and contains 45 % polar atoms. Dimerisation occurs through six hydrogen bonds connecting D strands so that the four-stranded β -sheets of each monomer form a single eight-stranded antiparallel β -sheet. In addition, side chains

* The difference in numbers (8.3 Å and 3.034/3.082 Å) is caused by a different method of superimposition. In (I) strand A of filamin C 24 was superimposed with strand H of gelation factor 6 to have the same number of atoms in each superimposition. Here less atoms were used for the corresponding superimpositions.

of Met2667 and Met2669 pack against Gly2671 of the adjacent monomer, creating hydrophobic interactions on the strand C (I, Fig. 4).

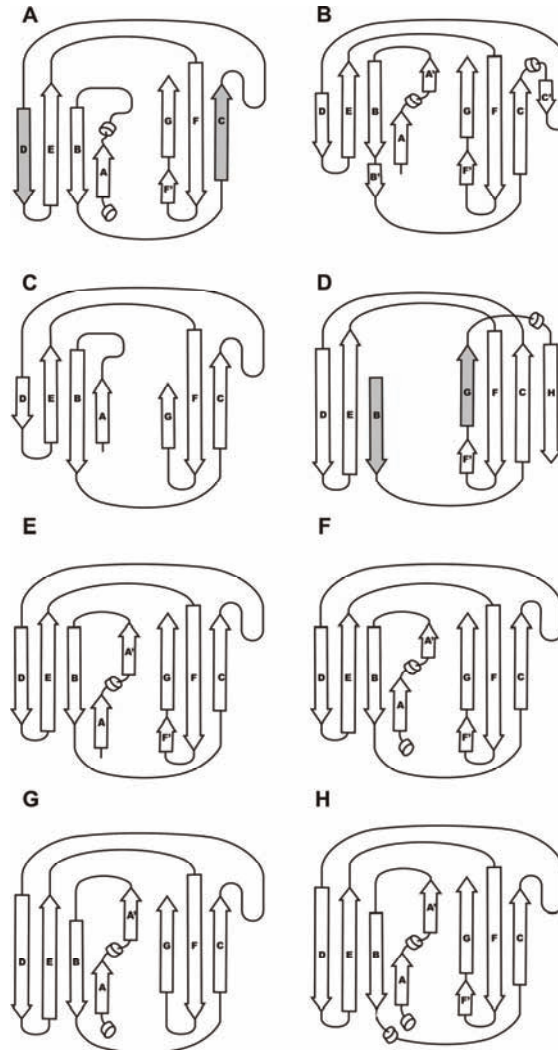


Fig. 3. Topology diagrams: (A) Human filamin C domain 24, (B) *Dictyostelium* filamin domain 5, (C) *Dictyostelium* filamin domain 4, (D) *Dictyostelium* filamin domain 6, (E) Human filamin domain 17, (F) Human filamin domain 21, (G) Human filamin domain 23, (H) Human filamin domain 19. β -strands are shown as arrows, 3_{10} helices as cylinders. In (A) and (D) β -strands forming the dimerization interface are coloured grey.

5.1.4 *The effect of the point mutations on the dimerisation (I)*

In order to confirm that dimerisation of filamin C 24 occurs in solution in the same way as seen in the crystal, we engineered mutants that would inhibit dimerisation. In one of the mutants a hydrophobic stack containing three consecutive methionine residues on the strand C was disrupted with a negatively charged residue: Met2669, the last of three, was mutated to Asp. In a second mutant a whole strand C (8 residues) was replaced with the corresponding strand from non-dimerising domain 20.

CD-measurements of filamin C 24 wild type and both mutants confirmed a folded β -structure, for all three proteins the deep minimum at 216 nm was recorded. However CD-spectra of both mutants were somewhat different from that of wild type in the range of 190-210 nm, possibly indicating a difference in the oligomerisation. The dimerisation status of wild type and mutants was studied by analytical ultracentrifugation and by gel-filtration. The results clearly showed that both mutations disrupted dimerisation (I, Figs.5 and 8).

5.1.5 *SAXS measurements of filamin C 23-24 (III)*

The dimerisation repeat of filamin is preceded by a 38 aa long hinge region with unknown structure. This hinge region has been shown to enhance the stability of the dimer and it is conserved in all filamin isoforms (Himmel *et al.* 2003). To perform further studies on an overall arrangement of a carboxy-terminus of filamin and to investigate if the domain 23 influences a formation of filamin dimer, a filamin C construct containing Ig-like domains 23 and 24 separated by hinge 2, was prepared. However, crystallisation experiments on this construct were not successful; possibly because of the presence of a flexible hinge region in between, and a small angle x-ray scattering (SAXS) experiment was performed.

The model of filamin C Ig-like domain 23-24 dimer obtained from SAXS experiment is presented on Figure 6B (III). The data confirmed that two molecules of the dimerisation domain are related by 2-fold rotation axis and showed, in accordance with the previous findings (I) that two Ig-like repeats 23 are not in contact with each other. An unexpected feature of the model was a very little interaction between domains 23 and 24, unlike it was hypothesized in (I) (I, Fig. 7). However, the flexibility between domains 23 and 24 seems to be restricted, and this must be controlled by the hinge sequence.

Although this SAXS study does not allow detailed modelling of the 38 amino acid hinge region due to limited resolution of the experiment, it provides a general view of the carboxy-terminus of the filamin molecule and gives an idea about overall domain arrangement (III, Fig. 6B). A spread-wings-like model allows proposing a looser packing of the domains than was suggested before (I).

5.2 Studies on filamin A 17 – glycoprotein Iba 556-577 interaction (II)

5.2.1 Mapping of the filamin A 17 – GPIba 556–577 interaction site

It has been previously reported that a 22-mer synthetic peptide from the cytoplasmic domain of GPIba (residues 556-577) binds to Ig-like domains 17-19 of filamin (Meyer *et al.* 1997, Feng *et al.* 2003, Cranmer *et al.* 2005).

In order to facilitate structural studies we further narrowed this interaction site to filamin A domain 17, although purified domain 19 also showed some affinity. However, point mutations in domain 17 of full-length filamin A completely inhibited its binding to the GPIba peptide (II, Figs.1, 3B).

The interaction between the GPIba peptide and filamin A domain 17 was further verified by isothermal titration calorimetry, and a K_d of 11 μM was calculated (Fig. 4).

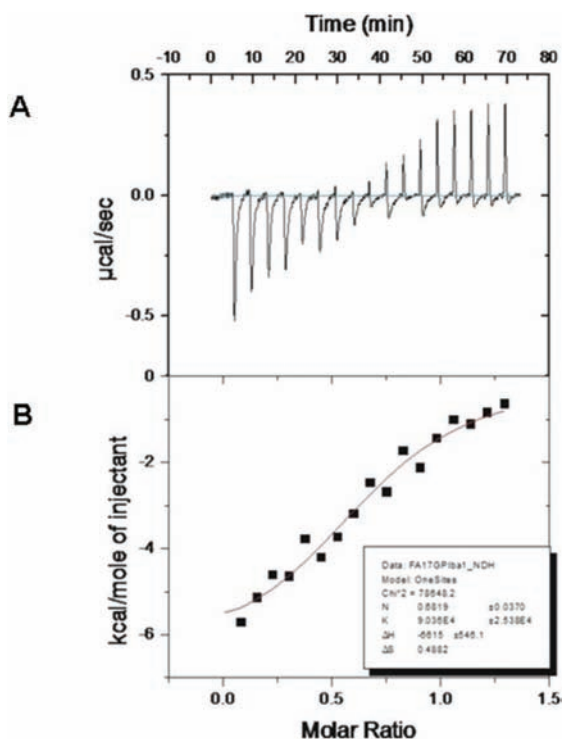


Fig. 4. Isothermal titration calorimetry results. (A) Primary data from the titration of GPIba peptide (1.2 mM) into filamin A17 (80 μM) at 25 $^{\circ}\text{C}$ in 100 mM Na phosphate, pH 7.4. (B) Data fitting on a model of a single set of sites to calculate a binding affinity K_d . All data is corrected for the heat of peptide dilution.

5.2.2 Crystal structure of GPIb α 556-577 – filamin A 17 complex

Co-crystallisation experiments with the recombinant filamin A 17 and GPIb α 556-577 peptide resulted after microseeding good quality crystals diffracting to 2.3 Å. The space group was P2₁2₁2₁. The structure of the complex was solved by molecular replacement, and the final model was refined to an R_f of 21.4 % and R_{free} of 25.6 %. The model contained two molecules in the asymmetric unit, each consisting of the filamin A 17 – GPIb α complex (Fig. 5). The longest peptide (chain T) contains 17 residues of the 21 in the original sequence (aa 557-574) and the longest filamin A 17 molecule (chain A) contains 90 of 94 residues present in the construct (aa 1863-1955). The following residues had side chains missing from the electron density map: A1881Asn, A1882Lys, A1892Asp, A1916Gln, B1891Lys, B1892Asp, B1909Glu, B1916Gln, B1917Asp, and B1940Glu. These residues are mostly located in flexible loop regions. The final model contains one molecule of glycerol, coming from the cryoprotectant solution. The first N-terminal Met belongs to the expression vector. The final model contains 87.7 % of the residues in the most favoured regions in the Ramachandran plot and 12.3 % of the residues in additionally allowed regions (II, Table 2).

There were small differences in the interaction of the two molecules of GPIb α with the two filamin A 17 molecules in the asymmetric unit of the crystal. Peptide T (Fig. 5) binds to molecule A and at the same time interacts through its N-terminus with the filamin molecule B. The N-terminus of peptide S, interacting with filamin molecule B, was not seen in the electron density map. It is likely that the physiological interaction between native proteins is represented by the interaction between filamin and the GPIb α peptide, which is same in both molecules (chain A – chain T and chain B – chain S). The binding of peptide T to chain B of filamin is therefore due to the arrangement within a crystal.

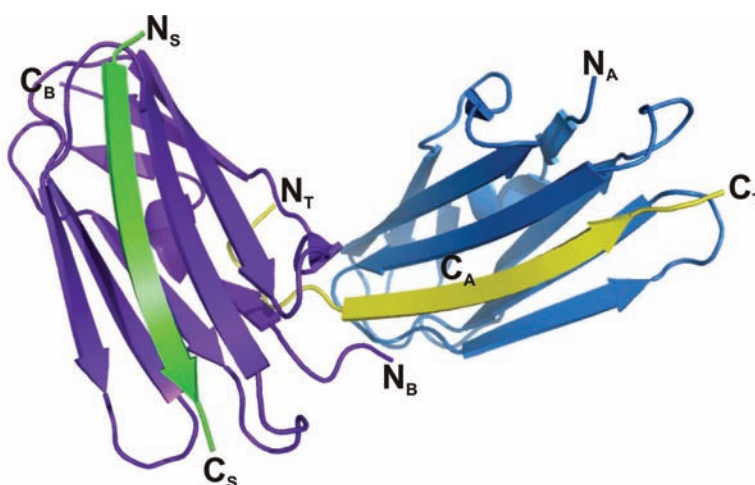


Fig. 5. Asymmetric unit of the crystal. Two molecules of filamin A 17 (monomer A in blue, monomer B in purple) and GPIb α (peptide T in yellow, peptide S in green) are shown.

The main interaction between filamin A and the GPIIb α peptide occurs through the 14 residues of GPIIb α (aa 560-573: LPTFRSSLFLWVRP) that bind to the β -strands C and D of filamin A (II, Figs. 2D-F). On the filamins side the interface contains 43 % polar and 57 % nonpolar atoms and occupies 14.1 % of the accessible surface area. Five residues on the strand C of filamin A form hydrogen bonds with the main chain of GPIIb α , which thus forms a β -strand interaction. This region of the peptide is flanked by two prolines (Pro561 and Pro573). In addition, a side chain OH-group of Ser565 forms a hydrogen bond to the main-chain O atom of Ala1908 (a loop between strands C and D).

The main hydrophobic contacts are represented by Ile1910 and Cys1912 of the strand D of filamin A and Leu567 of the peptide (13.3 % of the contact surface area). GPIIb α residues Val571 (13.7 %), Phe563 (11.2 %), Leu569 (9.7 %), Trp570 (6.5 %) and Phe568 (5.4 %) also form a vast hydrophobic interface with filamin A (II, Figs. 2D-F) This provides structural support for previous findings (Cranmer *et al.* 2005) that the GPIIb α residues Phe568 and Trp570 are crucial for its interaction with filamin A.

5.2.3 The effect of the point mutations in filamin A 17 on its interaction with GPIIb α

Point mutations were engineered to test whether the interaction seen in the crystal between filamin A 17 and GPIIb α also takes place in solution. The mutations were designed according to the interaction interface seen in the crystal structure of filamin A 17 – GPIIb α . The mutation of glycine 1897 to aspartic acid ought to disturb the packing beside GPIIb α residues Phe568 and Trp570, crucial for its interaction with filamin, by introduction of an amino acid with a side chain too large to fit between the two phenyl rings, and bearing at the same time a negative charge. Ile1910 and Cys1912, which form the main hydrophobic contacts with the peptide and are located on strand D, were mutated to Met and Asp, respectively (Fig. 6).

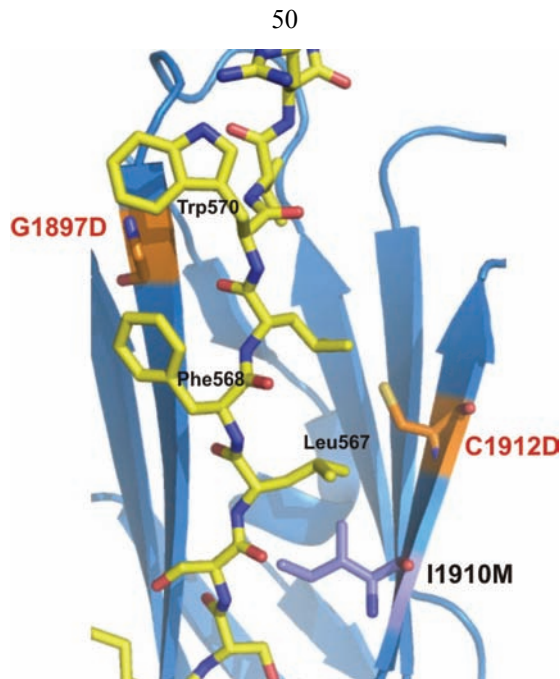


Fig. 6. Point mutations in filamin A 17. The filamin A 17 monomer A is shown in blue, GPIb α peptide T – in yellow. Mutated filamin A 17 residues are indicated. Mutations inhibiting filamin binding to GPIb α are shown in red.

Experiments on the effect of point mutations on the filamin A – GPIb α interaction *in vitro* and *in vivo* were performed by Dr. F. Nakamura, and the results obtained are described in detail in the original article II. In brief, *in vitro* protein interaction experiments showed that a mutation I1910M introduced into filamin A 17 did not have any effect on its interaction with the GPIb α 556-577 peptide. Mutations G1897D or C1912D reduced filamin A 17 binding, and the double mutant (G1897D and C1912D) did not bind at all. NMR spectroscopy experiments (performed by O. Heikkinen) confirmed that the filamin A 17 double mutant did not have any major defects in its β -folded structure. The mutations described above were also introduced into domain 17 of the full-length human filamin A, which was expressed as a GFP-tagged protein in the CHO-GPIb α β /IX cells that stably express the GPIb α β /IX complex (II, Fig. 3C). The double mutant GFP-filamin A did not associate with GPIb α β /IX in the cell, confirming that domain 17 of filamin A is the major GPIb α binding site *in vivo*.

6 Discussion

6.1 The human filamin dimerisation interface is common for all vertebrate filamins

The crystal structure of human filamin C 24, although similar to the *Dictyostelium* gelation factor dimerisation domain 6 by its global arrangement and by exact two-fold symmetry between monomers, proved to differ significantly in terms of topology. Particularly, it revealed a novel dimerisation interface fundamentally different from that found in domain 6 of *Dictyostelium* gelation factor. The human filamin C interface is formed by β -stands C and D in contrast to β -strands B and G in *Dictyostelium* (McCoy *et al.* 1999, Popowicz *et al.* 2004). The sequence alignment of C-terminal domains available for vertebrate filamins clearly demonstrate that strand C, containing the hydrophobic residues that form a dimerisation interface in human filamin C 24, is well conserved between species (I, Figs. 6A and B). Moreover, this sequence motif could not be found in other domains of vertebrate filamins. Based on this observation it could be proposed that all vertebrate filamins share a dimerisation interface analogous to that found in human filamin C.

It was proposed that the filamin dimerisation interface changed during evolution (Popowicz *et al.* 2006). A rigid and strong interaction between filamin monomers of lower organisms was replaced with a more flexible and weak dimerisation in higher organisms (dimerisation interface areas of 4501 \AA^2 and 1109 \AA^2 in *Dictyostelium* and human filamins, respectively).

6.2 Model of the molecular structure of vertebrate filamin

6.2.1 *A molecular leaf-spring vs tail-to-tail arrangement*

A comparison of the dimerisation Ig-like domains of human filamin and *Dictyostelium* gelation factor protein structures makes it clear that the dimerisation interface appears on the opposite edges of the β -sandwich. Furthermore, the interface in domain 6 of *Dictyostelium* could be formed only in the absence of the strand A, which also determines a different dimerisation mode for this protein.

However, despite the clear difference in the dimerisation mode between those two molecules, the common feature for human filamin C 24 and *Dictyostelium* filamin domain 6 structures is the antiparallel arrangement of the monomers. It has been shown, that in the *Dictyostelium* structure (McCoy *et al.* 1999, Popowicz *et al.* 2004) chains in the whole molecule are arranged in an antiparallel way, overlapping only at domain 6 (Fig. 7B). On the other hand, human native filamin A molecules purified from smooth muscle (Gorlin *et al.* 1990) and blood platelets (II), appear on the electron micrographs mostly as V-shaped molecules with different angles between the two monomers, providing evidence for a close-to-parallel chain arrangement. Moreover, Gorlin *et al.* (Gorlin *et al.* 1990) suggested the existence of another site where the domains of both arms of filamin interact. Based on the proposal discussed above, that vertebrate filamins share a dimerisation interface common to that in filamin C 24, a controversy exists regarding how to bring together an antiparallel chain arrangement within the dimerisation domain with the close-to-parallel arrangement of the rod regions? One way to resolve this dilemma is to propose a model of filamin's molecular structure, where the two-fold symmetry axis of the dimerisation domain is oriented parallel to the long rod region of the filamin molecule (Fig. 7A). SAXS experiments on longer filamin constructs were believed to provide some deeper insight into the way the filamin molecule is organised at its carboxy-terminus. Although the details of the interaction between the dimerisation domain and the next domain 23 through the flexible hinge region were not resolved, the V-shape of the molecule could be clearly seen. The extended wings-like appearance of the SAXS-generated model is more likely to support a leaf-spring like filamin organisation.

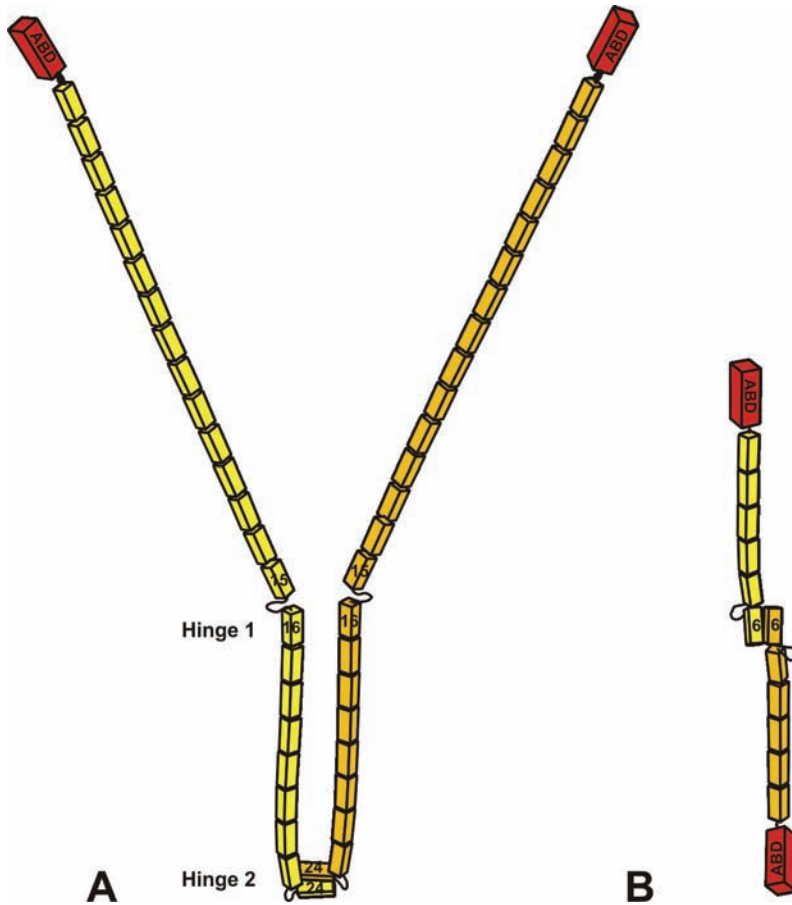


Fig. 7. Models of orientation of human filamin (A) and *Dictyostelium* filamin (B).

In a recent review (Popowicz *et al.* 2006) it was proposed that filamin repeats 16-24 bind larger proteins, such as receptors and serve as a capstan, whereas repeats 1-15 form a chain, binding smaller proteins that participate in signalling processes, and ABD carries an anchoring function. However, the amount of signalling proteins binding to the carboxy-terminus of filamin is significant (Table 1) and it is more likely that the C-terminal region serves as a platform where signalling molecules interact with each other, interconnecting multiple signalling pathways within the cell.

6.3 Ig-like domain 17 is a main interaction site of filamin A with the GPIb-IX-V receptor

Although the residues responsible for the GPIb-IX-V receptor – filamin A interaction were mapped on the cytoplasmic tail of the GPIb α subunit (Cranmer *et al.* 2005), the binding site on the filamin's side remained sufficiently broad encompassing domains 17-19 (Meyer *et al.* 1997). In this study the GPIb α -interaction site was narrowed to the single filamin A Ig-like repeat 17. Moreover, amino acid residues directly involved in that interaction were identified as glycine 1897 and cysteine 1912. Mutations in these two aminoacids together completely abolish an interaction between filamin A and GPIb α /IX *in vitro* and *in vivo*. Although both domains 17 and 19 of filamin A show affinity to GPIb α , isothermal titration calorimetry experiments demonstrated that the filamin A 17 – GPIb α binding constant is in the micromolar range. Moreover, domain 17 competed with full-length filamin for binding to the peptide.

6.3.1 How is the specificity of the filamin A – GPIb interaction defined?

The crystal structure of the complex of filamin A 17 with the GPIb α 556-557 peptide demonstrated that the interaction interface is formed by the extension of the β -sheet and involves hydrogen bonding between the β -strand formed by the peptide and the β -strand C of filamin. A hydrophobic interaction takes place between the peptide and both strands D and C.

The crystal structure of the repeat 21 of filamin A in complex with the integrin β 7 cytoplasmic tail peptide was also recently solved (Kiema *et al.* 2006), and that structure revealed a close resemblance to the filamin A 17 – GPIb α complex (Fig. 8). Superimposition of both structural complexes at their interaction interface reveals that topologically they are nearly identical (Figs. 3 and 8). Analysis of the residues crucial for the interaction on the filamin side in both structures shows that, although they are conserved, residues in the Ig-like repeat 21, which are critical for its interaction with integrin β 7, are not the same as the residues critical for the filamin A 17 – GPIb α interaction. (Ig21 – Ig17: Ile 2283 – Ile1910, strand D; Ala 2272 – Ser 1899; Ala2274 – Ala 1901, strand C). The Gly¹⁸⁹⁷ residue on the filamin A 17 β -strand C, crucial for filamin binding to GPIb α , is well conserved in odd-numbered domains (19, 21, 23) (Fig. 9). However, Cys¹⁹¹⁰, located on the D strand, shows a greater variability between domains, changing to Phe in 21, Thr in 19, but remaining conserved in repeats 23, 22 and 18.

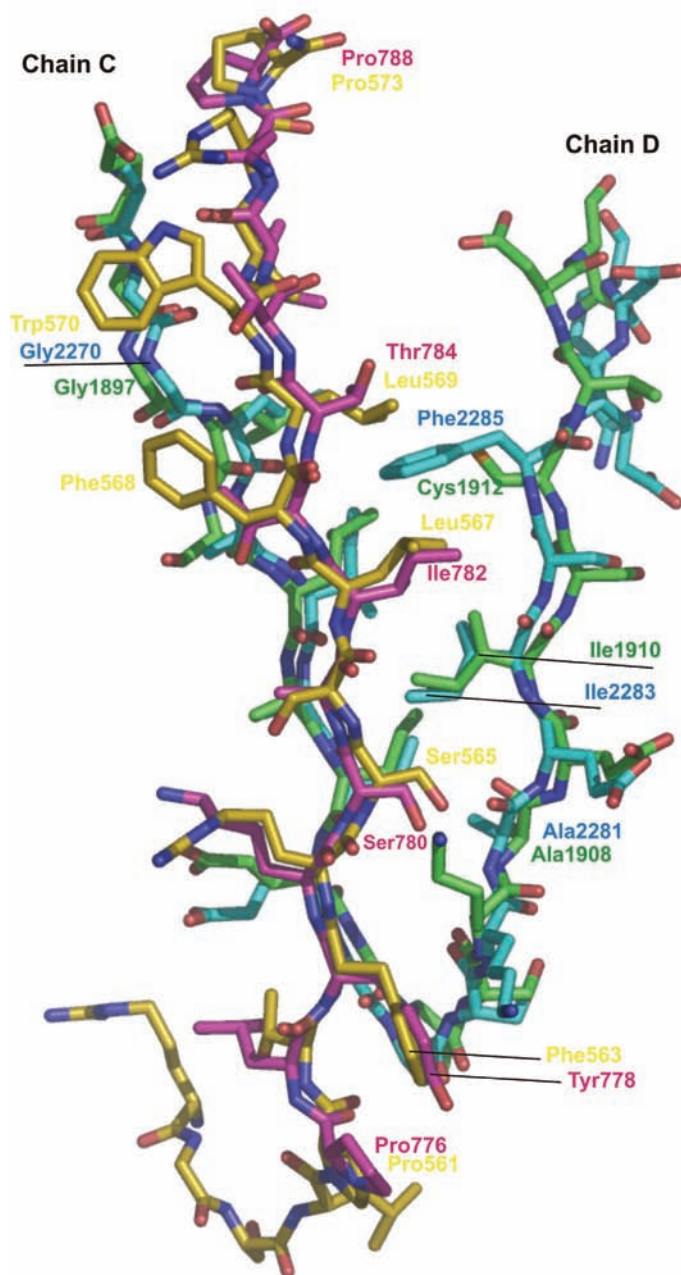


Fig. 8. Superimposition of filamin A 17 chains C and D (green) and the GPIβ 556-577 peptide (yellow) with filamin A 21 chains C and D (blue) and the integrin β7 cytoplasmic tail peptide (purple) (PDB id 2BRQ, Kiema *et al.* 2006).

Concerning the residues that form an interaction interface on the ligand side, few observations could be made. Although the similarity is low between the GPIIb α and integrin β 7 cytoplasmic domains, in both cases the peptide adopts the β -strand conformation and the region of the peptide which interacts with filamin domains is flanked with Pro residues and contains conserved Phe/Tyr and Ser residues (II, Fig. 5). The most remarkable feature of the filamin A 17 – GPIIb α complex is an interaction of Phe⁵⁶⁸ and Trp⁵⁷⁰ side chains with the main chain of filamin β -strand C. These residues are not conserved in integrin tails (II, Fig. 5). The stretch F⁵⁶⁸LW⁵⁷⁰ from GPIIb α corresponds to T⁷⁸³TT⁷⁸⁵ in integrin β 7, where Thr⁷⁸³ packs against Ala²²⁷² of filamin 21, critical for the filamin A 21 – integrin β 7 interaction, and superimposes with Phe⁵⁶⁸ of GPIIb α , one of the residues responsible for its interaction with filamin. At the same time Ile⁷⁸² and Thr⁷⁸⁴ of β 7 make a hydrophobic stack with ²²⁸⁵Phe of repeat 21, which superimposes with the stack formed by Leu⁵⁶⁷ and Leu⁵⁶⁹ with Cys¹⁹¹².

Overall, it appears likely that the specificity of filamin – ligand interactions based on the models available is defined by hydrophobic contacts formed between side chains of the ligand peptide and the filamin domain.

6.4 The hydrophobic CD face of Ig-like filamin domains is a common ligand binding site

Human filamins have multiple interaction partners (Table 1) and most of these interactions occur through C-terminal filamin domains 16-24, after a second hinge region. Currently three different crystal structures are available revealing interactions of human Ig-like filamin domains: filamin A 17 – GPIIb α 556-577 peptide (II); filamin A 21 – integrin β 7 cytoplasmic tail peptide (Kiema *et al.* 2006) and filamin C 24, which forms a homodimer (I). The interaction of filamin domains 17 and 21 with a ligand occurs through β -strands C and D. The ligand binding interface involves an extension of the β -sheet and the specificity of the interaction is determined mainly by hydrophobic interactions of side chains. In filamin C 24, dimerisation occurs also through strands C and D as well, and the prediction was previously made that the self-association found in human filamin C represents a common dimerisation interface for all vertebrate filamins. Further data allow even further generalisation and suggest that a CD face is a common filamin-ligand binding interface. Sequence alignment shows that strands C and D of domain 17 are highly conserved in all filamin isoforms (Fig. 9), and it is known that filamin B also interacts with GPIIb α (Xu *et al.* 1998, Takafuta *et al.* 1998).

The Gly¹⁸⁹⁷ residue on the filamin A 17 β -strand C that interact with GPIIb α is well conserved in odd-numbered domains (19, 21, 23) (Fig. 9). However, Cys¹⁹¹⁰, located on the D strand, shows the greater variability between domains, changing to Phe in 21, Thr in 19, but remaining conserved in repeats 23, 22 and 18. This possibly is a reason for the specificity of peptide recognition.

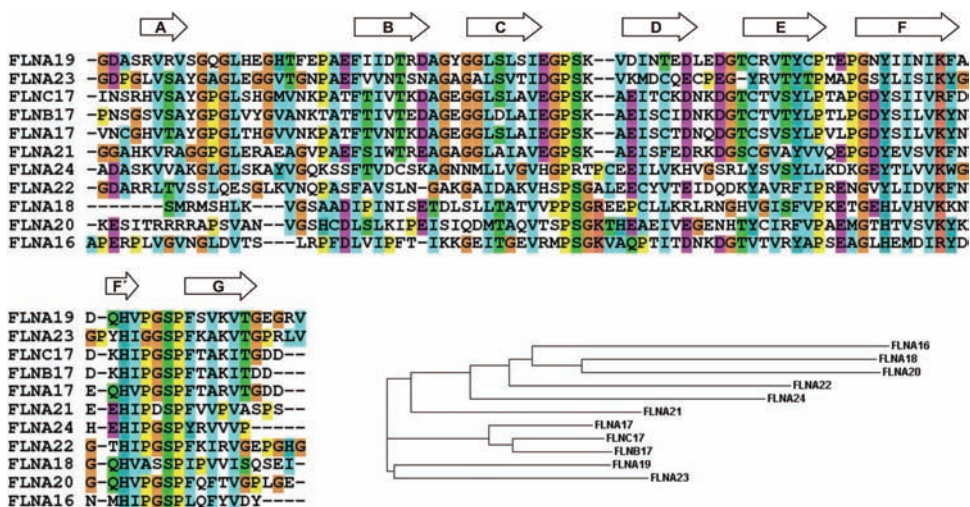


Fig. 9. Sequence alignment and phylogenetic tree of filamin A repeats 16-24 and repeat 17 of filamin isoforms B and C. β -strands are indicated. Conserved residues are coloured: orange – Gly; blue hydrophobic; green – polar; yellow – Pro; purple – acidic.

However, it is still too early to define a ‘Filamin-binding motif’ for filamin interaction partners, and further work in that direction is required. Nevertheless, current data provide helpful information for identifying target amino acids for mutagenesis experiments based on sequence homologies.

7 Conclusions

The present study provides a deeper insight into filamin structure. First, it reveals a fundamentally new mechanism of filamin dimerisation. The Ig-like domain 24 of human filamin dimerises through β -strands C and D, which are located on the opposite edge of the β -sandwich from strands B and G, which form a dimerisation interface in a *Dictyostelium* filamin analogue. Based on amino acid sequence similarities it could be proposed that all vertebrate filamins dimerise in the same way as human filamin. Mutagenesis studies confirmed that the dimer seen in the crystal structure is also present in solution. The overall structure of the C-terminal filamin domains 23 and 24 obtained during SAXS experiments confirmed the dimeric oligomerisation state of the protein.

Second, the study gives one of the first structural examples of a filamin-ligand interaction, shown by the crystal structure of the filamin A 17 – GPIb α 556-577 complex. Interaction between filamin and the GPIb-V-IX receptor is important for maintaining the integrity and shape of platelets, as well as for regulating the receptor adhesive function. The major interaction site between filamin A and the alpha subunit of the GPIb-V-IX was narrowed to the domain 17 of filamin in the present study. Moreover, residues crucial for that binding were identified. According to the present study, the specificity of filamin interactions is defined by hydrophobic contacts with the ligand molecule.

The study allows extending the hypothesis regarding a common vertebrate dimerisation interface even further to include the possibility that other filamin-binding proteins would form a β -strand interaction that involves a hydrophobic face of filamins, thus creating a common binding interface. However, taking into account the variety and diversity of filamin binding partners, it is still too difficult to predict a common filamin-binding motif by means of bioinformatics from the few structures available.

Taken together, current results provide useful information about molecular details of filamin structure and interactions.

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