STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE FOCAL ADHESION PROTEIN FAP52

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Abstract

FAP52 (focal adhesion protein, 52 kDa) is a focal adhesion-associated protein composed of a highly α-helical NH₂-terminus containing a poorly characterized FCH (Fes/CIP4 homology) domain, unstructured linker region and the COOH-terminal SH3 domain. FAP52 is also known as PACSIN 2 or syndapin II. Together with other PACSINs and syndapins FAP52 shares a common domain architecture.

The aim of this study was to characterize FAP52 in structural and functional terms. The function was pursued by identifying binding partners for FAP52, and by overexpressing the recombinant FAP52 in cultured cells. For the structural studies, various physico-chemical methods, such as chemical cross-linking, gel filtration chromatography, circular dichroism and X-ray crystallography were applied. In addition, the histological distribution of FAP52 in chicken tissues was explored.

FAP52 binds filamin, a protein that regulates the dynamics of the cytoskeleton by crosslinking actin filaments. The binding site in FAP52 was mapped to the NH₂-terminal 184 amino acids, of which the residues 146–184 form the core of the binding. In filamin, the binding site resides in the repeats 15–16 in the rod-like molecule encompassing 24 such repetitive domains. Overexpression of FAP52 or its filamin-binding domain in chicken embryo heart fibroblasts induced the formation of filopodial extensions on the cell surface and reduced the number of focal adhesions, suggesting a role in the organization of the cellular cytoskeleton and in cell adhesion machinery.

Experiments utilizing surface plasmon resonance analysis, size exclusion chromatography and chemical cross-linking showed that FAP52 self-associates in vitro and in vivo. The region responsible for the self-association was mapped to the amino acids 146–280, which is predicted to fold into a coiled-coil arrangement.

FAP52 was crystallized by using the hanging-drop vapor-diffusion method and ammonium sulfate grid screen. Native dataset was collected from two crystals, which diffracted to 2.8 Å and 2.1 Å resolution. For one form of crystals, phasing was performed using the native dataset and the datasets from two xenon-derivatized crystals. X-ray crystallography studies revealed a dimer in asymmetric unit.

Histological and in vitro studies showed that, in liver, FAP52 is preferentially expressed in bile canaliculi. In other tissues, FAP52 showed a specific staining pattern in gut, kidney, brain and gizzard.

Together, these data show that FAP52 self-associates in vivo and, probably via its interaction with its binding partner filamin, participates in the organization of the cytoskeletal architecture, especially of the cell surface protrusions, such as filopodia and microvilli of bile canaliculi.

Keywords: bile canaliculi, cytoskeleton, dimerization, filamin, focal adhesions
To Marja, Topias and Eelis
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Oulu, September 2004

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Abbreviations

aa   amino acid(s)
ABD  actin-binding domain
ABP-280 actin-binding protein, 280 kDa
BSA  bovine serum albumin
CEHF chicken embryo heart fibroblast
ECM  extracellular matrix
ECL  enhanced chemiluminescence
EGF  epidermal growth factor
EMBL European Molecular Biology Laboratory
FA(s) focal adhesion(s)
F-actin fibrous actin
FAP52 focal adhesion protein, 52 kDa
FCH  Fes-CIP4 homology
FPLC fast protein liquid chromatography
FPS  FAP52-PACSN-syndapin
G-actin globular actin
GP-Ibβ glycoprotein- Ibβ
GST  glutathione S-transferase
HA   hemagglutinin
HIP1R huntingtin-interacting protein 1-related protein
HRP  horseradish peroxidase
IPTG isopropyl-β-D-thiogalactopyranoside
LPA  lysophosphatidic acid
MAPK mitogen-activated protein kinase
MAYP macrophage-associated tyrosine-phosphorylated protein
mSos mammalian son-of-sevenless
NCBI National Center of Biotechnology Information
N-WASP neural Wiskott Aldrich syndrome protein
PBS phosphate-buffered saline
PCH  *pombe* Cdc15 homology
PCR  polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PEST</td>
<td>proline, glutamine/aspartic acid, serine, threonine</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>SAPKs</td>
<td>stress-activated protein kinases</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacryl amide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
</tbody>
</table>
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1 Introduction

Details of the regulation of many central cellular processes, such as cell division, migration and differentiation, are still only partially known. In order to clarify the molecular mechanisms of the processes, various schematic models are built with known proteins and arrows marking the interactions between them. However, the models still have gaps: proteins are missing and many interactions and functions unrevealed. The aim of basic research is to fill those gaps by completing the catalog of all the proteins and their domains, and by identifying their interactions and functions. Only detailed knowledge of the mechanisms of different cellular events enables us to understand the workings of the human body and, in the future, to develop therapeutic strategies and prophylactic measures against diseases.

Signaling proteins are typically constructed of distinct modules, domains, which occur in different combinations in different proteins. A domain can either have enzymatic activity, or it may be nonenzymatic and mediate interactions with specific sequences in specific proteins or other macromolecules. Currently, roughly 150 signaling domains have been identified (domain search by SMART; EMBL computational service at the network server http://smart.embl-heidelberg.de). A domain may be highly conserved among various proteins and between different species, such as SH3, which reveals a high degree of sequence similarity throughout the domain, or it may contain only a few conserved core residues, such as the WW domain, a 33-amino acid (aa)-long domain, which only has two conserved tryptophans. Examples of typical domains in signaling proteins are SH2- and SH3-domains. The SH2 domain binds to phosphotyrosine residues, and SH3 to specific proline-rich sequences (Cohen et al. 1995).

Focal adhesions are specialized structural entities that mediate adhesion of a cell to its substratum. Molecularly, they are composed of a large number of both structural and signaling proteins, which are linked together and coordinate their functions via specific domain-domain interactions.

This study focuses on the structure and function of the focal adhesion protein FAP52. FAP52 is an SH3 domain-containing, serine-phosphorylated protein, which in cultured fibroblasts localizes at focal adhesions (Meriläinen et al. 1997). It is expressed in most tissues.
2 Review of the literature

2.1 The FAP52/PACSIN/syndapin family

FAP52 (Meriläinen et al. 1997), PACSINs (Plomann et al. 1998) and syndapins (Qualmann et al. 1999) are closely related proteins that share a common domain architecture and are involved in actin organization and vesicular trafficking (Meriläinen et al. 1997, Ritter et al. 1999, Qualmann & Kelly 2000). FAP52 has been characterized from chicken (Gallus gallus). Its orthologs in other species have been named PACSIN 2 or syndapin II. In this thesis, the FAP52/PACSIN/syndapin proteins except for chicken FAP52 are called PACSINs. Three PACSIN genes have been identified. They encode the proteins called PACSIN 1, PACSIN 2 and PACSIN 3 (Ritter et al. 1999, Modregger et al. 2000). Based on the genebank search (program BLAST, NCBI network server at http://www.ncbi.nlm.nih.gov/BLAST/), all three PACSINs are found in all vertebrates, including mammals, birds, frogs and fishes. On the contrary, only PACSIN 2 is found in worms and insects. On the basis of sequence similarity, these related proteins form a novel family of proteins called the FAP52/PACSIN/syndapin (FPS) family. The known FPS proteins are listed in Table 1. FPS family is part of a larger protein family known as PCH, the name derived from their homology to the cdc15-protein of Schizosaccharomyces pombe (PCH= pombe cdc15 homology, Lippincott & Li 2000, see below).
2.1.1 The PCH family

The PCH family is an emerging group of proteins, which characteristically have a typical domain architecture, an NH₂-terminal coiled-coil and COOH-terminal SH3 domain, and which participate in actin-based cellular events, cytokinesis and vesicular traffic. However, the members show rather low sequence similarity (BLAST search). The current members of the PCH superfamily are Cdc15 (Fankhauser et al. 1995) and Imp2 (Demeter & Sazer 1998) of Schizosaccharomyces pombe, Hof1/Cyk2 (Kamei et al. 1998, Lippincott & Li 1998) of Saccharomyces cerevisiae, FPS proteins (Meriläinen et al. 1997, Plomann et al. 1998, Qualmann et al. 1999), and the mammalian proteins PSTPIP 1, a proline-serine-threonine phosphatase-interacting protein (Spencer et al. 1997), CD2BPI, a CD2 cytoplasmic tail-binding protein I (Li et al. 1998), and CIP4, a Cdc42-interacting protein 4 (Aspenström 1997). Mouse protein MAYP, the macrophage actin-associated tyrosine-phosphorylated protein (Yeung et al. 1998) and its human ortholog PSTPIP 2 (Wu et al. 1998) are similar to PSTPIP 1 except for the lack of the SH3 domain.
2.1.2 Structure

2.1.2.1 Domains and the sequence analysis of FPS family proteins

The FPS proteins share a common order of domains as schematically presented in Fig. 1. In the NH$_2$-terminus, there is an 89-aa long, highly $\alpha$-helical Fes-CIP4 homology (FCH) domain (Lippincott & Li 2000), followed by $\alpha$-helical coiled-coil domain, linker region, and the COOH-terminal SH3 domain. Additionally, FPS proteins contain consensus sequences for phosphorylation by serine/threonine kinases, PKA, PKC and casein kinase (Plomann et al. 1998).

The FCH domain contains a 20 aa-long RA EYL motif, named according to the conserved amino acid residues in its sequence (Plomann et al. 1998). The FCH domain is typically found in the NH$_2$-termini of proto-oncogene Fes/FES-related tyrosine kinases, RhoGAP-type GTPase activating proteins and in PCH family members, ranging from viruses and yeast to protozoans and mammals (domain search with the program SMART). The function of the FCH domain is largely unknown. Some recent results suggest, however, that it mediates interaction with microtubules (Tian et al. 2000, Fujita et al. 2002).

Most FCH domain-containing proteins also have a coiled-coil domain in their NH$_2$-terminal halves (analysis with the SMART program). The coiled-coil domain of FPS proteins has a propensity to mediate self-association, judged by computational analyses based on PairCoil- and Coils-programs (network servers at http://nightingale.lcs.mit.edu/cgi-bin/score and http://www.ch.embnet.org/software/COILS_form.html). The NH$_2$-ter-
minal region containing the FCH and coiled-coil domains is also called Cdc15-NT domain, where Cdc15 refers to *S. pombe* protein Cdc15 and NT to its NH$_2$-terminus (Ritter et al. 1999).

The linker region, which separates the NH$_2$-terminal FCH and coiled-coil domains from the COOH-terminal SH3 domain, is approximately 110 aa-long. It does not display any regular secondary structure or any specific function (Meriläinen et al. 1997). It does, however, contain a PEST sequence, a proline, glutamic/aspartic acid, serine and threonine-rich region, which is considered a signal for rapid degradation of proteins (Rogers et al. 1986, Rechsteiner & Rogers 1996). It also contains 2 or 3 asparagine-proline-phenylalanine (NPF) motifs (see Chapter 2.1.2.2). The NPF motif is a binding site for Eps15 homology domains (Paoluzi et al. 1998). Currently, no interactions mediated by NPF motifs of any FPS proteins have been described.

The SH3 domain is a widely occurring domain in a large variety of different proteins. Via binding to proline-rich target sequences, it participates in a wide variety of cellular events by mediating specific interactions between the components of signaling pathways and protein assemblies. One of its functions is to direct the localization of molecules to their specific compartments in the cell (for reviews, see Yu et al. 1994, Cohen et al. 1995, Mayer 2001). The SH3 domain of PACSIN isoforms has been found to bind to the following proteins (for references, see table II): dynamin I, synaptojanin, and synapsin I, an actin polymerization nucleating factor N-WASP, a cell surface metalloproteases MDC15, ADAM13 and ADAM13, the mammalian Son-of-sevenless (mSos), an apoptotic mediator CD95L and huntingtin. The known interactions are listed in Table 2.

**Table 2. Currently characterized binding partners of FPS proteins.**

<table>
<thead>
<tr>
<th>Binding partner</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamin I</td>
<td>Qualmann et al. 1999, Modregger et al. 2000</td>
</tr>
<tr>
<td>Synaptojanin</td>
<td>Qualmann et al. 1999, Modregger et al. 2000</td>
</tr>
<tr>
<td>Synapsin I</td>
<td>Qualmann et al. 1999, Modregger et al. 2000</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Qualmann et al. 1999, Modregger et al. 2000</td>
</tr>
<tr>
<td>MDC15</td>
<td>Howard et al. 1999</td>
</tr>
<tr>
<td>ADAM13</td>
<td>Cousin et al. 2000</td>
</tr>
<tr>
<td>mSos</td>
<td>Wasiak et al. 2001</td>
</tr>
<tr>
<td>CD95L</td>
<td>Ghadimi et al. 2002</td>
</tr>
<tr>
<td>Huntingtin</td>
<td>Modregger et al. 2002</td>
</tr>
<tr>
<td>ADAM12</td>
<td>Mori et al. 2003</td>
</tr>
</tbody>
</table>

**2.1.2.2 Sequence comparison**

Members of the FPS family are closely related proteins a with a high amino acid-sequence similarity. The identities among the mammalian and chicken orthologs are 81–98% for PACSIN 1, 85–98% for PACSIN 2, and 65–97% for PACSIN 3 (sequence comparison with BLAST). The relationship between FPS proteins is presented by a phylogenetic tree in Fig. 2.
Fig. 2. The phylogenetic tree of the FPS proteins of man, mouse, rat and chicken.

The NH$_2$-terminus (aa 1–340 in FAP52) and the SH3 domain (aa 390–448) are highly conserved regions in the members of the FPS family, typically having an identity of more than 90% among these proteins. One outstanding feature is the variation/alternative splicing in the region of four acidic residues EDDE/EDEE in the “linker” region (aa 302–305 in chicken FAP52). In addition to FAP52, PACSIN 2 isoforms IIaa and IIab in the rat have these four residues intact, whereas the isoforms IIba and IIbb, rat PACSIN 1 and all mouse PACSINs lack the first two residues (Qualmann & Kelly 2000; sequence analysis with the BLAST program). Alternative splicing of PACSIN 2 orthologs in humans, mouse, rat and chicken is schematically presented in Fig. 3.
Fig. 3. Presence of the alternative splicing sites in the PACSIN 2 isoforms in humans, mouse, rat and chicken. There are two alternative splicing sites in the polypeptides, the two acidic residues ED, and the 41-aa insert in the linker region. Two different splice variants have been found in humans, and four in rat. In mouse and chicken, only one form has been described.

The highest variation in the sequence of the FPS proteins is seen within the linker region. The two or three NPF motifs are fully conserved among the family, with the exception of PACSIN 3. Instead of NPF motifs, the linker region of Pacsin 3 contains two proline-rich sequences PxxP (P, proline; x, whatever aa), which are putative binding sites for SH3 domains. A distinguishing feature of the linker region of PACSIN 2 and syndapins Ilaa and IIba is an approximately 40 aa-long insert (see Fig. 3), which is not present in the other members. The insert also contains an additional NPF motif (Qualmann & Kelly 2000, analysis with BLAST). Interestingly, the NPF motifs throughout the FPS family are closely associated with acidic aspartic and glutamic acid residues. The consensus sequences for the three NPF regions are ZZZxxNPFxxxZ, NPFZZZ and NPFZZZZZ (Z, either aspartic or glutamic acid; x, whatever residue), as schematically presented in Table 3. Two of them contain the sequence NPFxD/E; NPFxD has been reported to serve as an endocytotic signal (Tan et al. 1996).

Table 3. Consensus sequences flanking the NPF motifs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>I (insert)</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAP52</td>
<td>deesNPFstd</td>
<td>NPFded</td>
<td></td>
</tr>
<tr>
<td>PACSIN 1</td>
<td>dessNPFggme</td>
<td>NPFded</td>
<td></td>
</tr>
<tr>
<td>PACSIN 2</td>
<td>NPFededd</td>
<td>ddesNPFstd</td>
<td>NPFded</td>
</tr>
<tr>
<td>Sdp I</td>
<td>dessNPFggme</td>
<td>NPFded</td>
<td></td>
</tr>
<tr>
<td>Sdp IIa/ba</td>
<td>NPFededd</td>
<td>ddesNPFstd</td>
<td>NPFded</td>
</tr>
<tr>
<td>Sdp IIb/bb</td>
<td>ddesNPFstd</td>
<td>NPFded</td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>NPFZZZZZ</td>
<td>ZZxxNPFxxxZ</td>
<td>NPFZZZ</td>
</tr>
</tbody>
</table>

* n, asparagine; p, proline; f, phenylalanine; e, glutamic acid; d, aspartic acid; Z, either e or d; x, whatever aa
2.1.3 Tissue expression

PACSIN 1 is expressed in the brain, as studied by Northern and Western blotting of various tissues, and by immunoelectron and immunofluorescence microscopy of mouse brain tissue and cultured rat forebrain neurons, respectively (Plomann et al. 1998, Qualmann et al. 1999). PACSIN 1 has also been found expressed in dorsal root ganglia neurons (Plomann et al. 1998). Human, but not mouse PACSIN 1 is also seen expressed in the heart, pancreas and liver (Sumoy et al. 2001). FAP52/PACSIN 2 is ubiquitously expressed in chicken, rat and mouse tissues (Meriläinen et al. 1997, Ritter et al. 1999, Qualmann & Kelly 2000). In rat, the PACSIN 2 splice variants IIaa and IIba have been found specifically in the heart when studied by Western blotting of a variety of rat tissues (Qualmann & Kelly 2000). PACSIN 3 has been detected in all examined tissues, but is expressed particularly strongly in the lung and muscle (Modregger et al. 2000, Sumoy et al. 2001).

2.1.4 Function

PACSINs have been implicated in endocytosis and actin organization (Plomann et al. 1998, Qualmann et al. 1999, Modregger et al. 2000, Qualmann & Kelly 2000). Overexpression of PACSINs induces a rearrangement of cortical actin cytoskeleton (Qualmann & Kelly 2000), and, on the other hand, inhibits endocytosis (Simpson et al. 1999, Modregger et al. 2000, Qualmann & Kelly 2000). FAP52 is phosphorylated in serine residues and associates with focal adhesions together with several other signaling proteins (Meriläinen et al. 1997).

2.1.4.1 FPS family proteins in neurons

PACSIN 1 is a highly brain-specific protein (Plomann et al. 1998, Qualmann et al. 1999). All PACSINs bind to the neuron-specific synaptic proteins dynamin I and synaptojanin (Qualmann et al. 1999, Modregger et al. 2000, Qualmann & Kelly 2000). In rat, PACSINs also bind to synapsin I, a protein implicated in the regulation of neurotransmitter release (Ferreira & Rapoport 2002). Expression of PACSIN 1 in the brain is developmentally regulated, so that the level of expression is increased from the 17th embryonal day to a fully differentiated, adult mouse. On the other hand, its expression is decreased within entorhinal-cortex lesion, suggesting that it negatively regulates self-repair system and regeneration (Plomann et al. 1998). Association of PACSIN 1 with neural differentiation is implicated by the finding that it is phosphorylated by casein kinase II, an enzyme known to play a role in neurogenesis (Diaz-Nido et al. 1994, Lim & Zaheer 1995). Histologically, PACSIN 1 is localized to the large neurons of the cortex and the brain stem, the pyramidal cells and astrocytes of the hippocampus. It is also seen in purkinje cells and in the molecular, but not the granular, layer of the cerebellum (Plomann et al. 1998).
A recent study has implicated PACSIN 1 in the pathogenesis of Huntington’s disease (Modregger et al. 2002) in that the SH3 domain of PACSIN 1 binds to mutated, but not to non-mutated, huntingtin. Huntington’s disease is a neurodegenerative disease, in which the gene encoding huntingtin is mutated to encode an abnormally long polyglutamate stretch. This is accompanied by neuronal loss in the striatal cortex, and, as a clinical manifestation, by progressive motor, psychiatric and cognitive dysfunctions (Young 2003). In the brain tissue in Huntington’s disease, the distribution PACSIN 1 is altered so that instead of axons, it is seen in the perinuclear cytoplasm already at the early stages of the disease (Modregger et al. 2002).

Also PACSINs 2 and 3 have recently been linked to neuronal differentiation via their binding to ADAM13. PACSIN 2 of *Xenopus laevis* (*X*-PACSIN 2) is suggested to serve as a downregulator of metallociprotease disintegrin ADAM13 (Cousin et al. 2000). ADAMs (also known as MDCs) are multifunctional transmembrane metalloglycoproteases, which are involved in a variety of biological processes, such as fertilization, neurogenesis, myogenesis, embryonic transforming growth factor-α release and the inflammatory response (Primakoff & Myles 2000). *X*-PACSIN 2 colocalizes with ADAM13 in migrating cephalic neural crest cells in the developing frog embryo, and, via its SH3 domain, binds to the proline-rich regions in the cytoplasmic domain of ADAM13 in vitro. In cultured *Xenopus* XTC cells, *X*-PACSIN 2 colocalizes with ADAM13 in membrane ruffles and cytoplasmic vesicles. Overexpressed *X*-PACSIN 2 also inhibits the developmental defects caused by ADAM13 overexpression, indicating that *X*-PACSIN downregulates the function of ADAM13 (Cousin et al. 2000).

### 2.1.4.2 Function in endocytosis

Endocytosis can be subdivided into five sequential steps: membrane invagination, coated pit formation, coated pit sequestration, detachment of the newly formed vesicle, and movement of the vesicle away from the plasma membrane. FPS proteins have been closely associated with the regulation of endocytosis via several interactions. They bind, via their SH3 domains, to dynamin I, synaptosomal and synapsin I, proteins that regulate vesicular traffic on plasma membrane (Modregger et al. 2000, Qualmann & Kelly 2000). Dynamin is a GTPase and plays an important role at the fission step of nascent clathrin-coated vesicles from plasma membrane (Schmid 1997). Synaptosomal, on the other hand, is a phosphatase and is present in endocytotic coated intermediates (McPherson et al. 1996) via interactions with various phosphoinositides (Guo et al. 1999). Synapsin I is a neuronal vesicle-associated phosphoprotein involved in exocytosis. It also regulates architecture of the actin cytoskeleton in the presynaptic nerve terminal (Sudhof et al. 1989, Greengard et al. 1993). Moreover, PACSINs bind to N-WASP and have been suggested to serve as a molecular link between the actin cytoskeleton and vesicle transport in presynaptic nerve terminal (see chapter 2.4; Qualmann et al. 1999, Modregger et al. 2000, Qualmann & Kelly 2000).

In cultured hippocampal neurons, PACSIN 1 has been shown to partially colocalize in vesicular structures with dynamin I, but not with clathrin (Modregger et al. 2000). Similar results were also obtained for PACSINs 2 and 3 in cultured NIH 3T3 fibroblasts and
C2F3 myotubes, respectively (Modregger et al. 2000). PACSIN 2 shows vesicular-like cytoplasmic staining also in migrating neural crest cells in the developing frog embryo (Ritter et al. 1999). Colocalization with dynamin I has also been seen for rat PACSIN 1 in the synaptic vesicles and growth cones of cultured neurons (Qualmann et al. 1999, Kes-sels & Qualmann 2002), and for rat PACSIN 2 in cultured rat PC12 cells (Qualmann & Kelly 2000). Thus, FPS proteins seem to interact with dynamin I in the dynamics of vesicle transport rather than to serve as structural components of clathrin-coated vesicles.

The function of FPS proteins in endocytosis has been studied in overexpression experiments in cultured cells. For instance, the SH3 domain of PACSIN 1 was transiently overexpressed in 3T3-L1 adipocytes, and endocytosis was monitored by detecting the internalization of transferrin (Simpson et al. 1999). The SH3 domain inhibited endocytosis at the fission step in a dose-dependent manner. In another assay, in which PACSIN isoforms were overexpressed in cultured cells, PACSINs were shown to block the endocytosis of transferrin (Modregger et al. 2000). The inhibitory effect was abolished when the function of the SH3 domain was abrogated by a specific point mutation. Several other SH3 domains also block endocytosis in vitro. For instance, overexpression of the NH₂-terminal SH3 domain of intersectin inhibits the intermediate phase of endocytosis, while that of endophilin I and amphiphysin II blocks the fission step (Simpson et al. 1999). Recently, a novel regulatory aspect in endocytosis was discovered involving increased association of PACSIN 1 with dynamin I upon phosphorylation of PACSIN 1 on serine by an inositol hexakisphosphate-regulated protein kinase (Hilton et al. 2001). The phosphorylated serine resides outside the SH3 domain that mediates the binding to dynamin I. Inositol hexakisphosphate is a phosphoinositol, which has been suggested to be involved in a wide variety of cellular processes, such as regulation of mRNA transport from nucleus, DNA repair, and control of phosphatase activity in pancreatic β cells (Shears 2001).

In addition to the interactions between FPS proteins and the endocytic proteins discussed above, the FPS protein-mediated inhibition of endocytosis may also be relayed via Rho proteins, multifunctional proteins that affect actin cytoskeleton, membrane trafficking, transcription, cell adhesion and cell cycle regulation (Hall 1998, Ridley 2001). To that effect, a recent study reveals direct SH3 domain-mediated binding of PACSINs to mSos, a guanine nucleotide exchange factor and activator of the Rho proteins Ras and Rac (Wasiak et al. 2001). Activated Rac, on the other hand, inhibits clathrin-coated vesicle formation and transferrin receptor-mediated endocytosis (Lamaze et al. 1996).

A recent study describes an interaction of PACSIN 2 with CD95 ligand (CD95L), a transmembrane receptor, which mediates cell death. PACSIN 2 has been suggested to regulate the expression of CD95L on plasma membrane by vesicle trafficking (Ghadimi et al. 2002).

### 2.1.4.3 Actin reorganization

FPS proteins have been suggested to regulate actin organization via two specific SH3 domain-mediated interactions. They include, first, the actin polymerization protein N-WASP, and, second, the ras- and rac-regulating protein Sos. FPS proteins bind to the polyproline region of the COOH-terminal half of N-WASP (Qualmann et al. 1999,
Modregger et al. 2000, Qualmann & Kelly 2000). N-WASP has an essential role in actin polymerization, which is the basis of the formation of filopodia and microspikes (Miki et al. 1998). In cultured HeLa cells and NIH 3T3 fibroblasts, overexpression of rat PACSINs 1 and 2 induced a rearrangement of the cortical actin and formation of long filopodia. In an experiment in which the COOH-terminus of N-WASP, containing the VCA region that is responsible for Arp2/3 binding, was overexpressed, these effects on actin organization were suppressed (Qualmann & Kelly 2000). Thus, the actin regulation is thought to be mediated via the Arp2/3 complex. N-WASP acts downstream of Cdc42 and phosphatidylinositol-4,5-bisphosphate (PIP2) (reviewed by Tekenawa & Miki, 2001). In resting cells, N-WASP is in autoinhibited state, where the VCA region is masked by an intramolecular interaction involving VCA and the Cdc42-binding domain, GBD/CRIB. Binding of Cdc42 brings about conformational changes in N-WASP, which unmasks the COOH-terminus and allows its binding to the Arp2/3 complex (Kim et al. 2000).

The route by which FPS proteins induce Arp2/3 activation is unclear. Several potential mechanisms have been suggested (Qualmann & Kelly 2000). They may work as upstream activators of the N-WASP activator Cdc42, behave like Cdc42 and cause the conformational change, recruit N-WASP to membrane, or directly activate the Arp2/3 complex. For further discussion of Arp2/3, see chapter 2.2.2.

There is also accumulating evidence that FPS proteins could regulate the dynamics of the cytoskeleton via mitogen-activated protein kinases (MAPK) (Wasiak et al. 2001). PACSINs 1 and 2 bind to the proline-rich sequence of mSos, activator for Ras and Rac, which regulate MAPKs and actin dynamics (Hall 1998). Activation of Rac has multiple effects, such as membrane ruffling and lamellipodia formation (Hall 1998, Ridley 2001). The interaction is mediated by the SH3 domain of PACSINs, and it is abolished by dominant negative proline-to-leucine mutation (P434L for PACSIN 1; P478L for PACSIN 2) in the SH3 domain (Wasiak et al. 2001).

A coupling of FPS proteins to actin dynamics is also suggested by the recent finding that PACSIN 3 binds to ADAM12 (Mori et al. 2003), an enzyme involved in the ectodomain shedding of the growth factor HB-EGF, and, thus, in the EGF receptor trans-activation. ADAM12 has also been implicated in actin reorganization and muscle development and regeneration (Gilpin et al. 1998, Bornemann et al. 2000, Kawaguchi et al. 2003).

2.2 Actin microfilaments and focal adhesions

2.2.1 Features of actin microfilaments

Actin microfilaments are responsible for the maintenance and regulation of cell shape, but they also play an essential role in dynamic processes, such as cell division, spreading and migration (Welch & Mullins 2002). Moreover, they participate in the subcellular processes, such as vesicular transport and cell organelle movement (Taunton 2001). Actin filaments display architectural polymorphism in cells. They can bind together parallelly to
form tight bundles (stress fibers), and thus provide the cell the ability to resist mechanical stress, but they can also form a loosely oriented network, as is the case in lamellipodia (Stryer 2000).

Actin filaments consist of fibrous actin (F-actin), which is a polymer of globular actin (G-actin) monomer. A single actin filament has the appearance of a two-stranded helix. They are polarized and dynamic structures, consisting of a barbed end (fast-growing end, positive end) facing the plasma membrane, and pointed end (slowly growing end, negative end) facing the cell center. Polymerization and depolymerization processes happen simultaneously in both ends. The barbed end favors polymerization, whereas depolymerization takes place in the pointed end. Actin is an ATPase, and in the cell the dynamics of actin filaments are partly driven by ATP hydrolysis. The speed of polymerization/depolymerization depends on the concentration of free G-actin molecules, on their bound ATP level, and on the presence of various regulatory proteins (see below). New actin subunits can be rapidly added to or removed from membrane-facing ends of the filaments in tightly regulated biological processes, such as cell migration and spreading. Rapid actin polymerization or depolymerization can be a response to an extra- or intracellular signal (Welch & Mullins 2002).

### 2.2.2 Proteins regulating actin polymerization

Numerous actin-binding proteins possess a propensity to modulate the actin organization in a cell. They cross-link actin filaments together, cap F-actin, sever the filaments to produce new barbed ends, bind free G-actin or link other proteins to F-actin (Hubberstey & Mottillo 2002, Remedios et al. 2002). The best-characterized and most important actors are discussed here.

Actin-binding proteins are a heterogeneous group of proteins, which do not share an individual actin-binding domain (ABD) with a common consensus sequence. However, certain subfamilies with a structural homology in ABDs have been identified. For instance, ABD of calponin, filamin, fimbrin and α-actinin consists of one to four calponin homology domains. They display a similar 3-D fold with 7 to 8 α-helical stretches and randomly coiled stretches between them, but have low sequence similarity (Winder 2003). The ABD of gelsolin superfamily proteins, which include e.g. gelsolin, villin and severin, on the other hand, is folded into several sandwiches consisting of numerous α-helices and β-sheets (Winder 2003).

Capping protein (Cap Z) is an important negative regulator of actin polymerization. Almost all barbed ends are capped with Cap Z. Dissociation of Cap Z is a prerequisite for polymerization or depolymerization to take place. (Cooper & Pollard 1985, Casella et al. 1987, Caldwell et al. 1989, Carlier & Pantaloni 1997). However, Cap Z is necessary for cell motility (Hug et al. 1995, David et al. 1998, Loisel et al. 1999). Binding of PIP2 to Cap Z leads to its dissociation from the barbed ends (Heiss & Cooper 1991).

Profilin is a small protein that binds to G-actin (Carlsson et al. 1977), PIP2 (Lassing & Lindberg 1985) and poly-L-proline containing proteins (Tanaka & Shibata 1985, Lindberg et al. 1988). Vasodilator-stimulated phosphoprotein (VASP; Reinhard et al. 1995), and possibly radixin (Funayama et al. 1991), zyxin (Sadler et al. 1992) and CAP-like pro-
teins (Vojtek et al. 1991) are the poly-L-proline-containing ligands of profilin. Profilin associates with microfilaments in the most dynamic areas of the cell, such as leading lamellae and newly formed attachment sites (Buss et al. 1992). The mechanism by which profilin regulates the dynamics of actin microfilaments is complex. By sequestrating G-actin, profilin decreases the accessibility of the building blocks of F-actin, thus leading to inhibition of actin polymerization and induction of depolymerization (Sohn & Goldschmidt-Clermont 1994). On the other hand, profilin has also been suggested to catalyze actin polymerization by delivering monomers to F-actin (Pring et al. 1992), and by converting ADP-actin to ATP-actin (Goldschmidt-Clermont et al. 1992). The profilin-G-actin complex can interact directly with actin filaments, and in that way counteract polymerization (Goldschmidt-Clermont et al. 1992, Pantaloni & Carlier 1993, Theriot & Mitchison 1993, Sohn & Goldschmidt-Clermont 1994). PIP$_2$, upon binding to profilin, inhibits its binding to actin. On the other hand, binding of PIP$_2$ to actin-bound profilin causes the dissociation of the complex (Katakami et al. 1992).

Gelsolins are a large family of actin-binding proteins (Janmey 1993) that have the capacity to bind to barbed ends of actin, nucleate new filament assembly, and bind to sides of filaments and sever them into smaller fragments (Lind et al. 1987, Hartwig 1992, Lamb et al. 1993, Allen & Janmey 1994). The capping and severing activity of gelsolin is activated by elevated concentration of Ca$^{2+}$-ions or a decrease of pH (Lamb et al. 1993), and inhibited by binding of PIP$_2$ (Janmey et al. 1987, Janmey & Stossel 1989). Severing produces new barbed ends, which can, after dissociation of gelsolin, serve as new foci of actin polymerization. In transformed fibroblasts, gelsolin has been found to be associated with podosomes (Wang et al. 1984), which are transient, highly dynamic types of focal adhesions along the edges of spreading cells (Petit & Thiery 2000). Due to the stronger binding of gelsolin than profilin to actin, its capping activity surpasses that of profilin (Ampe & Vandekerckhove 1994, Rozycki et al. 1994). Gelsolin is necessary for Rac-induced formation of filopodia (Azuma et al. 1998). Activation of Rac results in dissociation of gelsolin from F-actin (Arcaro 1998).

Adseverin/scinderin, fragmin, severin, villin and Cap G are close relatives of gelsolin. They show structural and functional similarity to gelsolin, with the exception that villin has the additional ability to merge actin filaments to bundles and that Cap G does not sever F-actin (Cant et al. 1998, Friederich et al. 1999, Hubberstey & Mottillo 2002). Two additional structural and functional relatives of villin, advillin (Marks et al. 1998) and supervillin (Wulfkuhle et al. 1999) have also been described. Tensin and its proteolytic fragment insertin bind barbed ends and decrease the polymerization rate of F-actin (Weigt et al. 1992, Lo et al. 1994a, Chuang et al. 1995). Tensin has also been shown to bind to the sides of actin filaments (Lo et al. 1994b).

Arp2/3 is a seven-subunit complex, which, in response of extracellular stimulus nucleates actin polymerization. The Arp2/3 complex consists of two actin-related proteins, Arp2 and Arp3, and of five additional proteins, named ARPC1-5 (Welch & Mullins 2002). Depending on the upstream signaling molecule, activation of the Arp2/3 complex can lead to the formation of either lamellipodia/membrane ruffling, or filopodia/microspikes. The former is a result of mesh-like actin filament organization, the latter of the formation of straight actin bundles (Takenawa & Miki 2001). Activation of the Arp2/3 complex by N-WASP directs actin polymerization to filopodia formation (Rohatgi et al. 1999). The mechanism behind this is unclear. One putative regulator could be the
actin-bundling protein filamin, which plays an important role in Cdc42-induced formation of filopodia (Ohta et al. 1999). Two mechanisms for Arp2/3 complex to activate actin polymerization have been suggested. First, the dendritic nucleation model, in which the activated Arp2/3 complex binds to the sides of existing filaments and nucleates the assembly of new filaments (Mullins et al. 1998, Blanchoin et al. 2000, Pollard et al. 2000, Millard et al. 2004). Second, the barbed end branching model, in which activated Arp2/3 nucleates branched filaments at existing barbed end of filament (Pantaloni et al. 2000, Millard et al. 2004). Arp2/3-induced actin polymerization is also important in the intracellular motility of Listeria monocytogenes, which utilizes its surface protein ActA as an activator of the Arp2/3 complex (Welch et al. 1998).

Several proteins are known to bind to sides or pointed ends of actin microfilaments and in this way to regulate actin polymerization or depolymerization. Tropomyosin, for instance, binds multiple subunits along the side of F-actin, and, when bound near the pointed end, prevents them from dissociation (Broschat 1990). Tropomodulin caps the pointed ends in striated muscle and participates in the assembly of sarcomers (Littlefield & Fowler 1998). Actin depolymerizing factor/cofilin-family members sever actin filaments into small pieces (Maciver et al. 1991, Du & Frieden 1998, Maciver et al. 1998, Blanchoin & Pollard 1999) and increase the loss of actin subunits from the pointed end (Carlier et al. 1997, Maciver et al. 1998).

Ezrin/radixin/moesin family members couple actin fibers to plasma membrane, probably by binding to the sides of the filaments (Pestonjamasp et al. 1995). Purified radixin also binds to the barbed ends in vitro (Tsukita et al. 1989). An integral membrane protein, ponticulin, also couples F-actin to plasma membrane by binding to the sides of microfilaments (Hill et al. 1994). In addition, it has the ability to nucleate actin assembly and create new barbed and pointed ends (Chia et al. 1993).

Fimbrin binds to the sides of microfilaments, and it is the major cross-linker of the filaments in the core of microvilli (Bretscher & Weber 1980). It contains two adjacent actin-binding domains and, thus, couples individual filaments to tight bundles (Matsudaira et al. 1983). In inner ear hair cell stereocilia it serves as an actin-bundling protein (Tilney et al. 1989).

### 2.2.3 Focal adhesions – structure and dynamics

#### 2.2.3.1 Structure of focal adhesions

The major adhesion sites between the cell and the extracellular matrix (ECM), termed focal adhesions (FAs), were initially observed by interference-reflection and electron microscopy as spots along the ventral plasma membrane of cultured fibroblasts (Abercrombie et al. 1971, Abercrombie & Dunn 1975, Izzard & Lochner 1976, Izzard & Lochner 1980). F-actin is present at early stages of FA-formation in chicken embryo fibroblasts (Depasquale & Izzard 1987), suggesting its important role in FA assembly. In fully developed FAs, the associated actin filaments merge to form tight parallel bundles.
Their barbed ends face FAs. New actin subunits can be rapidly added to or removed from membrane-facing ends of the filaments in tightly regulated biological processes, such as cell migration and spreading.

FAs couple the actin cytoskeleton of the cell to ECM, or, in cell cultures, to the growth substratum, and, thus, fix the cells to their environment. In addition to the role of FAs in connecting cells to the proper position in relation to their neighborhood and mediating mechanical force through plasma membrane, FAs serve as machinery for various extracellular stimuli to penetrate plasma membrane and to trigger and regulate various signaling cascades. Conversely, the cytoplasmic regulatory molecules also mediate the retrograde flow of signaling by modulating the function of FAs and e.g. the tenacity of the adhesion. FAs mediate numerous signals, such as those regulating cell adhesion, migration, proliferation and differentiation, apoptosis and gene expression. They play an essential role in tissue formation during embryogenesis and in biological regeneration processes, such as wound healing (for reviews of FAs, see Jockusch et al. 1995, Burridge & Chrzanowska-Wodnicka 1996, Petit & Thiery 2000, Juliano 2002).

FAs consist of transmembrane receptors and cytoplasmic proteins. Integrins are the main group of the transmembrane receptors segregated in FAs. The intracellular proteins include structural components, regulatory proteins, such as protein kinases and phosphatases and their substrates, as well as adapter proteins. Clear and exact distinctions between these groups cannot be made. The adapter protein paxillin, for instance, has also structural and regulatory features. Numerous FA components have been described, as listed in Table 4. The most important and best-characterized proteins with direct interaction with actin microfilaments are discussed in the following. For a detailed scrutiny, see the excellent reviews by Jockusch et al. (1995), Burridge and Chrzanowska-Wodnicka (1996), Petit and Thiery (2000), Zamir and Geiger (2001) and Juliano (2002).
### Table 4. List of known focal adhesion proteins.

<table>
<thead>
<tr>
<th>Transmembrane proteins</th>
<th>Regulatory proteins</th>
<th>Structural proteins</th>
<th>Adapter proteins or unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caveolin-1 (1)</td>
<td>AB (16)</td>
<td>actin (30)</td>
<td>CAP (69)</td>
</tr>
<tr>
<td>CD98/4F2Hc (2)</td>
<td>AND-34 (17)</td>
<td>α-actinin (59)</td>
<td>Cas (70)</td>
</tr>
<tr>
<td>EGF-receptor (3)</td>
<td>ASAP1 (18)</td>
<td>filamin (60)</td>
<td>Cbl (71)</td>
</tr>
<tr>
<td>insulin receptor (4)</td>
<td>β3-endodexin (19)</td>
<td>(acto)parvin (61)</td>
<td>CH-ILKBP (72)</td>
</tr>
<tr>
<td>integrins (18 α- and 8 β-subunits) (5)</td>
<td>calnexin (20)</td>
<td>ponsin (62)</td>
<td>CIB (73)</td>
</tr>
<tr>
<td>IAP/CD47 (6)</td>
<td>calpain II (21)</td>
<td>talin (63)</td>
<td>Cok (74)</td>
</tr>
<tr>
<td>LAR-PTP (7)</td>
<td>calreticulin (22)</td>
<td>tensin/insertin (64)</td>
<td>CRP-1/bombesin (75)</td>
</tr>
<tr>
<td>layilin (8)</td>
<td>Csk (24)</td>
<td>vinculin (66)</td>
<td>CSG (76)</td>
</tr>
<tr>
<td>TM4SF proteins/tetraspanins (14)</td>
<td>cytohesin-1 (25)</td>
<td>vincinex (67)</td>
<td>DOCK180 (77)</td>
</tr>
<tr>
<td>SHPS-1 (12)</td>
<td>gelsolin (29)</td>
<td>zyxin (68)</td>
<td>DRAL (78)</td>
</tr>
<tr>
<td>syndecan4/amphiglycan (13)</td>
<td>Graf (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIM4SF proteins/ tetraspanins (14)</td>
<td>ICAP-1 (31)</td>
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<tr>
<td>uPA-receptor (15)</td>
<td>ILK (32)</td>
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<td>PKC (38)</td>
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<tr>
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<td>PKG (39)</td>
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<tr>
<td>PKB/AKT (37)</td>
<td>PLC-γ (41)</td>
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<td></td>
</tr>
<tr>
<td>PP2A (42)</td>
<td>profiilin (43)</td>
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<tr>
<td>P53GAP (44)</td>
<td>PTEN (45)</td>
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<tr>
<td>PTP-PEST (46)</td>
<td>PTP1B (47)</td>
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<tr>
<td>Rac (49)</td>
<td>Rac1 (50)</td>
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<tr>
<td>Radixin (51)</td>
<td>RhoA (52)</td>
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<tr>
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<td>Sox (54)</td>
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<tr>
<td>Src (55)</td>
<td>TAP20 (56)</td>
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<tr>
<td>Trio (57)</td>
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Alpha-actinin is an antiparallelly organized rod-like homodimer, which cross-links actin filaments and serves as a spacer between actin filaments (Jockusch & Isenberg 1981, Blanchard et al. 1989, Meyer & Aebi 1990). It mediates the binding of actin filaments to FAs by binding to β1, β2 and β3 integrins (Otey et al. 1990, Burridge & Chrzanowska-Wodnicka 1996). It also binds to vinculin (Kroemker et al. 1994) and zyxin (Crawford et al. 1992), other important structural components of FAs. Overexpression of α-actinin in cultured cells causes the formation of more stable cell attachment sites, whereas suppression results in more motile cells (Gluck et al. 1993, Gluck & Ben-Ze'ev 1994). Its expression is quickly increased upon accelerated FA assembly (Gluck et al. 1992).


Talin is an FA-specific homodimer, constructed of two antiparallelly organized 270-kDa polypeptides. The dimer is a rod-shaped molecule with globular ends, which are formed of the NH2-termini of the subunits (Rees et al. 1990, Goldmann et al. 1994). The NH2-terminus binds to the focal adhesion kinase and β integrins, and interacts with phospholipids and membranes, whereas the COOH-terminus binds actin, β integrin and vinculin (reviewed by Petit & Thiery 2000). In vitro studies reveal that talin can cap and cross-link actin filaments and nucleate the formation of new filaments (Muguruma et al. 1990, Muguruma et al. 1992, Kaufmann et al. 1991, Goldmann et al. 1992), and possibly link actin filaments to plasma membrane (Goldmann et al. 1992, Niggli et al. 1994). Reducing the level of active talin-1 in fibroblasts, HeLa cells or undifferentiated embryonic stem cells by different techniques results in the inhibition of cell spreading and migration as well as disassembly of FAs and stress fibers (Nuckolls et al. 1992, Albiges-Rizo et al. 1995, Bolton et al. 1997, Priddle et al. 1998). However, a similar effect was not observed in differentiated stem cells (Priddle et al. 1998). During platelet activation, talin undergoes an increase in its phosphorylation state (Bertagnolli et al. 1993) and redistribution to newly formed adhesion sites (Beckerle et al. 1989).

Tensin is a homodimeric actin filament-capping protein, which has three actin-binding sites per subunit (Lo et al. 1994a). This gives the protein a propensity to cap or cross-link actin microfilaments. By capping of barbed ends, tensin regulates actin polymerization (Lo et al. 1994a,b). In addition, tensin has a vinculin-binding domain (Lo et al. 1994b). This enables tensin to anchor the microfilaments to FAs and the SH2 domain (Davis et al.
1991), which gives regulatory features to tensin. Recently, participation of tensin in the regulation of cell migration has been described (Chen et al. 2002).

VASP is an F-actin binding protein, which has been shown to localize at nascent FAs (Reinhard et al. 1992). VASP contains two conserved domains (EVH1 and EVH2) in the NH$_2$-terminus and the COOH-terminus, respectively, and a proline-rich region between them (Haffner et al. 1995). The proline cluster region is involved in the binding of profilin (Tanaka & Shibata 1985, Reinhard et al. 1995). Thus, VASP could serve as a mediator of profilin’s regulatory function against actin. EVH1 is responsible for binding to vinculin and zyxin, while EVH2 mediates tetramerization of VASP, F-actin binding and actin filament bundling (Bachmann et al. 1999, Calderwood et al. 2000).

Several other novel proteins have been documented to modulate actin filaments. 0-nexilin, for instance, is an actin-crosslinking protein, which is located at FAs of rat fibroblasts (Ohtsuka et al. 1998). Parvin/actopaxin modulates cytoskeletal organization at FAs by mediating functions of several proteins, such as ILK, paxillin, vinculin, nck2 and guanidine nucleotide exchange factors (reviewed by Brakebusch & Fässler 2003).

2.2.3.2 Dynamics of focal adhesions

During the dynamic cellular processes, such as cell migration and adhesion, FAs are quickly assembled and disassembled. Their dynamics, assembly in the forward-moving end and disassembly at the dorsal surface, allow cells to migrate. Contact of ECM components with their transmembrane receptors, integrins, or stimulation of a cell by growth factors results in integrin clustering (Schmidt et al. 1993, Felsenfeld et al. 1996) and subsequent association of the cytoskeletal FA elements, such as focal adhesion kinase, tensin, talin, vinculin, α-actinin and paxillin with the cytoplasmic domains of the integrins. The small GTPase Rho is a major regulator of the process (Hall 1994, Hotchin & Hall 1995, Nobes & Hall 1995, Takai et al. 1995, Ren et al. 1999). Of the other regulatory molecules Src kinase, Rac, Ras, MAPK cascade proteins, and cortactin are needed for assembly (Miyamoto et al. 1995a, Miyamoto et al. 1995b). Specific tyrosine phosphorylations are prerequisites for FA assembly (Kornberg et al. 1991, Burridge et al. 1992, Miyamoto et al. 1995a, Miyamoto et al. 1995b, Nobes et al. 1995, Craig & Johnson 1996).

Disassembly of FAs includes weakening of the interactions between FA components, severing of portions of FAs and the associating membranes, dispersion of integrins along the plane of the membrane, or moving them to new adhesion sites (Chen et al. 1981, Regen & Horwitz 1992, Palecek et al. 1996). Also extracellular anti-adhesive components, such as thrombospondin, tenascin and SPARC, are involved in cell migration, and in the formation and disassembly of FAs (Murphy-Ullrich et al. 1991, Sage & Bornstein 1991, Murphy-Ullrich et al. 1996, Greenwood & Murphy-Ullrich 1998). Stimulation with epidermal growth factor (EGF), for instance, promotes cell migration via regulated disassembly of FAs (Xie et al. 1998).
2.3 Filamin

2.3.1 Splice variants, domain structure and tissue expression

Filamins are a family of homodimeric, high molecular-weight proteins, which are involved in organizing actin filaments into a loose network of non-parallel fibers or tight parallel stress fibers. In addition to actin orchestration, filamins link various transmembrane proteins to the actin cytoskeleton and serve as scaffolds for many cytoplasmic signaling molecules (van der Flier & Sonnenberg 2001).

Filamin shows filamentous staining along actin fibers, hence the name (Wang et al. 1975). Three filamin genes have been identified in humans: filamin A (filamin-1, α-filamin, ABP-280, 2647 aa; Gorlin et al. 1990), filamin B (filamin-3, β-filamin, ABP-278/276, 2602 aa; Takafuta et al. 1998, Xu et al. 1998) and filamin C (filamin-2, γ-filamin, ABPL, 2705; Xie et al. 1998). They display a high (~70%) sequence similarity, except for the hinge regions H1 and H1 (see below), which have about 45% similarity. Filamin C has an additional 81-aa insert in the repeat 20. Vertebrates seem to have three filamin genes. In worms and insects, two long and two shorter filamin proteins have been found, probably originating from alternatively spliced transcripts (databank search by BLAST; van der Flier & Sonnenberg 2001).

The NH2-terminal 267-aa region of human filamins represents a typical α-actinin-like ABD (Matsudaira 1991), which contains two calponin homology domains (CH1 and CH2). ABD is followed by a rod-like region, which is formed of 24 approximately 100 aa-long repetitive segments (filamin repeats), each consisting of immunoglobulin-like β-sheet sandwiches (Tyler et al. 1980, Gorlin et al. 1990). Filamin contains two flexible hinge regions, H1 and H2, between the repeats 15 and 16, and 23 and 24, respectively (Gorlin et al. 1990, Hock et al. 1990). Filamin forms a V-shaped dimer where the repeats 24 of the two polypeptides interact noncovalently with each other (Gorlin et al. 1990).

The expression pattern of filamin is complex due to alternative gene promoters and alternative splicing. Splice variants for all human filamins have been detected (Gorlin et al. 1990, Takafuta et al. 1998, Xie et al. 1998). Variants that lack the repeat 15 of filamin A, H1 of filamins B and C, the 41-aa region between the repeats 19 and 20 of filamins A and B, and the four COOH-terminal repeats of filamin B have been identified (for domain architecture, see below). Alternative splicing affects the properties of filamins. Thus, the splice variant of filamin B with deletions of H1 and the 41-aa region [filamin-Bvar-1(ΔH1)] binds more strongly to integrins and localizes to focal adhesions (van der Flier et al. 2002). Furthermore, filamin-Bvar-1(ΔH1), but not the other variants of filamin B, accelerates the differentiation of myoblasts into muscle cells in vitro (van der Flier et al. 2002).

Taking together all variants, filamins A and B are expressed in most human tissues. At the level of variants, on the basis of RT-PCR, filamin-Avar-1 and filamin-Bvar-1 are weakly expressed in all tissues examined (van der Flier et al. 2002). Regarding the presence of H1, on the basis of RT-PCR, the expression level of wild type filamin B is the predominant form in the prostate, uterus, lung, liver, thyroid, stomach, lymph node, small intestine and spleen, while filamin-B(ΔH1) dominates in the spinal cord and in Daudi lymphoma.
cells. Both forms are seen in the placenta, bone marrow, brain, umbilical vein endothelial cells, retina and skeletal muscle (Xu et al. 1998). A variant lacking four COOH-terminal repeats is expressed in cardiac muscle (van der Flier et al. 2002). The expression of filamin C, mostly as a filamin-C(ΔH1) splice variant, is restricted to skeletal and cardiac muscle (Xie et al. 1998), in which it is enriched in Z-lines and in myotendinous junctions of skeletal muscle, Z-lines and intercalated discs of heart muscle. It is also present in dense plaques and dense bodies of smooth muscle. In cultured cells, filamin A has been localized to stress fibers, cortical actin network, membrane ruffles and cleavage furrow (van der Flier & Sonnenberg 2001).

### 2.3.2 Functions of filamin

#### 2.3.2.1 Actin cross-linking and organization

Filamin organizes actin filaments into parallel bundles or different types of network. In the networks, filamin is located at the crossroads/intersections of the actin filaments, or at the membrane contact points (Hartwig & Shevlin 1986). Filaments from different sources differ in their actin cross-linking activity. For instance, macrophage filamin forms tighter networks than chicken gizzard filamin (Brotschi et al. 1978). The type of the actin network depends on the filamin-to-actin ratio. At high filamin excess, parallel bundles are formed, whereas smaller amounts result in loose, orthogonal networks (Brotschi et al. 1978, Niederman et al. 1983, Dabrowska et al. 1985).

In vitro, the type of filamin-based network is also influenced by the presence of other actin cross-linking proteins. When pure filamin and actin are mixed, an orthogonal network is produced, while the presence of α-actinin results in the formation of dense cables (Schollmeyer et al. 1978). Lack of the H1 hinge region, which contributes to a less flexible filamin dimer, seems to favor formation of parallel bundles (van der Flier & Sonnenberg 2001).

#### 2.3.2.2 Filamin as an anchoring protein for transmembrane receptors

Filamins A and B bind to the cytoplasmic tail of the glycoprotein (GP)-Ibα subunit of the heteropentameric, platelet-specific von Willebrand factor (vWF) receptor (Ezzell et al. 1988, Andrews & Fox 1991, Meyer et al. 1997, Takafuta et al. 1998, Xu et al. 1998). In platelets, joining of GP-Ibα to the receptor complex triggers rearrangement of the cytoskeleton, as well as platelet aggregation. The repeats 17–19 mediate the binding (Andrews & Fox 1991). Expression of filamin in filamin-A-deficient melanoma cells (M2 cells), which have been stably transfected with the vWF receptor, leads to an increase of the level of vWF receptor on the plasma membrane (Meyer et al. 1998).
Via its four and a half COOH-terminal repeats, filamin interacts with the cytoplasmic tails of integrins \( \beta_1 \) (Loo et al. 1998, Pfaff et al. 1998, Zent et al. 2000), \( \beta_2 \) (Sharma et al. 1995), \( \beta_3 \) and \( \beta_7 \) (Liu et al. 2000). Filamin-B_var-1, the variant with a 41-aa deletion between the repeats 19 and 20, also binds to integrin \( \beta_1 \) and shows increased binding affinity to integrin \( \beta_1 \) (van der Flier et al. 2002). Similarly to the case of GB-Ib, the expression of filamin in M2 cells elevates the level of integrin \( \beta_1 \) in the plasma membrane (Meyer et al. 1998), suggesting that filamin has a role in the retention of transmembrane receptors (van der Flier et al. 2002).

Filamin also interacts with the cytoplasmic tails of other transmembrane proteins such as \( \gamma \)- and \( \delta \)-sarcoglycans (Thompson et al. 2000), presenilins (Zhang et al. 1998, Guo et al. 2000), furin (Liu et al. 1997), Fc\( \gamma \)RI (Ohta et al. 1991), tissue factor (Ott et al. 1998), and probably the acetyl choline receptor as well (Shadiack & Nitkin, 1991). Furthermore, filamin binds and regulates the function of dopamine D2 receptor (Li et al. 2000) and the androgen receptor (Ozanne et al. 2000).

### 2.3.2.3 Filamin as a scaffolding protein for signaling molecules

Filamin has been suggested to serve as docking site for signaling molecules and, thus, to direct them to proper localization and orientation for actin filament nucleation, actin dynamics and vesicle transport (van der Flier & Sonnenberg 2001).

Several members of the Ras superfamily of GTPases bind to the COOH-terminal repeats of filamin (Ueda et al. 1992). Among them, the binding of RalA is GTP-dependent, while that of the Rho-like GTPases Cdc42 and Rac1 is not (Ohta et al. 1999). Also Trio, a guanine nucleotide exchange factor for RhoG, Rac and RhoA, binds, via its pleckstrin homology domain, to filamin (Bellanger et al. 2000). Transfection and microinjection experiments with Swiss 3T3 fibroblasts and filamin A-deficient human melanoma M2 cells show that the formation of filopodia, induced by RalA and Cdc42, unlike that induced by RhoA and Rac1, is dependent on filamin (Ohta et al. 1999).

Filamin A also binds SEK-1 (MKK-4, JNKK), a kinase that activates several stress-activated protein kinases (SAPKs) (Marti et al. 1997). In filamin A-deficient M2 cells, TNF\( \alpha \) and lysophosphatidic acid (LPA)-induced activation of SAPKs is reduced, suggesting that appropriate TNF\( \alpha \)- and LPA-mediated SAPK activation is dependent on the presence of filamin. Expression of dimerization-deficient mutant filamin fails to normalize LPA-activation of SAPK, whereas it corrects the defective SAPK response to TNF\( \alpha \). Moreover, interaction of filamin-A with tumor necrosis receptor-associated factor-2 is essential for TNF\( \alpha \)-mediated activation of SAPKs and of the transcription factor NF-\( \kappa \)B (Marti et al. 1997, Leonardi et al. 2000). Filamin's role in TNF-receptor signaling has been supported by studies in Drosophila. In Drosophila, filamin binds to Toll, a TNF receptor superfamily member, which regulates the development of dorsal-ventral polarity of the fruit fly, and to Tube, a signaling protein downstream of Toll (Edwards et al. 1997).
2.3.2.4 Regulational aspects of filamin function

The regulation of filamin function is poorly known. However, some regulatory mechanisms have been suggested (Fig. 4). One mechanism could be the occupancy of the transmembrane receptors that are/become coupled to filamin. Thus, for instance, binding of immunoglobulin to FcγRI receptor causes the dissociation of filamin from FcγRI and, thus, release of FcγRI receptor from the cortical actin cytoskeleton (Ohta et al. 1991). FcγRI is a receptor in the plasma membrane of hematopoietic cells, in which it binds to the Fc domain of the antigen-bound IgG (Strzelecka et al. 1997). In contrast, in the case of tissue factor, the cellular receptor for Factor VII, occupancy of the receptor is needed for filamin-A binding (Ott et al. 1998). Binding of Factor VII to tissue factor upon tissue injury triggers the blood coagulation process (McVey 1999).

In cells with high activity of Ras-related GTPases, filamin is found to be strongly phosphorylated (Yada et al. 1990, Ueda et al. 1992). Phosphorylation, on the other hand, has an influence on its actin binding and actin cross-linking activities (Zhuang et al. 1984, Ohta & Hartwig 1995). At least EGF, platelet-derived growth factor and LPA induce serine/threonine phosphorylation of filamin (van der Flier & Sonnenberg 2001). The known kinases responsible for filamin phosphorylation are ribosomal S6 protein kinase-2 (Ohta & Hartwig 1996), PKA, PKC and CaM-kinase II (Wallach et al. 1978, Kawamoto & Hidaka 1984, Chen & Stracher 1989, Ohta & Hartwig 1995).

The hinge regions H1 and H2 contain calpain cleavage sites. Cleavage in H2 results in the formation of filamin that is unable to dimerize. Thus, it lacks the ability to cross-link actin filaments (Davies et al. 1978, Gorlin et al. 1990). Cleavage by calpain is probably regulated by phosphorylation of filamin (Chen & Stracher 1989, Gorlin et al. 1990, Wu et al. 1994, Jay et al. 2000). Upon platelet activation, GP-Ibα is released from filamin after cleavage of filamin by calcium-dependent protease (Fox 1985). In addition, cleavage of filamin by m-calpain and granzyme is associated with myotube differentiation process (Kwak et al. 1993a, b) and caspase-independent apoptosis (Browne et al. 2000), respectively.
Fig. 4. Suggestions for the regulation of the function of filamin. A. Binding of a ligand to the filamin-bound receptor causes the dissociation of filamin from the receptor. B. Phosphorylation of filamin enhances the capacity of filamin to bind and cross-link F-actin. C. Cleavage of the repeat 24 by calpain protease cuts the dimerization domain loose, which causes the loss of the actin cross-linking capacity of filamin.

### 2.4 Endocytosis and the cytoskeleton

Endocytosis is a complex process that includes the endocytic machinery itself as well as its regulatory system. Currently, the regulatory mechanisms are largely unrevealed. During the past few years, a linkage between endocytosis and the actin cytoskeleton has been established. Disturbance of normal actin cytoskeleton by actin-perturbing drugs or mutant
Rho-family proteins disrupts endocytosis in some, but not in all assays (Gottlieb et al. 1993, Jackman et al. 1994, Lamaze et al. 1997, Fujimoto et al. 2000, da Costa et al. 2003). Recent studies have revealed several proteins that are associated both with endocytosis and actin dynamics (Qualmann & Kessels 2002).

2.4.1 Actin and endocytosis

Currently, the regulatory role of actin cytoskeleton cannot be conclusively assigned to any specific stage of endocytosis. Also the molecular mechanisms are largely unknown. Building upon variable models the following modes of involvement of the cytoskeleton in the specific steps of endocytosis have been presented (Qualmann et al. 2000):

First, the network of actin filaments could localize the endocytotic machinery to specific compartments by setting physical barriers or by directly associating with endocytotic proteins. This is supported by the observations that coated pit formation in a defined location was altered and led to diffusion of clathrin-coated pits from the place of their origin upon disrupting the actin architecture by monomer-sequestering drugs (Gaidarov et al. 1999).

Another possibility is that actin filaments facilitate invagination of the planar membrane, and, in this way, make it more available for endocytotic machinery to attach.

In a third model, actin filaments play a role in the membrane scission event upon which the newly formed vesicle is liberated from the plasma membrane. In experiments utilizing multicolor real-time fluorescence microscopy, rapid actin polymerization has been seen to take place at the sites of vesicle pinching (Kaksonen et al. 2003). On the other hand, inhibition of actin polymerization arrests the endocytotic process to the stage of invaginated coated pit (Lamaze et al. 1997).

Finally, actin polymerization might serve as a motor, which moves the newly formed vesicle through the cytoplasm. This theory is supported by the observation that different vesicles are associated with actin comet tails (Frischknecht et al. 1999, Merrifield et al. 1999, Rozelle et al. 2000, Taunton et al. 2000).

As opposed to these views of an intact actin network as a facilitator of endocytosis, there is also evidence that actin could suppress it. Some assays suggest that the cortical actin cytoskeleton has to be removed prior to initialization of membrane trafficking. A rigid cortical cytoskeleton has been observed to inhibit endocytosis (Trifaró & Vitale 1993). Electron microscopy studies of Cos-7 cells have shown that the immediate vicinity of the coated pits is almost free of fibrous actin (Fujimoto et al. 2000).

2.4.2 Proteins acting at the interface between endocytosis and the actin cytoskeleton

Dynamin is a GTPase that regulates the fission stage of endocytosis (for a review, see Sever et al. 2000). Some studies suggest dynamin to be coupled to actin dynamics. For
instance, overexpression of the mutant dynamin, which is unable to bind GTP, causes not only inhibition of endocytosis, but also a redistribution of actin stress fibers and alteration of cell shape (Damke et al. 1994). On the other hand, downregulation of dynamin I in hippocampal cultures led to inhibition of the actin-associated, neurite outgrowth (Torre et al. 1994). Low concentrations of overexpressed dynamin 2 were shown to enhance actin nucleation, while higher concentrations had an inhibitory effect. Recently, expression of mutant Vps1p, the yeast dynamin-related protein, has been shown to result in both actin abnormalities and impaired vesicular protein sorting in Saccharomyces cerevisiae (Yu et al. 2004). Vps1p binds to the actin-regulating protein Sla1p (Yu et al. 2004). Moreover, dynamin has been found to regulate the dynamics of actin-based comet tails, which provide mechanical force for cellular organelles and Listeria monocytogenes to move inside the cells (Lee & Camilli 2002, Orth et al. 2002).

PACSINs represent novel linkers between endocytosis and actin cytoskeleton. They bind vesicle-trafficking proteins dynamin I, synapsin I and synaptojanin, as well as N-WASP, a nucleator of actin polymerization (Qualmann & Kelly 2000, Modregger et al. 2000). Exogenous expression of the SH3 domain of rat PACSINs in cultured cells leads to a block in receptor-mediated endocytosis. It affects the dynamin-regulated step, in which the vesicles are pinched off, and leads to accumulation of clathrin coated pits and vesicles at the apical plasma membrane of the cells (Simpson et al. 1999, da Costa et al. 2003). Co-overexpression of the VCA domain of N-WASP, the crucial region for the initiation of actin polymerization, cancels the disruption. On the other hand, overexpression of PACSINs induces Arp2/3 complex activation, probably via interaction with N-WASP, resulting in the reorganization of actin cytoskeleton and the formation of filopodia (Qualmann & Kelly 2000, Kessels & Qualmann 2002). PACSIN 1 also binds to mSos, a guanine nucleotide exchange factor for the cytoskeletal regulators Ras and Rac, suggesting another link between the cytoskeleton and endocytosis (Wasiak et al. 2001). Overexpressed rat PACSIN locates at the sites of high actin turnover, such as lamellipodia and filopodia. Chicken FAP52 has been localized to focal adhesions, which also represent the sites of dynamic actin at the plasma membrane (Meriläinen et al. 1997). Thus, FPS proteins have been suggested to couple actin polymerization to dynamin function in the fission reaction (Qualmann et al. 1999). The putative mechanisms by which FPS family proteins affect endocytosis are presented in Fig. 5.
Fig. 5. FPS proteins and endocytosis. The mechanism of the FPS proteins in the regulation of endocytosis may be mediated by its interaction partners, such as N-WASP, dynamin I, synaptojanin, or huntingtin and its interactor Hip1.

Mammalian Abp1 binds to F-actin by its NH2-terminal binding domains and colocalizes with highly dynamic actin in lamellipodia (Kessels et al. 2000, 2001). Its involvement in actin dynamics is dependent on the GTPase Rac1. In yeast, Abp1 binds both to the Arp2/3 complex and F-actin (Goode et al. 2001). It has been suggested to recruit Arp2/3 complex to sides of actin filaments. Recently, Abp1 has been described to interact, via its COOH-terminal SH3 domain, with dynamin (Kessels et al. 2001). Its overexpression leads to reduction of receptor-mediated endocytosis (Kessels et al. 2001). Thus, Abp1 might serve as a physical link between endocytosis and the actin cytoskeleton.

Similarly to Abp1, the Src kinase substrate cortactin (Wu et al. 1991) binds to F-actin (Wu & Parsons 1993), Arp2/3 complex (Urno et al. 2001), and dynamin (McNiven et al. 2000). Cortactin and dynamin colocalize with F-actin in lamellipodia (Kaksonen et al. 2000, McNiven et al. 2000), and promote actin polymerization activity of Arp2/3 and stabilize the actin filament network (Weaver et al. 2001). Cortactin has been suggested to regulate actin assembly, which propels the movement of endosomes through the cytoplasm (Kaksonen et al. 2000).

WASP family members enhance the ability of the Arp2/3 complex to nucleate actin polymerization by binding via its COOH-terminus to Arp2/3 (Machesky & Insall 1998,
Welch 1999). Platelets and lymphocytes of Wiskott-Aldrich syndrome patients show a decreased number of microspikes and abnormal actin organization (Snapper & Rosen 1999). In addition to regulation of actin organization, WASPs have been shown to be involved in endocytosis. Upon EGF treatment of Cos-7 cells, neuronal N-WASP forms a complex with the EGF receptor, probably via the adaptor protein Grb2 (Miki et al. 1996). WASP-knockout lymphocytes exhibited, in addition to impaired actin polymerization, reduction in T cell receptor endocytosis (Zhang et al. 1999). Moreover, N-WASP binds to PACSINs, which are also implicated in both actin dynamics and endocytosis (Simpson et al. 1999, Qualmann & Kelly 2000). Recently, endosomal and lysosomal vesicles have been observed to move in cell-free extracts via actin propulsion, an event that exhibited N-WASP staining in the interface of the vesicle and the actin tail (Taunton et al. 2000).

Synaptojanin is a phosphoinositide phosphatase (McPherson et al. 1996, Woscholski et al. 1997), which is also implicated in both endocytosis and actin dynamics. Synaptojanin binds several SH3 domain-containing proteins, such as PACSINs (Qualmann et al. 1999, Qualmann & Kelly 2000), which are involved in endocytosis. It plays a role in uncoating of coated vesicles (Cremona et al. 1999). *In vitro*, synaptojanin is able to hydrolyze PIP2, which is bound to profilin, coflin and α-actinin. Overexpression of synaptojanin causes rearrangement of actin stress fibers (Sakisaka et al. 1997). This is well in line with the observation that a decrease in the level of PIP2 causes actin depolymerization.

Sla2 of budding yeast is a component of the cytoskeleton and participates in the internalization step of endocytosis (Wesp et al. 1997). In cells lacking Sla2, non-motile endocytotic vesicles with actin-comet tails are seen (Kaksonen et al. 2003), suggesting its regulatory role in endocytosis-associated actin events. Similarly to Sla2, lowering the level of its mammalian homolog HIP1R by RNAi causes the stable association between the actin assembly and the endocytotic machineries (Engqvist-Goldstein et al. 2004). Via direct binding to clathrin and α-adaptin, HIP1R closely associates with clathrin and clathrin-coated pits and vesicles (Engqvist-Goldstein et al. 1999, Gaidarov et al. 1999, Waelter et al. 2001). The talin-like COOH-terminus of HIP1R binds F-actin. The binding site is necessary for its colocalization with cortical actin (Engqvist-Goldstein et al. 1999). HIP1R promotes clathrin cage assembly and links it to F-actin *in vitro* (Engqvist-Goldstein et al. 2001). The dual function of HIP1R may be needed for spatial organization of endocytosis or actin-dependent movement of newly formed vesicles (Engqvist-Goldstein et al. 1999).
3 The aims of the present study

A novel SH3 domain-containing protein, FAP52, was originally characterized by Meriläinen et al. (1997). In cultured fibroblasts, FAP52 is localized in focal adhesions. In Northern blotting, its transcript has been detected in most tissues. On the basis of computational analyses of its primary structure, the NH2-terminus of FAP52 is highly α-helical, and it has a tendency to fold into α-helical coiled-coil and form higher-order oligomeric complexes. The specific aims of the present study were:

1. to further characterize FAP52 in structural terms,
2. to describe novel binding partners of FAP52,
3. to determine the function of FAP52,
4. and to determine the distribution of FAP52 in different tissues.
4 Materials and methods

The detailed methods are described in the original articles I–IV.

4.1 General procedures and computer programs (I–IV)

The standard solutions, buffers and procedures for the purification and precipitation of DNA, restriction enzyme digestions, ligation reactions, SDS-PAGE runs, immunohistochemical and histochemical reactions, and stainings were as described in Sambrook et al. (1989). DNA sequencing was carried out on an automated ABI Prism 377XL DNA Sequencer (PerkinElmer, Branchburg, NJ). For sequence alignment, the program Clustal X, version 1.8 (Thompson et al. 1997), and for sequence comparisons, the program BLAST at the network server http://www.ncbi.nlm.nih.gov/BLAST/ were utilized. For the prediction of coiled-coil arrangements, the programs PairCoil (Berger et al. 1995) and MultiCoil (Wolf et al. 1997) were employed at the network servers http://nightgale.lcs.mit.edu/cgi-bin/score and http://nightgale.lcs.mit.edu/cgi-bin/multicoil, respectively.

4.2 Materials (I–IV)

The plasmid DNA purification kits were from Qiagen (Hilden, Germany) and Promega (Madison, WI), and the DNA gel extraction kit from Qiagen. *E. coli* strain BL21(DE3) cells, expression vectors pGEX-2T, pGEX-2TK and pGEX-4T-1, glutathione-Sepharose 4B, thrombin, Rainbow High Molecular Weight Markers, and synthetic oligonucleotides were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), isopropyl-β-D-thiogalactopyranoside (IPTG) from MBI Fermentas (Vilnius, Lithuania), Site-Directed Mutagenesis kit, *pfu* polymerase and T4 DNA ligase from Stratagene (La Jolla, CA), restriction enzymes from MBI Fermentas and Promega, and chicken gizzard filamin from Progen Biotechnik (Heidelberg, Germany). For blot overlay
assay, chicken gizzard filamin was purified according to the method described by Feramisco and Burridge (1980). Fugene 6-transfection reagent, reduced glutathione, protease inhibitors aprotinin, leupeptin and phenylmethylsulphonyl fluoride were obtained from Boehringer Mannheim (Mannheim, Germany), and ampicillin, bovine serum albumin (BSA) and benzamidine, disuccinimidyl suberate, Nondenatured Protein Molecular Weight Marker kit, urease, and chicken egg albumin from Sigma Chemicals (St. Louis, MO). Triton X-100 was from Merck (Darmstadt, Germany), γ-globulin from Calbiochem-Novabiochem (Darmstadt, Germany), and nitrocellulose membranes from Schleicher & Schuell (Keene, NH). HeLa cells were purchased from the American Type Culture Collection (Rockville, MD), Immu-Mount mounting medium from Shandon (Pittsburgh, PA), and cell culture media and fetal calf serum from Hyclone Laboratories (Logan, UT).

4.3 Antibodies (I, II, IV)

Rabbit polyclonal antibody to bacterially produced FAP52, denoted Affi-K7, was produced and affinity-purified as described (Meriläinen et al. 1997). Rabbit polyclonal anti-hemagglutinin (HA) antibody (anti-HA) was from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal anti-GST antibody from Biacore (Uppsala, Sweden), mouse monoclonal anti-filamin antibody from Chemicon International (Temecula, CA), and mouse monoclonal anti-paxillin antibody from Zymed Laboratories (San Francisco, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse immunoglobulins, and goat anti-rabbit and anti-mouse IgG-agarose were purchased from Sigma Chemicals, and rhodamine-phalloidin, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG from Molecular Probes (Eugene, OR). For immunoelectron microscopy, Protein A-gold particles (⌀ 5 nm and 10 nm), prepared by Slot and Geuze (1985), were used.

4.4 cDNA constructs (I–III)

All the cDNA constructs and corresponding polypeptides were designated as “FAP52” or “Fil” for chicken FAP52 and human filamin A, respectively, followed by subscripted numbers indicating the first and the last aa residues represented.

To express the recombinant full-length FAP52 and its fragments, the corresponding cDNAs were generated by polymerase chain reaction (PCR) utilizing the oligonucleotide primers with BamHI and EcoRI restriction sites. The resulting cDNAs were inserted into the corresponding restriction sites of pGEX-2T or pGEX-2TK vectors. COOH-terminal fragments of filamin were expressed by pGEX-4T-1 vectors with the cDNAs encoding for the fragments Fil11524–2283, Fil12283–2490 and Fil22495–2647 (a kind gift from Dr. T.P. Stossel, Division of Hematology, Brigham and Women’s Hospital, Boston, USA; Ohta et al. 1999). Further COOH-terminal truncation mutants were generated from the construct
encoding for the fragment Fil_{1524-2283} by using Site-Directed Mutagenesis kit and oligo-
nucleotide primers to generate stop codons at appropriate sites of the cDNA.

To express FAP52 or its fragments in cultured eukaryotic cells, the corresponding
cDNA-constructs were produced by PCR with engineered restriction sites. They were cut
with EcoRI and EcoRV and subcloned into EcoRI/EcoRV cloning site of pRK5-vector (a
kind gift from Dr. Joseph Schlessinger, New York University, Medical Center, New York,
NY). An HA-tag was engineered to the COOH-termini of the constructs by primer
design. The following constructs were generated: FAP52-HA (FAP52_{1–448} plus HA-tag);
FAP52_{Nt}-HA (FAP52_{1–293} plus HA-tag); FAP52_{SH3}-HA (FAP52_{390–448} plus HA-tag).

4.5 Expression and purification of the GST fusion proteins (I–III)

GST fusion proteins were expressed in *E. coli* BL21(DE3) cells according to standard
protocols, purified from the cell lysates on glutathione-Sepharose 4B beads, and liberat-
ed from the beads by incubation with reduced glutathione. For some experiments, FAP52
or its mutant forms were released from their fusion partners by incubation with thrombin.
For crystallization, FAP52 released from GST was further purified by using a Mono Q
anion-exchange chromatography column (Amersham-Pharmacia Biotech) in 20 mM Bis-
Tris, pH 6.0, connected to an ÄKTA fast protein liquid chromatography (FPLC) system
(Amersham Pharmacia Biotech), and eluted with increasing NaCl concentration. For the
control experiments, GST was expressed from the plasmid pGEX-2TK and purified as
above.

4.6 Cell cultures, transfections and immunofluorescence
microscopy (I, II, IV)

Chicken embryo heart fibroblasts (CEHF) were prepared and grown as described previ-
ously (Meriläinen *et al.* 1997). HeLa-cells were grown according to the same protocol
with the exception that Dulbecco’s modified Eagle’s medium was used as a growth medi-
um.

Transfections were carried out with the cells grown on glass coverslips and by using a
Fugene 6-transfection reagent and following the manufacturer’s instructions.

For immunofluorescence microscopy, the cells were fixed, stained and viewed as
described in the original articles I and II. Briefly, the cells grown on coverslips were
washed with Hank’s salt solution, fixed with paraformaldehyde solution, and post-fixed
with methanol or ethanol. After immersing fetal calf serum, the cells were incubated with
the primary antibodies, washed, incubated with appropriate fluorochrome-conjugated sec-
dary antibodies, washed again, and mounted on an object glass. Viewing was under a
Zeiss LSM510 laser scanning microscope equipped with a Zeiss Axiovert 110M inverted
microscope (Carl Zeiss Microscopy, Oberkochen, Germany). The images were processed
by using LSM 3D software, version 5.2. (Carl Zeiss Microscopy).
4.7 Immunoelectron microscopy (I, IV)

Immunoelectron microscopy was carried out as described in the original article I. Briefly, the cells were washed with Hank’s salt solution, fixed with paraformaldehyde-sucrose solution, harvested by scraping, and pelleted. The pellet was then resuspended in a sucrose solution and frozen in liquid nitrogen. Cryosections were cut on a microtome and placed on Butvar-coated nickel grids. They were then washed and blocked with 5% BSA with 0.1% ColdWaterFishSkin gelatin.

In the double labeling experiments, the sections were first incubated with monoclonal antibodies targeted to a protein of interest, followed by incubation with rabbit anti-mouse IgG, and then with Protein A-gold (Ø 10 nm). After this, the sections were fixed in glutaraldehyde solution and blocked with BSA. They were then incubated with Affi-K7 followed by Protein A-gold (Ø 5 nm). The sections were then post-fixed in glutaraldehyde, washed, counterstained with uranyl acetate, and coated with 1.8% methyl cellulose with 2% uranyl acetate. The sections were viewed using a Philips 410 LS transmission electron microscope (Philips, Eindhoven, Netherlands).

4.8 Cell lysates and immunoprecipitation (I, IV)

The cells grown to confluency were harvested by a scraper, suspended in the lysis buffer and incubated on ice bath for 20 min. The lysates were clarified by centrifugation at 12,000 x g.

The whole cell (unfractionated) lysates were incubated with the primary antibodies (Affi-K7 or mouse anti-filamin antibodies), followed by incubation with agarose-coupled anti-rabbit or anti-mouse IgG antibodies, respectively. The control reactions were carried out with the same protocol, but in the absence of the primary antibodies. The precipitates were washed and subjected to SDS-PAGE, followed by immunoblotting.

4.9 Affinity chromatography and pull-down experiments (I)

Affinity chromatography was carried out with CEHF lysates, which were incubated with the recombinant GST-FAP52 or GST alone, followed by incubation with the glutathione-Sepharose 4B beads. The beads were then washed, and the proteins were liberated from the beads by adding the SDS-PAGE loading buffer and boiling. The proteins were then subjected to SDS-PAGE and coomassie brilliant blue staining.

For pull-down experiments, GST fusions constructs of the fragments of FAP52 or filamin were immobilized on glutathione-Sepharose 4B beads and incubated with chicken gizzard filamin or recombinant FAP52, respectively. For the controls, GST was immobilized on the beads instead of the FAP52 or filamin fusion proteins. The proteins were released from the beads by adding the SDS-PAGE loading buffer and boiling. They were then subjected to SDS-PAGE and immunoblotting.
4.10 Immunoblotting and blot overlay assay (I, II, IV)

For immunoblotting, the electrophoretically separated proteins were transferred to nitrocellulose membranes, which were then blocked in nonfat dried milk powder in tris-buffered saline. Thereafter, the filters were incubated with Affi-K7 or mouse anti-filamin. After a 3-hour or overnight washing, the filters were incubated with either HRP-conjugated goat anti-rabbit or anti-mouse IgG, respectively. After washing, the blot was developed by the enhanced chemiluminescence (ECL) detection system.

Blot overlay assays were carried out with bacterially produced FAP52 and GST, or with GST-fusions of filamin mutants. They were separated on SDS-PAGE and transferred to nitrocellulose membranes. The filters were incubated with nonfat dried milk powder in tris-buffered saline, and then overlaid with the purified filamin or recombinant FAP52, respectively. For the visualization of the bound proteins, the overlays were washed and then incubated with either the mouse anti-filamin antibodies or Affi-K7, respectively, as above, followed by HRP-conjugated goat anti-mouse or anti-rabbit IgG, respectively, and ECL method.

4.11 Electrospray ionization mass spectrometry (I)

For mass-spectrometry, pieces of polyacrylamide gels corresponding to the protein bands of interest were excised from the coomassie brilliant blue-stained gels and processed as described by Shevchenko et al. (1996). Briefly, the proteins were in-gel reduced, alkylated, and digested with trypsin. The supernatant obtained was acidified with formic acid, loaded onto a Poros R2 microcolumn (Perseptive Biosystems, Framingham, MA), and desalted according to Gobom et al. (1999). The peptides eluted with methanol/formic acid solution were introduced into a nanoelectrospray needle (Protona, Odense, Denmark). Nanoelectrospray tandem mass spectrometry was performed on an API III mass spectrometer (PerkinElmer Instruments, Shelton, CT) equipped with a nanoelectrospray source, as described elsewhere (Wilm et al. 1996).

4.12 Surface plasmon resonance analysis (I, II)

Surface plasmon resonance (SPR) measurements were performed on a BIACORE 3000 analyzer under control of BIACORE control software, version 3.1.1. (Biacore). A carboxymethyl-coated CM5 sensor chip was prepared by immobilization of anti-GST antibody on the surface of the chip by utilizing the GST Capture and the Amine Coupling kits (Biacore). As ligands, GST-FAP52 or GST-Fil1524–2283, or mutants thereof were coupled to the immobilized anti-GST. In order to keep GST-FAP52 a monomer, mildly acidic buffer (Bis-Tris, pH 6.0), a condition in which FAP52 exists mainly as monomer (data not shown), was utilized during the coupling procedure. For the binding assays, chicken gizzard filamin or the recombinant FAP52 were then passed over the chip at varying con-
centrations. The binding of the analyte to the ligand was detected from the change in the sensorgram. In order to correct the sensorgram for background binding and bulk refractive index changes, a different flow cell without the GST-fusion protein was used as a reference. As controls, GST-coated chips were used, over which filamin or FAP52 was passed. Kinetics was analyzed by BIAevaluation software, version 3.1 (Biacore).

4.13 Chemical cross-linking (II)

For chemical cross-linking, the bacterially produced protein or the CEHF lysate clarified by centrifugation was incubated in the presence or absence of disuccinimidyl suberate. The reaction was terminated by adding SDS-PAGE loading buffer into the reaction mixture. The samples were run on SDS-PAGE and visualized by coomassie brilliant blue staining or immunoblotting.

4.14 Gel filtration chromatography (I, II)

Bacterially produced FAP52 or CEHF lysate, clarified by centrifugation, was applied on a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) connected to an ÄKTA FPLC system. The column was pre-equilibrated with PBS and calibrated with the molecular weight markers urease (hexamer 545 kDa, trimer 272 kDa), γ-globulin (150 kDa), BSA (dimer 132 kDa, monomer 66 kDa) and chicken egg albumin (45 kDa). The sample was eluted with PBS and the run was monitored by absorbance at 280 nm. The peak fractions were collected and analyzed by SDS-PAGE and immunoblotting.

4.15 Far UV circular dichroism (II)

Determination of the circular dichroism (CD)-spectra was carried out by using Jasco J-715 spectropolarimeter (Jasco Research, Brentwood Bay, B.C., Canada) under control of J-700 Hardware Manager, version 1.50.00 (Jasco Research). For the experiments, the protein was dialyzed against 50 mM potassium phosphate buffer, pH 7.4. The CD value scans were measured from 190 to 260 nm, at a step resolution of 2 nm, and with a scanning rate of 20 nm/min. The CD spectra were the average of 10 scans. The data were processed with Standard Analysis Program, version 1.50.01 (Jasco Research). The content of α-helicity was calculated by Protein Secondary Structure Program, Model SSE338 (Jasco Research; Yang et al. 1986).
4.16 Crystallization of FAP52 (III)

For crystallization, bacterially produced GST-FAP52 was purified by affinity and anion-exchange chromatography as described above. Purified FAP52 was then subjected to buffer change and desalting by performing three cycles of concentration-dilution in 20 mM Tris, pH 8.0 and by using Centriprep concentrating column (Millipore Corp, Bedford, MA). Finally, FAP52 was adjusted to 13.5 mg/ml.

The crystals were grown by the hanging drop vapor diffusion technique (Hampton Research, Laguna Niguel, CA). An ammonium sulphate grid screen was utilized to find proper crystallization conditions. The drops were prepared by mixing 1 µl of the protein with 1 µl of the reservoir solution over the 1-ml volume of the reservoir. After one-day of equilibration, small stick-like crystals quickly appeared. Subsequently, polyethylene glycol PEG2kMME in concentrations ranging from 10 to 30% was combined with ammonium sulphate ranging from 100 to 400 mM. The pH was buffered to 8.0 by 100 mM HEPES. One condition, 20–22% PEG2kMME, 350 mM ammonium sulphate, 100 mM HEPES, pH 8.0, yielded crystals of two different morphologies. Form I displayed a shape of thick needles or columns, and the form II appeared as prism/plate-like crystals.
5 Results

5.1 Interaction of FAP52 with filamin (I)

To identify new binding partners for FAP52, coimmunoprecipitation and pull-down (affinity binding) experiments were carried out by using the lysates of cultured CEHF's as a target. For co-immunoprecipitation experiments, the lysates were incubated with the Affi-K7 antibodies, followed by agarose-bound anti-rabbit IgG antibodies. The beads along with their lysate-derived bound materials were then collected by centrifugation, and subjected to SDS-PAGE and silver staining. For pull-down experiments, GST-FAP52 was coupled to the reduced glutathione-conjugated sepharose beads in a column, and the cell lysates were passed over the column. The attached proteins were then eluted from the beads and subjected to SDS-PAGE and coomassie brilliant blue staining. The immunoprecipitation and the pull-down experiments both revealed a 280-kDa polypeptide band, which was present in the experimental but not in the control assay [Fig. 1 in the original article I (Fig. 1/I)]. In pull-down experiments, another prominent band of about 220 kDa was seen.

The 280-kDa band from the pull-down experiments was excised from the gel and subjected to in-gel trypsin digestion. The extracted peptides then underwent analysis by nanoelectrospray tandem mass spectrometry. The partial primary structures of several peptides in the mass spectrum of the 280-kDa band were compared with the sequences in the databases. In five cases there was a match with GST, in four cases with FAP52, in one case with myosin, and in one case with filamin. We concluded that the myosin sequence among the peptides derived from the 280-kDa band originated from the more abundant material of the 220-kDa band corresponding to the MW of myosin heavy chain.

5.1.1 Association between FAP52 and filamin in cells in vivo

The association of FAP52 with filamin was further explored by immunoprecipitation of FAP52 from CEHF-lysates, followed by immunoblotting for coprecipitation of filamin.
Reciprocal experiments were carried out by immunoprecipitation of filamin from FAP52+HA–transfected HeLa cells and immunoblotting to probe for the coprecipitation of FAP52+HA. Filamin was present, as judged by immunoblotting, in the Affi-K7 precipitates. Reciprocally, FAP52+HA was found in the precipitates obtained by immunoprecipitation with anti-filamin. Thus, FAP52 associates with filamin in vivo. These experiments did not resolve whether the interaction is a direct one or mediated by some other component (Fig. 2/I).

5.1.2 Direct binding of FAP52 to filamin

In order to explore whether the association between FAP52 and filamin is due to direct interaction, blot overlay assays were carried out. Bacterially produced FAP52 or the COOH-terminal fragments of filamin were subjected to SDS-PAGE and transferred to a nitrocellulose filter, which was then incubated with chicken gizzard filamin or recombinant FAP52, respectively. Binding of filamin to FAP52 was assayed by using anti-filamin antibody or Affi-K7, respectively, followed by the ECL method. Indeed, filamin was seen to directly bind FAP52 (Fig. 3a,b/I).

Surface plasmon resonance is a technique that permits determination of the dynamics of the interactions between various biomolecules. In the experiments, one interactor is anchored to the solid phase, the sensor chip. The other interactor is then passed over its immobilized binding partner. The dynamic association and dissociation can be detected as changes in the response in the sensorgram. The dissociation and association rates are determined from the response profile, and the dissociation constant (K_D) can then be derived from their binding equations.

We utilized SPR to further demonstrate the interaction between FAP52 and filamin. Bacterially produced GST-FAP52 was immobilized to the sensor chip, and chicken gizzard filamin was passed over the chip. In the sensorgram, a distinct change was seen upon filamin injection. On the other hand, a return to base level was seen upon washing the chip after the injection of filamin (Fig. 3c/I). This indicates a direct and reversible binding of FAP52 to filamin. The K_D was determined to be 9.64 x 10^{-9} M, suggesting a high-affinity binding between FAP52 and filamin.

5.1.3 Filamin-binding site in FAP52

Next we wanted to map the filamin-binding site in FAP52. For that purpose, the sequentially truncated NH2- and COOH-terminal mutant forms of FAP52 in GST fusion were utilized in SPR and blot overlay experiments and assayed for the binding of filamin. Both methods showed a binding of filamin to FAP52 polypeptides corresponding to residues 1–184, 1–219, 1–359 and 1–448, but not to 1–145, 146–448, 185–448, 220–448, 281–448, 360–448 and 390–448 (Fig. 4/I). These results point to the region 146–184 of FAP52 as a filamin-binding site. However, no binding was seen for the isolated polypeptide corresponding to the aa 146–184 (GST-FAP52146-184), indicating that the region 146–184 is
necessary, but not sufficient for filamin binding. For binding in vivo, the residues in the NH$_2$-terminal region 1–145 are probably also needed.

### 5.1.4 FAP52-binding site in filamin

Similar but reciprocal experiments were then performed in order to map the FAP52-binding site(s) in filamin. Both SPR and blot overlay assay affirmatively indicated the polypeptides corresponding to the aa 1524–1858, 1524–1956, 1524–2064, 1524–2132, 1524–2172 and 1524–2283 of filamin as being capable of binding FAP52 (Fig. 5/I). SPR but not overlay assay also showed binding to the fragment 1524–1780 and weakly to fragment 1524–1664. This disaccord could very well be due to a higher sensitivity of the SPR method. From these results it can be concluded that the residues 1664–1858, corresponding to the repeats 15–16 of filamin, are responsible for binding of FAP52.

### 5.1.5 Colocalization of FAP52 with filamin in cultured cells

The localization of FAP52 in naive CEHFs in relation to filamin distribution was explored by immunofluorescence microscopy and immunoelectron microscopy by using double immunolabeling technique. In immunofluorescence microscopy (Fig. 6/I), filamin showed mostly a distinct filamentous staining along actin stress fibers. FAP52, on the other hand, was seen to reside in FAs (Fig. 6a/I). At the sites where the actin fibers abut the FAs there was a distinct overlap of these otherwise distinct staining patterns. More closely, the overlap was seen in a distinct region corresponding to the hinge region comprising the more central (proximal) part of the FA (Fig. 6a/I). Colocalization was also seen in small punctates along the very edges of the cell periphery especially at sites corresponding to the dynamic, fast-moving, forward-pushing edge of spreading fibroblasts (Fig. 6b/I).

For immunoelectron microscopy, cryosections of cultured fibroblasts were double-labeled with antibodies to FAP52 and filamin, which were then visualized by using appropriate secondary antibody conjugated with small and large gold particles, respectively (Fig. 7/I). Labeling indicating the presence of filamin was seen in a linear fashion, most prominently in the cell periphery. FAP52, on the other hand, was only localized to peripheral parts of the cell, at the elongated structures, which, on the basis of their morphology and localization, represent FAs (Fig. 7a/I). At a higher magnification, colocalization of FAP52 and filamin was seen (Fig. 7b,c/I).
5.2 Overexpression of FAP52 in cultured cells (I, II)

Overexpression of protein by using an exogenously introduced vector has become a valued means to explore a role of the given protein in cellular structure and function. We overexpressed FAP52 and its truncated forms as HA-tagged fusion proteins, a technique that allow easy visualization of the fusion products in the cell by using anti-HA antibodies. Due to strong overexpression, FAP52 was seen in most cytosolic compartments. There was, however, also a distinct accumulation in surface protrusions and along the edge of the cell (Fig. 8/I). Moreover, fibroblasts overexpressing FAP52-HA displayed several types of abnormal morphologies. A normal, nontransfected cell has an elongated or polygonal, smooth-contoured morphology (Fig. 8a/I). Some cells transfected with FAP52-HA displayed an elongated appearance with several surface protrusion representing filopodia (Figs 8a/I; 9a/II). More typical were roundish transfectants with abnormal or arrested spreading (Fig. 8b/I). In some cells, ruffling edge-typed formations were seen (Fig. 8c,d/I). Actin, as visualized by phalloidin, and FAP52-HA were seen accumulated in such membrane ruffles. The cells with a slender appearance displayed attenuated actin staining (Figs 8e,f/I; 9b/II), indicating the disturbance of actin filament network. As a conclusion from these studies, it can be said that FAP52 colocalizes with filamentous actin-based structures, and, upon overexpression, FAP52 modulates the actin cytoskeleton. FAP52 may disturb normal actin orchestration by stimulation of actin polymerization in the cell periphery, resulting in filopod formation, and, on the other hand, by disruption of the actin filament network, probably via accelerated actin depolymerization or inhibited actin polymerization.

We also wanted to see how the overexpression of FAP52 would affect the distribution of filamin. For that purpose, FAP52-HA was transiently expressed in CEHFs, and anti-HA and anti-filamin antibodies were utilized for the visualization of FAP52 and filamin, respectively. The FAP52-HA transfectants displayed an abnormal filamin distribution (Fig. 8a/I). Instead of the normal linear organization along actin fibers, dot-like densities close to the cell surface were seen. Also FAP52 and actin were present in the dots. There were only thin actin filaments connecting the dots to each other. This suggests that FAP52 associates with filamin in vivo, and that the modulation of the actin cytoskeleton upon overexpression of FAP52 may be mediated via filamin.

In order to try to resolve what functions the specific domains of FAP52 could serve in cells we also carried out transfections by using FAP52NT-HA and FAP52SH3-HA constructs. The cells overexpressing FAP52NT-HA mostly showed a similar distribution of the exogenously expressed FAP52 as seen in the cells overexpressing the full-length protein. However, also some very long filopodia were seen in FAP52NT-HA-transfectants (Figs 8e,f/I; 9a,c/II). Overexpressed FAP52SH3-HA was mostly located in the perinuclear region and was not associated with any major detectable changes in the overall cell morphology, as verified by visualization of actin filaments (data not shown).

Since FAP52 and filamin are closely associated with FAs, we paid special attention to the number and morphology of FAs in transfected cells. Double labeling experiments revealed that in the cells overexpressing FAP52-HA or FAP52NT-HA, the FAs were small and randomly oriented, while in naive cells they were radially oriented and regularly spaced in the periphery of the cell (Figs 8g/I; 9d/II). On average, FAs in transfected cells
were also fewer in number than in naive cells (40 vs. 61). Thus, overexpression of FAP52 distorts the structure and reduces the number of FAs in cultured fibroblasts.

5.3 Self-association of FAP52 (II)

5.3.1 Computational analysis and circular dichroism

Secondary structure prediction of FAP52, based on its primary structure, indicated that it has in its NH$_2$-terminus a long stretch with an $\alpha$-helical arrangement with a high-degree of propensity to coiled-coil arrangements. This could be fulfilled either by intra- or inter-molecular interactions. In order to distinguish between these possibilities we started to analyze the capacity of FAP52 to self-associate. The PAIRCOIL program predicted that FAP52 includes three regions comprising the residues 146–179, 185–219 and 248–280 with the probability of 53%, 100%, and 9%, respectively, to form coiled-coils (Fig. 2/II). Another program, MULTICOIL, further discriminates the propensity for dimeric and higher oligomeric arrangements, giving the likelihoods of dimer formation of 3%, 74% and 0%, and of trimer formation of 4%, 10% and 0.5% for the same regions, respectively (I). These analyses suggest a high propensity for FAP52 to self-associate and for the region spanning the aa 146–280 to represent the coiled-coil domain.

The content of $\alpha$-helicity of the wild-type FAP52 and FAP52$_{Nt}$ was also estimated by circular dichroism, and found to be 25.8% and 57.7%, respectively. The results are well in line with the computational analyses that predict the $\alpha$-helicity to reside in the NH$_2$-terminus of FAP52.

5.3.2 Self-association in vitro and in vivo

In order to resolve whether FAP52 forms dimers, trimers or higher order oligomers in vitro we decided to apply cross-linking of the bacterially produced FAP52, followed by SDS-PAGE and coomassie brilliant blue staining. After covalent cross-linking, FAP52 migrated as two separate bands with the estimated molecular weight of 170 kDa and 63 kDa. They correspond roughly to the trimultiple and the unit weight of FAP52. The non-crosslinked FAP52 was seen as a single band of 63 kDa. This suggests that FAP52 has the capacity to self-associate in vitro and, most probably, to arrange trimers.

In order to study whether FAP52 occurs as oligomers also in vivo, we utilized chemical cross-linking, and, on the other hand, gel filtration chromatography, of the lysates of cultured CEHFs. For chemical cross-linking experiments, the cross-linked cell lysates were subjected to SDS-PAGE and coomassie brilliant blue staining. FAP52 migrated as a single band of 170 kDa (Fig. 5/II). For gel filtration chromatography, the cell lysates were applied in the calibrated gel filtration column, and, upon elution, fractions were collected. In order to determine the elution profile of the endogenous FAP52, the fractions
were then subjected to SDS-PAGE and immunoblotting. The majority of FAP52 eluted in a fraction corresponding to the molecular weight of 170 kDa, whereas the minority showed a mass below 60 kDa (Fig. 6/II). Taken together, FAP52 predominantly occurs in oligomeric, most probably in trimeric form in vivo.

5.3.3 Kinetics of self-association

We utilized SPR to look at the dynamics of the self-association of FAP52. For that purpose, series of concentrations ranging from 20 nM to 400 nM of recombinant FAP52 were passed over GST-FAP52 coupled to the sensor chip, and the binding profile was detected as changes in the RU values in the sensorgram. The association and dissociation rates were estimated to be $1.15 \times 10^5$ M$^{-1}$ s$^{-1}$ and $5.47 \times 10^{-4}$ s$^{-1}$, respectively, which gives a $K_D$ of $4.7 \times 10^{-9}$ M, indicating high affinity between the subunits.

5.3.4 Mapping of the self-association site

Secondary structure prediction strongly implicated the NH$_2$-terminal $\alpha$-helical domain as the self-association site of FAP52. The verification for the self-association site was experimentally pursued by carrying out SPR experiments with GST-FAP52 immobilized on the sensor chip. Use of the sequentially truncated 5'- and 3'-deletion mutants of FAP52 in the mobile phase also allowed a more accurate mapping of the association site(s) within this defined region. The NH$_2$-terminal fragments encompassing the residues 1–184, 1–219, 1–359, but not the one representing the residues 1–145 were seen to bind to the chip. A distinct binding was also seen with the COOH-terminal fragments with the residues 146–448, 185–448 and 220–448. These results indicate that several distinct regions within a span of 146–280 contribute to self-association.

5.4 Crystallization and phasing of FAP52 (III)

In order to determine the 3-D structure of FAP52, we decided to resort to crystallization of FAP52 and its X-ray diffraction analysis. For crystallization, the hanging drop technique was used. Two crystal forms were detected. The space group of the crystal form I was $P2_12_12_1$, having the cell dimensions $a=101.4$, $b=105.9$, $c=126.3$ Å. The best crystal diffracted to a resolution of 2.8 Å. The crystals were column-shaped and their average volume was $500\text{x}60\text{x}60$ μm$^3$. The unit cell volume was $1.36 \times 10^6$ Å$^3$. The asymmetric unit contains two or three protein molecules, suggesting $V_M$ of 3.3 Å$^3$Da$^{-1}$ or 2.2 Å$^3$Da$^{-1}$, respectively.

The crystals of the form II were prisms or rhomboidal plates and they represent the $C2$ space group. The unit cell dimensions were $a = 164.7$, $b = 102.2$, $c = 107.3$ Å, $\beta = 131.2^\circ$. 

The best crystal diffracted to a resolution of 2.1 Å. In addition, a 3.27 Å low-resolution dataset of 54 frames with 3 degrees oscillation angle was collected to supplement the missing low-resolution reflections lost due to overload. The average dimension of the crystals was 250x250x100 µm³. The unit cell volume was 1.36 x 10⁶ Å³, consistent again with 2 or 3 molecules per asymmetric unit. These crystals were fragile and broke easily during mounting and pressurization, and the trials to obtain xenon-derivatized crystals failed utterly, or the datasets showed radiation damage. Therefore 30 frames at the end of the dataset were omitted from the processing.

The datasets of two different xenon-derivatized crystals were collected using wavelengths of 1.5 and 1.9 Å, and the maximum resolution of 3 Å was reached. Multiwavelength anomalous dispersion phasing was successfully performed. Preliminary structural analysis revealed a dimer in the asymmetric unit.

5.5 FAP52 in bile canaliculi

The distribution of FAP52 in chicken tissues was studied by using the Affi-K7 antibodies and immunofluorescence microscopy. A positive staining reaction was seen in several tissues. Intriguingly, a very distinct staining pattern was seen in the liver, where FAP52 was seen almost exclusively in close proximity to bile canaliculi (Fig. 1/IV). Due to this association of FAP52 with a highly differentiated and specific structure, we decided to look more closely at the role of FAP52 in the structure and function of bile canaliculi.

For closer scrutiny, cultured human hepatoma HepG2 cells, Affi-K7 antibodies, and immunofluorescence and immunoelectron microscopy were utilized. In immunofluorescence microscopy, vacuolar-like structures resembling bile canaliculi were seen when the actin microfilaments were visualized by rhodamine-phalloidin. Their authenticity was judged by labeling for the canaliculus-specific component, CEA (not shown). FAP52 localized along the basal domains of the cells and, more specifically, in subdomains corresponding to CEA-positive bile canaliculi (Fig. 3/IV). FAP52 was especially strongly present in the bases of microvilli, which were visualized in double-labeling experiments by rhodamine-phalloidin. In immunoelectron microscopy, FAP52 was present under the plasma membrane along the canaliculi and, occasionally, in microvillar projections. The authenticity of the bile canaliculi was again judged by labeling for CEA (not shown).
6 Discussion

6.1 FAP52 as a multidomain protein

FAP52 was originally described as a novel SH3 domain-containing protein (Meriläinen et al. 1997). Analysis of the primary structure revealed the presence of a unique SH3 domain in the COOH-terminus of the protein and a very highly \( \alpha \)-helical stretch with a high propensity to coiled-coil arrangement in the NH\(_2\)-terminus. In the middle, an unstructured “linker” region was predicted.

Currently, more than 400 SH3 domain-containing proteins are known (search from human protein database at the network server http://pfam.wustl.edu). Therein, the SH3 domain serves the function of binding various proteins with a proline-rich consensus recognition motifs (Cesareni et al. 2002). In many cases, the SH3 proteins also contain other protein-protein interaction motifs, such as the SH2 domain (Pawson et al. 2001).

Sequences with a propensity to coiled-coil arrangements are known to mediate both homo- and hetero-oligomerization of proteins. Thus, we set out to study whether FAP52 and especially its NH\(_2\)-terminal part was capable of forming dimers or higher order oligomers. First, we resorted to computational analysis in order to determine the nature of \( \alpha \)-helicity in FAP52. For that purpose we utilized the programs PAIRCOIL and MULTI-COIL. The results showed the presence of three distinct regions with a high tendency to fold in a coiled-coil arrangement and probably in higher order oligomeric complexes. These regions, spanning the aa residues 146–179, 185–220 and 248–280, reside in the NH\(_2\)-terminus. Dimer was a preferred form in the calculations based on MULTI-COIL, which estimates probabilities for various degrees of oligomerization.

The content of \( \alpha \)-helicity was verified also empirically by utilizing circular dichroism analysis. In full-length FAP52 and in its NH\(_2\)-terminal 359 aa it was estimated to be 25.8% and 57.7%, respectively, which is well in line with the prediction by the PHD program (Meriläinen et al. 1997) and with the results of the PAIRCOIL and MULTI-COIL programs.
6.2 FAP52 self-associates

The capacity of FAP52 to self-associate was tested by utilizing chemical cross-linking, gel filtration chromatography and SPR analyses. All methods concertedly showed FAP52 to have a capacity to oligomerize. In the cross-linking and gel filtration experiments, FAP52 appeared as species of 63 kDa and 170 kDa, corresponding roughly to the monomeric and trimeric forms of the protein. The kinetics of the self-association was estimated by SPR analysis. The results showed the dissociation constant $K_D$ to be $4.7 \times 10^{-9}$ M, which indicates high affinity between the subunits. The observation that, despite such high affinity, FAP52 also exists as a monomer in cell extract, may be due to the interference of the self-association by other proteins of the extract, or to the putative effect of phosphorylation on self-association.

The region in FAP52 that is responsible for the self-association was also determined by utilizing SPR with the sequentially truncated mutants of FAP52. The results revealed three distinct regions, encompassing the aa residues 146–179, 185–220 and 248–280 most likely responsible for the self-association. Thus, the empirical data completely match the predictions based on the PAIRCOIL and MULTICOIL programs.

In order to determine the detailed 3-D structure of FAP52, we utilized X-ray diffraction analysis. The preliminary structural analysis revealed an antiparallel dimer in the asymmetric unit. The discrepancy between the results of X-ray crystallography and chemical cross-linking/gel filtration, which suggested the presence of trimers rather dimers, could be due to the abnormal migration of FAP52 in gel electrophoresis and size exclusion chromatography. The calculated molecular mass of FAP52 is 52 kDa, whereas its migration in SDS-PAGE corresponds to the molecular mass of 63 kDa. The difference in the calculated mass and the molecular weight determined by migration in gel may be due to an exceptionally rigid secondary structure of FAP52.

The NH$_2$-terminal region is highly conserved among the members of the FPS protein family. In a comparison with the BLAST program at the web server http://www.ncbi.nlm.nih.gov/BLAST, their similarity with FAP52Nt ranges from 72% to 91% (data not shown). Among PACSIN 2 proteins, the human, rat and mouse orthologs are 91% identical with the NH$_2$-terminal region of chicken FAP52. In addition to FAP52, all three PACSINs have a tendency to self-associate, but also to form heteroaggregates with the other PACSINs (Modregger et al. 2000).

Several FA components, such as $\alpha$-actinin (Blanchard et al. 1989) and talin (Goldmann et al., 1994) have been shown to exist as antiparallel dimers. The rod-shaped core of native $\alpha$-actinin is made up of two $\alpha$-actinin subunits, in which the coiled-coil regions mediate dimerization (Djinovic-Carugo et al. 1999). Due to self-association, proteins may have a capacity to cross-link actin filaments ($\alpha$-actinin) or to serve as a docking site for various structural or signaling molecules to FAs (talin). In the case of FAP52, dimerization could bring proteins that bind to the SH3 of FAP52 to close proximity of each other. The SH3 domain of FPS proteins binds to a number of proteins involved in vesicular trafficking and actin dynamics, such as synapsin I, dynamin I, synaptojanin, N-WASP and mSos (Qualmann et al. 1999, Modregger et al. 2000, Wasiak et al. 2001). Thus, FAP52 may serve as a coordinator of their proper spatial placement in the cellular processes.
6.3 FAP52 as a filamin-binding protein

Elucidation of the function of FAP52 was pursued by determining its binding partners, and by in vivo studies, in which FAP52 or its fragments were overexpressed in cultured cells. By using affinity chromatography, immunoprecipitation, blot overlay and SPR analyses, FAP52 was here found to bind to filamin, a dimeric, high-molecular weight protein, which cross-links actin filaments and organizes them into parallel bundles or nonparallelly to form orthogonal networks. The COOH-terminus of filamin is composed of 24 repeats, which form a rod-like appearance. The 24th repeat forms the self-association domain. In the NH2-terminus, there is an actin-binding region. The FAP52-binding site in filamin was mapped to the repeats 15–16. This region of filamin also serves as the binding site for e.g. TNF receptor-associated factor 2 (TRAF2; Leonardi et al. 2000), dopamine D2-receptor (Li et al. 2000), and calcium-sensing receptor (CaR) (Awata et al. 2001). The filamin-binding site in FAP52 overlaps with the self-association site. The question of how the self-association of FAP52 and the interaction with filamin are related is the subject of further studies.

Filamin has been suggested to have a crucial role in mediating inflammatory stimuli via the TNF receptor for the activation of SAPKs and NF-κB (Leonardi et al. 2000). Filamin binds to TRAF2, an intracellular adaptor in TNF receptor signaling (Leonardi et al. 2000). Overexpression of filamin blocks the TNF-induced SAPK and NF-κB activation. On the other hand, in M2 melanoma cells, which lack filamin, TNF fails to activate SAPKs and NF-κB (Leonardi et al. 2000). Filamin is also needed in the signaling from CaR to MAPK cascade activation. By experiments in which CaR was transiently expressed in M2 cells and in A7 melanoma cells, which stably express filamin, it was shown that CaR activates ERK only in the presence of filamin (Awata et al. 2001, Pi et al. 2002). Filamin also enhances dopamine D2-receptor signaling. Dopamine D2-receptor mediates signaling in a multitude of neurobiological and endocrine processes, e.g. by inhibiting adenylate cyclase (Li et al. 2000). Cells with mutant filamin display attenuated inhibition of adenylate cyclase (Li et al. 2000).

In the studies discussed above, filamin is suggested to serve as a scaffold and docking molecule for the signaling proteins. Filamin also binds same small-molecular weight modulators of the actin cytoskeleton, such as RhoA, Rac1 and Cdc42, and actin itself (Ueda et al. 1992, Ohta et al. 1999). Due to a multitude of binding sites of signaling molecules in filamin, it may have the capacity to “trap” the entire repertoire of signaling molecules needed for a given cellular process within narrow confines and to guarantee their proper orientation. It may also bring about a high local concentration of signaling molecules involved in a specific cellular process at a focal point.

Filamin also binds to integrins β1A, β1D, β2, β3 and β7 (Pfaff et al. 1998, Takafuta et al. 1998, Liu et al. 2000). Integrin β1, the receptor for the ECM component fibronectin, has been shown to fix the cytoskeleton to cell surface, and, on the other hand, the cell surface to ECM in stationary or less motile cells (Critchley 2000). β2, on the other hand, plays a similar role in more motile cells, such as leukocytes (Arnaout 1990, Sharma et al. 1995). Tight integrin-filamin interaction has been shown to inhibit cell migration (Calderwood & Ginsberg 2003). Thus, by linking actin microfilaments to integrins, filamin regulates the physical properties of cells. The lymphocyte-specific integrin β7 mediates the contact of the cell with ECM and the vascular wall. In these cells filamin is suggested to
contribute the targeting of lymphocytes (Pfaff et al. 1998). In platelets, filamin binds to β3 integrin (Goldmann 2000). The significance of the interaction is not known.

6.4 FAP52 as a regulator of the cytoskeleton

Overexpression of FAP52 or its filamin-binding region in cultured fibroblasts induces reorganization of the actin cytoskeleton and the formation on filopodia to the cell surface. Similar effects were recently demonstrated also for rat PACSIN 2 (Qualmann & Kelly 2000).

Formation of filopodia is mediated by the Arp2/3 complex, a nucleator of actin filament polymerization (Miki et al. 1998, Qualmann & Kelly 2000). Its activation leads to formation of either filopodia or lamellipodia, depending on the nature of the activator molecule. Activation by N-WASP leads to formation of filopodia. PACSINs (Qualmann & Kelly 2000, Modregger et al. 2000) bind N-WASP. Thus, the formation of filopodia by FAP52 is probably mediated by N-WASP.

The basis of the formation of the filopodia is the reorganization of the dendritic actin filaments into parallel bundles facing the plasma membrane (Svitkina et al. 2003). Our hypothesis is that FAP52, via binding to filamin, controls the actin-filamin interaction in a way that enhances the bundling event. This could happen e.g. by bringing the two “arms” of the filamin dimer into a more parallel orientation.

In fibroblasts, overexpression of FAP52 or its NH2-terminal fragment, which contains the filamin-binding site, led to a distinct reduction in the number of FAs. Whereas the naive cells expressed on average 61 FAs per cell, the transfected fibroblasts had only 40 FAs. Also the morphology and orientation of FAs in FAP52-overexpressing cells was abnormal.

Currently, no interactions of FAP52 with any FA-specific proteins have been described. Thus, the mechanism responsible for the localization at FAs is not clear. We are currently working to identify putative interactions of FAP52 with FA-proteins to elucidate the molecular mechanisms of its targeting.

Recently, a novel, FA-specific splice variant for filamin B [Filamin Bvar1(ΔH1)] was described (van der Flier et al. 2002). It lacks 41 aa between the repeats 19 and 20, and has a deletion between the repeats 15 and 16, corresponding to the hinge region H1. Interestingly, the FAP52-binding site in filamin A spans from the repeat 15 to repeat 16, and, thus, overlaps with the deletion site in filamin B. Whether the reduction of the number of FAs is due to an interference of the excess FAP52 with the normal molecular architecture is a subject of additional research.
6.5 FAP52 as a component of the pericanalicular web of the bile canaliculi

To expand our insight of the significance of FAP52, we screened its expression in various tissues by immunohistochemistry and FAP52-specific antibodies (Affi-K7). Apart from a fairly wide-spread expression in many tissues we also found a distinct and prominent expression present in bile canaliculi.

The liver produces and secretes bile, which is a mixture of various end products of the metabolism of the organism. Bile is secreted by hepatocytes to bile canaliculi, which form an anastamosing network within plates of hepatocytes (Young & Heath 2000). Bile canaliculi are formed of the apical regions of the plasma membranes of the adjacent hepatocytes, which are highly specialized, polarized cells. Apical membrane extends small microvilli to the lumens of canaliculi (Young & Heath 2000).

Human hepatoma HepG2 cells are an ideal model for studies on bile canaliculi. They grow to islets and spontaneously form short, round bile canalicular structures. In cultures, they retain the biosynthetic capability and the specialized functions of normal liver parenchymal cells (Chiu et al. 1990, Sormunen et al. 1993).

In immunofluorescence microscopy of cultured HepG2 cells, bile canaliculi, which were visualized by rhodamine-phalloidin, were seen as circular structures with the microvilli directed towards their lumens. FAP52 was seen to localize in the walls of the bile canaliculi. FAP52 was especially strongly present in the bases of microvilli. In electron microscopy studies, FAP52 was localized just below the lateral plasma membrane. Moreover, it was also present in microvilli.

The canaliculus-associated cytoskeleton consists of three distinct zones of actin microfilaments (Tsukada et al. 1995; see Fig. 6). First, the parallel bundles of actin microfilaments form the core of the microvilli. Actin filaments are there associated with villin, an actin-splicing protein (Craig & Powell 1980). Second, the membrane-associated actin microfilament band is attached to the membrane. It is associated with myosin II, and is probably involved in the maintenance of the elasticity and the structural integrity of the membrane, and in the regulation of the vesicle transport of the canaliculus (Tsukada et al. 1995). Third, the contractile pericanalicular actin filament band is a circumferential belt around the canaliculus. The contraction and relaxation, which is due to the organization of actin microfilaments with myosin II and tropomyosin, allows the narrowing and dilatation, respectively, of bile canaliculi.
Fig. 6. Actin microfilament architecture in pericanalicular web. Actin filaments are organized into three distinct zones. 1, cores of microvilli; 2, membrane-associated microfilaments; and 3, circumferential microfilament band. BC, bile canaliculus; C, cytoplasm; APM, apical plasma membrane; M, microvillus. (Modified from Tsukada et al. 1995).

As judged by immunofluorescence microscopy and immunoelectron microscopy, FAP52 is located in the regions where the actin filaments of microvilli intersect the perpendicular pericanalicular actin filament band. The capacity to bind filamin, an actin-orchestrating protein, and the ability to modulate actin organization on cultured cells suggests the involvement of FAP52 in the regulation of actin architecture in these nodes of the actin network.
7 Conclusions

FAP52 binds directly to filamin. The binding site was mapped to the α-helical NH₂-terminus of FAP52, and to repeats 15–16 of filamin.

FAP52 self-associates in vitro and in vivo. The site responsible for the self-association resides in the α-helical region spanning the aa residues 146–280, which also contains the filamin-binding site. The preliminary X-ray analysis suggests FAP52 to exist as dimer.

Cultured fibroblasts overexpressing FAP52 or its filamin-binding NH₂-terminus undergo dramatic morphological alterations. These cells typically extend numerous filopodia on their surfaces. The distribution of filamin was also changed to the filopodia. In addition, instead of normal, filamentous organization in naive cells, numerous dot-like densities were seen along the actin fibers. It seems that FAP52 is important in organizing the actin filaments to parallel stress fibers.

FAP52 has a specific pattern of localization in the liver. Both chicken liver and cultured human hepatocytes showed an accumulation of FAP52 under the plasma membrane of bile canaliculi and, especially, at the intersection of the microvillous cores and the pericanalicular actin microfilaments. Thus, FAP52 may have an important role in organizing the actin architecture of the pericanalicular web.

Conclusively, FAP52 participates in the organization of the actin cytoskeleton, probably via its direct interaction with filamin. FAP52 self-associates in vivo, which may provide FAP52 the ability to cross-link filamin molecules or regulate the flexibility of the filamin dimer, or to serve as a coordinator of the cytoskeleton-associated proteins.
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